Osmotic regulation of the *Streptomyces lividans* thiostrepton-inducible promoter, *ptipA*

Nasima Ali,[†] Paul R. Herron, Meirwyn C. Evans and Paul J. Dyson

Author for correspondence: Paul Dyson. Tel: +44 1792 295667. Fax: +44 1792 295447.
e-mail: p.j.dyson@swansea.ac.uk

Molecular Biology Research Group, School of Biological Sciences, University of Wales Swansea, Singleton Park, Swansea SA2 8PP, UK

Transcriptional activation of the thiostrepton-inducible promoter, *ptipA*, in *Streptomyces lividans* is mediated by TipAL. This transcriptional activator belongs to the MerR/SoxR family that characteristically binds an operator sequence located between the –10 and –35 hexamers normally occupied by RNA polymerase. As for the *Escherichia coli merT* promoter, the *ptipA* hexamers are separated by a long 19 bp spacer and hence a topological transition of the DNA is likely to be a requisite for alignment with RNA polymerase. Growth conditions that could facilitate this conformational change were investigated using transcriptional fusions of *ptipA* with reporter genes. Adjustment of growth medium osmolarity led to increased and prolonged TipAL-dependent expression, both with and without the inducer, thiostrepton. These effects correlated with increases in negative DNA supercoiling. Moreover, an inability to induce the promoter with thiostrepton in strain TK64 was corrected by increasing the concentration of osmolyte, compensating for an apparent reduced level of negative DNA supercoiling in the strain. Prolonging the time of activation of *tipA* in the wild-type by manipulating growth conditions revealed that mycelial autolysis could be induced by thiostrepton in 4-d-old cultures.

Keywords: *Streptomyces*, gene expression, morphological development, DNA supercoiling

INTRODUCTION

Streptomycetes are mycelial Gram-positive bacteria of particular interest for their complex differentiating morphology and ability to produce a wide variety of bioactive compounds including antibiotics, immunomodulators, enzyme inhibitors and hydrolytic enzymes. Understanding the various mechanisms that contribute to the regulation of both morphological and physiological differentiation has obvious importance. An example is the potential for both overproduction and synthesis of novel antibiotics via genetic manipulation (Hutchinson, 1994). Moreover, the ability to manipulate gene expression has been invaluable in exploiting *Streptomyces lividans* as a host for secretion of heterologous recombinant proteins into the culture broth (Binnie *et al.*, 1997; Pozidis *et al.*, 2001).

One of the most widely used promoters used to drive inducible overexpression of cloned genes in *Streptomyces* is the *tipA* promoter, *ptipA* (Takano *et al.*, 1995; Yu & Hopwood, 1995; Enguita *et al.*, 1996). In its normal context in *S. lividans*, this promoter controls the synthesis of an mRNA transcript that itself encodes two different proteins: the 31 kDa TipAL and the 17 kDa TipAS (Murakami *et al.*, 1989; Holmes *et al.*, 1993). The former is an autogenously regulated transcriptional activator that specifically binds thiostrepton, a cyclic peptide produced as a secondary metabolite by diverse bacteria including *Streptomyces* (*Streptomyces azureus*), *Bacillus* and *Micrococcus*. This compound has two distinct activities. It is best known as an inhibitor of the prokaryotic ribosome (Cundliffe, 1971) and thus has antibiotic activity typically at concentrations of $>10^{-7}$ M. However, even at extraordinarily low sub-inhibitory concentrations ($<10^{-9}$ M), thiostrepton can induce expression of the *tipA* gene of *S. lividans*. It does so by covalent binding to TipAL, thereby increasing the affinity for binding of the transcriptional activator to regions of dyad symmetry contained within *ptipA* (Chiu *et al.*, 1996, 1999). To avoid runaway *tipA* expression,

[†] **Present address:** Division of Gastroenterology, University Hospital, Nottingham NG7 2UH, UK.

Abbreviations: NE, nutrient extract; RNAP, RNA polymerase; TPE, Tris-phosphate-EDTA buffer.

activation is modulated by the smaller TipAS protein that shares the same C-terminal thiostrepton-binding domain as TipAL. The alternative translational initiation site for TipAS permits synthesis of this protein in vast molar excess to TipAL, and hence TipAS can inhibit TipAL-mediated transcription by sequestering thiostrepton.

TipAL belongs to a family of transcriptional activators characterized by a conserved N-terminal motif that interacts with promoter sequences, coupled to various C-terminal domains that are specific for diverse substrates including Hg(II) (MerR; Helmann *et al.*, 1990; Ansari *et al.*, 1992), genistein (NolA; Sadowsky, 1991), rhodamine 6G and tetraphenylphosphonium chloride (BmrR and BltR, respectively; Ahmed *et al.*, 1994) or an unknown mediator of the superoxide response (SoxR; Gort & Imlay, 1998). Of particular note are some unusual features of transcriptional activation shared by TipAL and MerR. The latter regulates the *merT* and *merR* mercury resistance operons of a wide variety of eubacteria (Summers, 1992). Between the -10 and -35 hexamer motifs of promoters controlled by TipAL and MerR are found exceptionally long, 19 bp (rather than 17 bp) spacers containing inverted repeat sequences. These proteins exist in solution as dimers and bind to the regions of dyad symmetry. Both bind in the presence or absence of inducer and activate transcription in combination with the ligand. This is unusual with respect to other prokaryotic transcriptional activators that interact with sequences within or upstream of the -35 hexamer and do not have an inactive promoter-bound form (Collado-Vides *et al.*, 1991).

MerR facilitates binding of RNA polymerase (RNAP) *in vivo* to allow formation of a transcriptionally repressed RNAP-MerR promoter complex primed for rapid induction (Heltzel *et al.*, 1990). *In vitro* experiments have shown that upon exposure to Hg(II), MerR-Hg(II) facilitates a topological transition of the promoter to its active form (Ansari *et al.*, 1992; Parkhill *et al.*, 1993). In the *merT* and *tipA* promoters, the 19 bp spacing puts the -35 and -10 hexamers out of phase with respect to the RNAP amino acid residues with which they must interact. MerR, upon binding Hg(II), induces a 33° local underwinding of the promoter spacer region and thus allows functional alignment (Ansari *et al.*, 1992). Given that untwisting of the promoter is the mechanism of activation, global alterations of supercoiling can also be expected to influence expression. Indeed, increased negative supercoiling *in vitro* can alter DNA pitch and increase the helix torsional energy, permitting expression driven by promoters containing 19 bp spacing (Aoyama & Takanami, 1988). Negative supercoiling can also be increased *in vivo*, induced by low concentrations of coumermycin which serve to increase gyrase expression (Franco & Drlica, 1989). Using these growth conditions, an increase both in MerR-Hg(II) activation and MerR-Hg(II)-independent expression was measured, resulting from supercoiling-dependent reorientation of the *pmerT* -35 and -10 hexamers (Condee & Summers, 1992).

There are also some fundamental differences between the MerR and TipAL regulatory proteins. MerR acts as a repressor and binds to its recognition site in *pmerT* less tightly in the presence of Hg(II) (O'Halloran *et al.*, 1989). Addition of the ligand both destabilizes the MerR-DNA complex, relaxing a MerR-induced DNA bend (Ansari *et al.*, 1995), and converts MerR from a repressor into an activator. In contrast, at least *in vitro* with a non-supercoiled substrate, TipAL can bind to its target site and activate transcription whether thiostrepton is present or not (Holmes *et al.*, 1993). Its affinity for the site is increased by an order of magnitude when the ligand is present. Here we investigate the kinetics of activation of *ptipA* *in vivo* and show that elevated external osmolarity increases intracellular negative DNA supercoiling and thereby enhances *ptipA* expression. A functional consequence of the increased expression of the *tip* operon at high medium osmolarity is the induction of mycelial autolysis.

METHODS

Bacterial strains and culture conditions. Wild-type *Streptomyces lividans* strain 1326 (SLP2⁺ SLP3⁺) and the plasmid-free derivative TK64 (*pro-2 str-6* SLP2⁻ SLP3⁻) (Hopwood *et al.*, 1983) were originally obtained from the John Innes Culture Collection (Norwich, UK). The *tipA* null mutant strain KT (*tipA::hyg*) (Chiu *et al.*, 1999) was provided by C. J. Thompson, University of Basel, Switzerland. *Escherichia coli* JM109 [*recA1 supE44 endA1 hsdR17 gurA96 relA1 thi Δ(lac-proAB)* F' (*traD36 proAB⁺ lac^q lacZ ΔM15*)] (Yanisch-Perron *et al.*, 1985) was used as a host for plasmid constructions. *E. coli* ET12567(pUB307) (*dam-13::Tn9 dcm-6 hsdM*) was employed as the plasmid donor strain for intergeneric conjugation with *S. lividans* as described by Flett *et al.* (1997). Media, culture conditions and DNA manipulations for *E. coli* were carried out as described by Sambrook *et al.* (1989). Streptomycetes were transformed by the method of Thompson *et al.* (1982). To obtain reproducible results it was necessary to work with newly obtained transformants containing pAK114 (Murakami *et al.*, 1989); older cultures tested after storage of spores at -20°C tended to exhibit heterogeneity with regard to the behaviour of the *ptipA-aphII* fusion. Mannitol soya flour solid medium (Hobbs *et al.*, 1989) was used for raising streptomycete spores that were harvested and purified as described by Kieser *et al.* (2000). Supplemented minimal medium (SMM) and the conditions used to allow reproducible exponential growth and measurement of ODs, were developed by Strauch *et al.* (1991). In addition, for semi-quantitative growth studies and plasmid isolation, a tryptic soy complete medium (TSB) was used, containing (per litre) 30 g tryptic soy broth, 10 g yeast extract and 5 mM MgCl₂. The osmolarity of both SMM and TSB was adjusted by adding an appropriate volume of either 3 M sucrose or 5 M NaCl solutions prepared and autoclaved separately to double strength SMM or TSB, together with an appropriate volume of H₂O. For TSB cultures, baffled flasks containing stainless steel springs and media were inoculated with a tenth volume of a 24 h pre-culture grown in TSB.

Luciferase assays. Light (485 nm), generated by strains harbouring *ptipA-lux* fusions, was monitored by using an Anthos Lucy 1 luminometer with 0.1 s integration. For each assay, 300 µl culture was combined with 1 µl n-decanal (Sigma) in the wells of black microtitre plates. Samples were incubated

for 2 min at ambient temperature before taking readings. Measurements were made from triplicate samples obtained from three independent cultures.

Plate assays for *ptipA* induction. Assay plates contained nutrient extract (NE) agar containing, per litre, 10 g glucose, 2 g yeast extract, 2 g meat extract, 2 g Casamino acids, adjusted to pH 7.0 with KOH (Murakami *et al.*, 1989). Spores (approx. 10^8) of *S. lividans* strains were suspended in 3 ml soft agar (NE agar diluted 1:2 in H₂O) and spread over the surface of a plate. Plates used to test expression of *aphII* contained 15 µg kanamycin ml⁻¹. Thiostrepton (Sigma), dissolved in dimethyl sulphoxide, was applied to the surface of the top agar on discs. Plates were incubated at 30 °C and growth scored at 48 h, and subsequently at 96 h to monitor autolysis.

Plasmid constructions. To initially test the behaviour of the *ptipA-luxAB* fusion, it was engineered into an autonomously replicating shuttle vector, pND6a. This was constructed by fusing two plasmids at their unique *Bam*HI sites to create pND5a:pTipK, a derivative of pBGS19 (Spratt *et al.*, 1986) containing *ptipA* subcloned from pAK113 (Murakami *et al.*, 1989) on a *Sac*I-*Hind*III fragment, and pND4, a pUC18 (Yanisch-Perron *et al.*, 1985) derivative containing the *luxAB* cassette subcloned on a *Hind*III-*Sac*I fragment from M13-1201 (K. Chater, unpublished). pND6a was then made by fusing pND5a and pSIT12 at their respective *Sac*I sites, the latter plasmid being derived from pGM9 (Muth *et al.*, 1989) by replacing a 160 bp *Eco*RI-*Bam*HI fragment with a 3.5 kb fragment of pJD19 (Dyson & Evans, 1996) cut with the same enzymes. To subsequently introduce the *ptipA-luxAB* fusion into the Φ C31 *att* site of the chromosome, a derivative of the conjugative integrating plasmid pIJ8660 (Sun *et al.*, 1999) was constructed by replacing the *egfp* gene flanked by *Kpn*I and *Not*I sites with a 2975 bp *Kpn*I-*Eag*I fragment from pND6a. In the resulting plasmid, pAL122, the *ptipA-luxAB* fusion is flanked by *to* and *tfd* transcriptional terminators. A copy of the *tsr* gene was then subcloned as a *Kpn*I-*Xba*I fragment from pND6a into the adjacent *Kpn*I and *Xba*I sites of pAL122 to give the final 10.7 kb plasmid pNA1. The *tsr* gene was introduced into streptomycete strains analysed for *tipA*-dependent autolysis using a version of the conjugative integrating plasmid pIJ8600 (Sun *et al.*, 1999). The plasmid used, pME25, was constructed by deleting the approximately 900 bp *Kpn*I fragment of pIJ8600, thereby removing *ptipA*. Both pNA1 and pME25 were introduced into *S. lividans* strains by intergeneric conjugation, selecting for resistance to apramycin.

Separation of topoisomers. Supercoiled plasmid DNA was isolated from TSB cultures supplemented with thiostrepton (25 µg ml⁻¹) using an alkaline lysis technique described by Kieser *et al.* (2000). For each growth condition under analysis, the isolation was performed from three independent cultures. Plasmid supercoils were further enriched using Wizard plasmid purification columns according to the manufacturer's instructions (Promega). Agarose gels (1%, w/v) were made up in 1× TPE (45 mM Tris-phosphate and 0.87 mM EDTA, pH 7.2) and contained 5 µg chloroquine ml⁻¹. The 1× TPE running buffer contained, in addition, 100 µM thiourea. Supercoiled plasmid DNA was electrophoresed in the dark for 30 h at 1.5 V cm⁻¹ while the buffer was being recirculated to maintain a temperature of 14 °C. After electrophoresis, gels were rinsed in H₂O and then stained for 1 h in 1 µg ethidium bromide ml⁻¹, followed by destaining for 30 min in H₂O. Gel images were captured with a gel documentation system and densitometry performed on each lane using Quantity One software (Bio-Rad).

RESULTS

Behaviour of the *ptipA-lux* fusions

A *ptipA-luxAB* fusion was integrated into the chromosome of *S. lividans* 1326. Submerged cultures were grown in a supplemented minimal medium used for physiological studies (Strauch *et al.*, 1991), permitting accurate measurement of both cell densities and the effects of medium additions. Under non-induced conditions, the luciferase fusions had readily detectable activity, particularly during the early phase of growth (Fig. 1). Addition of 10^{-5} M thiostrepton at different time points indicated that the level of induction was very much dependent on growth phase (Fig. 1), with maximal induced/uninduced ratios of over 15 being observed during exponential phase.

When the fusion was introduced into a *tipA* null mutant, *S. lividans* KT (Chiu *et al.*, 1999), the luciferase activity detected in both the presence and absence of thiostrepton was barely above background levels (<0.004 luminometer counts per OD₄₅₀ unit). Comparison of the uninduced levels of expression in *S. lividans* KT and the wild-type, with up to 1.4 luminometer counts per OD₄₅₀ unit, indicated that constitutive activity of the promoter in the latter is dependent on TipAL. This confirms the conclusion from *in vitro* data that, even in the absence of thiostrepton, TipAL can activate expression (Holmes *et al.*, 1993).

Luciferase expression was also measured with respect to the concentration of thiostrepton added to the wild-type at the beginning of the growth phase. For a range of concentrations between 10^{-10} and 10^{-4} M, greatest induction was again observed during exponential phase, with maximal induction obtained with a 10^{-5} M concentration (results not shown). Unless stated otherwise, a 10^{-5} M concentration was used in all subsequent experiments requiring addition of the inducer, when it was added at the beginning of the growth phase.

Response of *ptipA* to changes in osmolarity

In other non-mycelial bacteria, an increase in extracellular osmolarity can affect the activity of promoters influenced by DNA supercoiling (Higgins *et al.*, 1988; Hsieh *et al.*, 1991; Sheehan *et al.*, 1992). We found that constitutive activity of the *ptipA-lux* fusion in wild-type *S. lividans* increased in relation to increasing the osmolarity of supplemented minimal medium by addition of sucrose (Fig. 2). Growth rate itself was not affected by increases in osmolarity of up to 0.7 M sucrose. At mid-exponential phase, for sucrose concentrations between 0.3 M and 0.7 M, the promoter activity was up to five times greater than for cultures grown in the absence of sucrose. A measurable increase in thiostrepton-independent expression due to the increase in osmolarity extended into stationary phase. A higher medium osmolarity (0.9 M sucrose) retarded growth, but resulted in a maximum tenfold increase in non-induced activity of the promoter during exponential

Fig. 1. Thiostrepton induction of a chromosomally integrated *ptipA-luxA* gene fusion. Thiostrepton was added to independent cultures grown in supplemented minimal medium at four different time points indicated by the differently shaded arrows beneath the x axis. Luciferase expression was quantified in relation to the biomass at each time point and the values for each culture are represented by bars whose shading corresponds with that of the relevant arrow indicating time of addition of thiostrepton. Unshaded bars represent luciferase expression in the absence of inducer. The results plotted are mean values derived from three independent cultures; standard deviations were $\leq 15\%$ of these values.

Fig. 2. Effect of sucrose on constitutive and induced *ptipA* activity. Luciferase activity was quantified as in Fig. 1 for induced (white symbols) and non-induced (black symbols) cultures grown in supplemented minimal medium in the presence of various sucrose concentrations: circles, 0.7 M; squares, 0.3 M; triangles, 0 M.

phase (data not shown). Increased medium osmolarity with the same range of sucrose concentrations served to both increase the level of thiostrepton induction to more than double, and also extend the period of induction well into stationary phase (Fig. 2). Parallel semi-quantitative assays in the complete medium used subsequently in DNA supercoiling assays showed similar effects of increasing osmolarity (results not shown).

Iso-osmotic concentrations of NaCl were found to severely retard growth in submerged cultures grown in

Fig. 3. Effect of NaCl on constitutive and induced *ptipA* activity. Luciferase activity in 300 μ l aliquots of cultures was quantified at the time points indicated. Cultures were grown in complete medium with (white symbols) and without thiostrepton (black symbols) containing 0.0 M NaCl (triangles) or 0.5 M NaCl (squares).

supplemented minimal medium. On the other hand, increasing medium osmolarity with just 0.1 M NaCl resulted in a small but reproducible increase in constitutive activity of the *ptipA-lux* fusion, although there were no significant effects on thiostrepton induction (data not shown). Growth was largely unaffected in complete medium supplemented with 0.5 M NaCl, which resulted in a large increase in thiostrepton-independent expression of *luxAB*. Moreover, addition of 0.5 M NaCl also increased and extended the period of Tsr-induction well into stationary phase (Fig. 3).

Table 1. Effect of medium supplements on expression of the *ptipA-aphII* gene fusion

Strain	Growth medium supplement*	Maximum [Km] permitting confluent growth ($\mu\text{g ml}^{-1}$)†
1326	No addition	2.5
1326	1.0 M sucrose	2.5
1326	0.6 M NaCl	5
1326(pAK114)	No addition	5
1326(pAK114)	1.0 M sucrose	100
1326(pAK114)	0.6 M NaCl	100
1326(pAK114)	25 $\mu\text{g Tsr ml}^{-1}$	200

* Supplements were added to NE agar; Tsr, thiostrepton.

† Km, kanamycin.

The effects of changing medium osmolarity were also investigated with a qualitative assay of *ptipA* activity involving growth on solid media (NE agar) supplemented with either sucrose or NaCl. In this case, a plasmid-borne copy of the promoter controlled expression of a kanamycin-resistance gene (Murakami *et al.*, 1989). The level of kanamycin resistance is proportional to the expression of the *aphII* gene (Ward *et al.*, 1986). The results indicated a significant increase in expression of *aphII* as a consequence of growth in the presence of external added osmolyte (Table 1). With assays in which thiostrepton was applied to discs, the diameter of the zone of kanamycin ($50 \mu\text{g ml}^{-1}$)-resistant growth after 24 h incubation increased from 35 mm on standard media to 40 mm on medium containing 0.72 M sucrose and 45 mm on medium containing 0.4 M NaCl.

External osmolarity and the growth cycle affect DNA supercoiling

To examine if the effects of increased osmolarity on gene expression could be attributed to changes in DNA supercoiling, we used the plasmid pAK114 to monitor DNA topology. Electrophoresis of supercoiled plasmid DNA in chloroquine-agarose gels separates topoisomers differing by one linking number. This indicated that addition of osmolyte to the growth medium increased global negative supercoiling as revealed in plasmid isolated from cultures harvested at mid-exponential phase (Fig. 4, lanes 1–3). Moreover, we noted a decrease in negative superhelicity in older cultures. The most abundant topoisomer in plasmid isolated from stationary phase cultures contained a reduction in negative supercoiling of up to two linking numbers, but this decrease was offset by supplementing the growth media with either 0.72 M sucrose or 0.5 M NaCl (data not shown).

Inefficient *ptipA* induction in *S. lividans* strain TK64 is associated with relaxed control of DNA supercoiling

The *ptipA-luxAB* fusion was introduced into the plasmid-free *S. lividans* strain TK64. Despite possessing an intact copy of the *tip* operon as judged by Southern hybridization (results not shown), we could detect little or no luciferase activity in cultures grown in minimal medium even with addition of the inducer thiostrepton. The promoter was induced, however, by a combination of increasing the external osmolarity and addition of thiostrepton. In this case, a maximum of 10.6 lumino-meter counts per OD_{450} unit was measured in exponential phase, equivalent to an induced/uninduced ratio of 35. Similar effects were observed with TK64 transformed with pAK114. With the disc assay, after 48 h incubation on standard medium, a number of discrete colonies grew in the proximity of the disc, rather than a confluent zone observed with 1326(pAK114). With plates containing either sucrose or NaCl, confluent zones of kanamycin-resistant growth were induced by thiostrepton (Fig. 5). Plasmid pAK114 DNA was isolated from mid-exponential cultures grown in standard medium and medium adjusted by addition of either sucrose or NaCl. Comparison of the superhelical densities of plasmid populations revealed that, under standard growth conditions, the most abundant plasmid topoisomer from TK64 was less highly supercoiled than that from the wild-type (Fig. 4). Moreover, a greater range of topoisomers was evident, with the distribution varying from a typical Gaussian one. The changes in superhelical densities as a result of adjusting medium osmolarity were more dramatic in TK64 than in the wild-type, with a greater shift towards increased negative superhelicity, especially in the case of plasmid isolated from cultures grown in 0.5 M NaCl.

Thiostrepton-induced mycelial autolysis

After 5 d incubation of disc assay plates of wild-type *S. lividans* carrying pAK114 grown on NE media supplemented with 0.72 M sucrose, a marked change in the appearance of the zones of kanamycin-resistant growth was noted. A 12 mm diameter zone of autolysis occurred around the thiostrepton-impregnated disc. To investigate this further, and to rule out any possible involvement of kanamycin present in the assay plates, we tested derivatives of both the wild-type and the *tipA* null mutant strain, KT, containing a copy of the *tsr* gene integrated at their respective $\Phi\text{C31 att}$ sites. After 4 d incubation, zones of autolysis formed around the discs placed on lawns of the *tip*⁺ strain grown in the presence of sucrose. Smaller and less distinct zones formed on medium supplemented with 0.5 M NaCl. No autolysis zones were detected surrounding the discs on standard medium. The *tipA* null mutant strain KT failed to produce zones of autolysis when grown in conditions of either low or high medium osmolarity, even after 14 d of extended incubation. For standard assays, 25 μg thiostrepton was added to each disc. By reducing this to non-selective amounts, the wild-type and KT strains could be

Fig. 4. The effect of osmolarity on *in vivo* plasmid supercoiling. (a) Plasmid pAK114 DNA was isolated from strains grown in complete medium with osmolyte addition. Topoisomers were resolved by electrophoresis in 1% agarose gels containing $5 \mu\text{g}$ chloroquine ml^{-1} . Under these conditions more negatively supercoiled topoisomers migrate faster. Topoisomer distribution of plasmid DNA isolated from 1326 and TK64 strains, respectively, when grown in complete

Fig. 5. Thiostrepton disc assays with strain TK64(pAK114). Discs impregnated with 25 µg thiostrepton were placed on the surface of kanamycin-containing plates (NE agar) seeded with equal amounts of spores of strain TK64 containing pAK114. The growth medium contained: (a) no added osmolyte; (b) 0.72 M sucrose; (c) 0.5 M NaCl.

tested directly. The autolysis response could be manipulated by adjusting the concentration of sucrose: with addition of 0.36 M sucrose, as little as 50 ng thiostrepton was found to promote autolysis in the wild-type (Fig. 6).

Fig. 6. Thiostrepton induction of autolysis. Discs with the indicated number of nanograms of thiostrepton were placed on the surface of NE agar plates seeded with wild-type (a, b) or the *tipA* null mutant KT (c). The growth medium contained 0.36 M sucrose (a, c) or no added osmolyte (b). After 24 h there was confluent growth on all plates. The zones of autolysis observed in (a) appeared after 4 d incubation.

DISCUSSION

Streptomycetes have evolved as ecological generalists, capable of growth in widely fluctuating environments in the soil and marine sediments. Investigations into their physiology need to account for this and, as we demonstrate here, reproducing conditions of soil water depletion by increasing external osmolarity can reveal unexpected aspects of gene function. To date, studies on thiostrepton induction of gene expression in *S. lividans* have focused on growth in complete liquid medium containing 1 M sucrose (although plate assays of *tipA* promoter activity used medium lacking additional

medium containing: lanes 1 and 4, no added osmolyte; lanes 2 and 5, 0.7 M sucrose; lanes 3 and 6, 0.5 M NaCl. (b) Quantitation of the intensities of the bands corresponding to individual topoisomers in each lane. The most abundant topoisomer in each lane, with linking number Lk_m , is indicated by the arrow. The change in Lk_m , ΔLk , is indicated for each lane taking the Lk_m of pAK114 from 1326 grown without additional osmolyte as the reference point. ΔLk values in parentheses represent changes in Lk_m of TK64 samples isolated from cultures grown with either additional sucrose or NaCl with respect to the Lk_m of plasmid from non-amended medium. The results illustrated were representative of analysis of plasmid DNA isolated from three independently grown cultures.

osmolyte), revealing that at least eight proteins are synthesized in response to addition of the antibiotic (Chiu *et al.*, 1999). Analysis of only one of these proteins, TipAL, has given an insight into the response. Indeed, this transcriptional activator is responsible for overall control of a thiostrepton-inducible regulon, although *tipA*-independent thiostrepton responses are also observed (Chiu *et al.*, 1999).

The *tipA* promoter shares structural similarity with *pmerT* from *E. coli*, as do their respective transcriptional activators. By studying the kinetics of induction in defined conditions, we show that this similarity extends to the sensitivity of activation of both promoters to changes in DNA supercoiling. The thiostrepton-independent level of activity of *ptipA* *in vivo*, which is dependent on TipAL function, can be elevated tenfold by increased external osmolarity. Moreover, with addition of osmolyte, thiostrepton induction is doubled and extended over a far greater period of the growth cycle. As these changes in gene expression are correlated with an increased level of negative DNA supercoiling, it suggests that, as for *pmerT*, the effects could result from reorientation of the *ptipA* -35 and -10 hexamers allowing functional alignment with RNAP. However, in contrast to the case for *pmerT*, experimental reduction of the 19 bp spacer of *ptipA* to 17 bp inactivates the promoter rather than increasing constitutive activity (C. Thompson, personal communication). A further difference in the two inducible systems is that the inducing ligand complexed to the transcriptional activator serves to increase RNAP affinity for the promoter in the case of *ptipA*, but activates transcription from a RNAP-promoter complex preformed at *pmerT*. Thus the difference in the responses observed due to changes in DNA conformation may be a consequence of distinctive protein-DNA interactions at the two promoters.

The sensitivity of the promoter to global changes in supercoiling is also revealed in strain TK64. This is one of a handful of *S. lividans* strains, belonging to a common genealogy, in which poor thiostrepton induction of *ptipA* has previously been noted (Kieser *et al.*, 2000). The luciferase assays revealed no induction of the chromosomally integrated gene fusion by thiostrepton alone, whereas the results of disc assays suggested that, for a plasmid-borne fusion, a subpopulation of plated spores could be induced. We have subsequently retested colonies growing in the proximity of the thiostrepton discs and shown that they can give confluent induction zones. For the plasmid-borne *ptipA-aphII* gene fusion, the presence of an induction-competent subpopulation may contribute to the observed atypical distribution of topoisomers. The inability of TipAL or a TipAL-thiostrepton complex to activate the chromosomal promoter is presumably due to the lower overall level of negative DNA supercoiling in TK64, implying that a mean threshold level is necessary to untwist the promoter. This threshold is attained under conditions of increased external osmolarity that result in large incremental increases in negative supercoiling. These large shifts in supercoiling suggest that TK64 is unable to

maintain as tight homeostatic control on DNA supercoiling as the wild-type. The exact nature of the mutation causing this relaxed control is as yet unknown but has presumably been acquired early on in the derivation of the TK strains.

Analysis of changes in protein expression patterns due to thiostrepton induction previously revealed noticeably different, but biologically undefined, responses in stationary phase as compared to exponential submerged cultures (Chiu *et al.*, 1999). Here we reveal that surface-grown cultures, in which *tipA* is induced by a combination of thiostrepton and high osmolarity, undergo mycelial autolysis following vegetative growth. In the absence of osmolyte or in a *tipA* mutant background, no autolysis was observed, suggesting that it is a consequence of prolonged induction of *tipA* due to increased negative DNA supercoiling. All the assays reported here were conducted using NE agar medium on which sporulation of the wild-type is poor. To obtain a clearer understanding of how this autolysis is related to development, we are currently undertaking microscopic investigations of mycelia grown on a variety of other media. Programmed autolysis of substrate mycelia is a normal event in morphological development of a streptomycete colony (Miguélez *et al.*, 1999). Recycled macromolecules are utilized to fuel upward growth of the aerial mycelia. Although the control of this process is largely unresearched, it can be expected to be under tight repression. It is tempting to speculate that an active thiopeptide such as thiostrepton is not only an antibiotic but may serve as, or perhaps mimic, a physiological or developmental signal that triggers cell death. The low thiostrepton concentration required to induce the response supports this possibility. In the habitat of a drying soil, this signal coupled with an increase in osmolarity could induce lysis of the vegetative mycelia to fuel development of the spores that can survive periods of desiccation. The timing of *tipA*-dependent autolysis is clearly developmentally linked but it may also be a response to the acquisition of cell defects. It has been suggested that thiostrepton can reduce levels of intracellular thiols responsible for maintaining the cytoplasmic redox potential (Chiu *et al.*, 1999). This can affect protein folding, the scavenging of oxygen radicals, the metabolic activity of redox-dependent enzymes, and levels of NADPH and NADH pools. Cell death may be the ultimate stress response to rid a colony of defective mycelia.

In conclusion, the *tipA* promoter is clearly responsive to changes in external osmolarity. When the promoter is used to experimentally regulate expression of cloned genes in *S. lividans*, it is important to appreciate that basal level expression is already increased in the presence of levels of sucrose typically included in media used to regenerate protoplasts after transformation or for growth of submerged cultures. Likewise, when high induced expression is required over an extended period of the life cycle, addition of osmolyte is necessary, although it should be noted that the resulting phenotype may be influenced by upregulation of *tipA* itself.

ACKNOWLEDGEMENTS

We thank Anna Schnitger, Raul Gonzalez and Masum Ahmed for their contributions in measuring promoter activity. N.A. was supported by a BBSRC studentship. We are grateful to Charles Thompson for helpful comments on the manuscript.

REFERENCES

- Ahmed, M., Borsch, C. M., Taylor, S. S., Vazquezlaslop, N. & Neyfakh, A. A. (1994). A protein that activates expression of a multidrug efflux transporter upon binding the transporter substrates. *J Biol Chem* **269**, 28506–28513.
- Ansari, A. Z., Chael, M. L. & O'Halloran, T. V. (1992). Allosteric underwinding of DNA is a critical step in positive control of transcription by Hg-MerR. *Nature* **355**, 87–89.
- Ansari, A. Z., Bradner, J. E. & O'Halloran, T. V. (1995). DNA-bend modulation in a repressor-to-activator switching mechanism. *Nature* **374**, 371–375.
- Aoyama, T. & Takanami, M. (1988). Supercoiling response of *Escherichia coli* promoters with different spacer lengths. *Biochim Biophys Acta* **949**, 311–317.
- Binnie, C., Jenish, D., Cossar, D., Szabo, A., Trudeau, D., Krygsmann, P., Malek, L. T. & Stewart, D. I. H. (1997). Expression and characterization of soluble human erythropoietin receptor made in *Streptomyces lividans* 66. *Protein Express Purif* **11**, 271–278.
- Chiu, M. L., Folcher, M., Griffin, P., Holt, T., Klatt, T. & Thompson, C. J. (1996). Characterization of the covalent binding of thiostrepton to a thiostrepton-induced protein from *Streptomyces lividans*. *Biochemistry* **35**, 2332–2341.
- Chiu, M. L., Folcher, M., Katoh, T., Puglia, A. M., Vohradsky, J., Yun, B. S., Seto, H. & Thompson, C. J. (1999). Broad spectrum thiopeptide recognition specificity of the *Streptomyces lividans* TipAL protein and its role in regulating gene expression. *J Biol Chem* **274**, 20578–20586.
- Collado-Vides, J., Magasanik, B. & Gralla, J. D. (1991). Control site location and transcriptional regulation in *Escherichia coli*. *Microbiol Rev* **55**, 371–394.
- Condee, C. W. & Summers, A. O. (1992). A *mer-lux* transcriptional fusion for real-time examination of *in vivo* gene-expression kinetics and promoter response to altered superhelicity. *J Bacteriol* **174**, 8094–8101.
- Cundliffe, E. (1971). The mode of action of thiostrepton *in vivo*. *Biochem Biophys Res Commun* **44**, 912–917.
- Dyson, P. J. & Evans, M. (1996). pUCS75, a stable high-copy-number *Streptomyces-Escherichia coli* shuttle vector which facilitates subcloning from pUC plasmid and M13 phage vectors. *Gene* **171**, 71–73.
- Enguita, F. J., de la Fuente, J. L., Martin, J. F. & Liras, P. (1996). An inducible expression system of histidine-tagged proteins in *Streptomyces lividans* for one-step purification by Ni²⁺ affinity chromatography. *FEMS Microbiol Lett* **137**, 135–140.
- Flett, F., Mersinias, V. & Smith, C. P. (1997). High efficiency intergeneric conjugal transfer of plasmid DNA from *Escherichia coli* to methyl DNA-restricting streptomycetes. *FEMS Microbiol Lett* **155**, 223–229.
- Franco, R. J. & Drlica, K. (1989). Gyrase inhibitors can increase *gyrA* expression and DNA supercoiling. *J Bacteriol* **171**, 6573–6579.
- Gort, A. S. & Imlay, J. A. (1998). Balance between endogenous superoxide stress and antioxidant defenses. *J Bacteriol* **180**, 1402–1410.
- Helmann, J. D., Ballard, B. T. & Walsh, C. T. (1990). The MerR metalloregulatory protein binds mercuric ion as a tricoordinate, metal-bridged dimer. *Science* **247**, 946–948.
- Heltzel, A., Lee, I. W., Totis, P. A. & Summers, A. O. (1990). Activator-dependent preinduction binding of sigma-70 RNA polymerase at the metal-regulated *mer* promoter. *Biochemistry* **29**, 9572–9584.
- Higgins, C. F., Dorman, C. J., Stirling, D. A., Waddell, L., Booth, I. R., May, G. & Bremer, E. (1988). A physiological role for DNA supercoiling in the osmotic regulation of gene expression in *S. typhimurium* and *E. coli*. *Cell* **52**, 569–584.
- Hobbs, G., Frazer, C. M., Gardner, D. C. J., Cullum, J. A. & Oliver, S. G. (1989). Dispersed growth of *Streptomyces* in liquid culture. *Appl Microbiol Biotechnol* **31**, 272–277.
- Holmes, D. J., Caso, J. L. & Thompson, C. J. (1993). Autogenous transcriptional activation of a thiostrepton-induced gene in *Streptomyces lividans*. *EMBO J* **12**, 3183–3191.
- Hopwood, D. A., Kieser, T., Wright, H. M. & Bibb, M. J. (1983). Plasmids, recombination and chromosome mapping in *Streptomyces lividans* 66. *J Gen Microbiol* **129**, 2257–2269.
- Hsieh, L. S., Rouviere-Yaniv, J. & Drlica, K. (1991). Bacterial-DNA supercoiling and [ATP]/[ADP] ratio – changes associated with salt shock. *J Bacteriol* **173**, 3914–3917.
- Hutchinson, C. R. (1994). Drug synthesis by genetically engineered microorganisms. *Biotechnology* **12**, 375–380.
- Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F. & Hopwood, D. A. (2000). *Practical Streptomyces Genetics*. Norwich: John Innes Foundation.
- Miguélez, E. M., Hardisson, C. & Manzanal, M. B. (1999). Hyphal death during colony development in *Streptomyces antibioticus*: morphological evidence for the existence of a process of cell deletion in a multicellular prokaryote. *J Cell Biol* **145**, 515–525.
- Murakami, T., Holt, T. G. & Thompson, C. J. (1989). Thiostrepton-induced gene expression in *Streptomyces lividans*. *J Bacteriol* **171**, 1459–1466.
- Muth, G., Nussbaumer, B., Wohlleben, W. & Puhler, A. (1989). A vector system with temperature-sensitive replication for gene disruption and mutational cloning in streptomycetes. *Mol Gen Genet* **219**, 341–348.
- O'Halloran, T. V., Frantz, B., Shin, M. K., Ralston, D. M. & Wright, J. G. (1989). The MerR heavy-metal receptor mediates positive activation in a topologically novel transcription complex. *Cell* **56**, 119–129.
- Parkhill, J., Ansari, A. Z., Wright, J. G., Brown, N. L. & O'Halloran, T. V. (1993). Construction and characterization of a mercury-independent MerR activator [MerR(Ac)] – transcriptional activation in the absence of Hg(II) is accompanied by DNA distortion. *EMBO J* **12**, 413–421.
- Pozidis, C., Lammertyn, E., Politou, A. S., Anne, J., Tsiftoglou, A. S., Sianidis, G. & Economou, A. (2001). Protein secretion biotechnology using *Streptomyces lividans*: large-scale production of functional trimeric tumour necrosis factor alpha. *Biotechnol Bioeng* **72**, 611–619.
- Sadowsky, M. J., Cregan, P. B., Gottfert, M., Sharma, A., Gerhold, D., Rodriguez-Quinones, F., Keyser, H. H., Hennecke, H. & Stacey, G. (1991). The *Bradyrhizobium japonicum nola* gene and its involvement in the genotype-specific nodulation of soybeans. *Proc Natl Acad Sci USA* **88**, 637–641.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sheehan, B. J., Foster, T. J., Dorman, C. J., Park, S. & Stewart,

- G. S. A. B. (1992).** Osmotic and growth-phase dependent regulation of the *eta* gene of *Staphylococcus aureus* – a role for DNA supercoiling. *Mol Gen Genet* **232**, 49–57.
- Spratt, B. G., Hedge, P. J., Heesen, S. T., Edelman, A. & Broome-Smith, J. K. (1986).** Kanamycin-resistant vectors that are analogs of plasmids pUC8, pUC9, pEMBL8 and pEMBL9. *Gene* **41**, 337–342.
- Strauch, E., Takano, E., Baylis, H. A. & Bibb, M. J. (1991).** The stringent response in *Streptomyces coelicolor* A3(2). *Mol Microbiol* **5**, 289–298.
- Summers, A. O. (1992).** Untwist and shout – a heavy metal-responsive transcriptional regulator. *J Bacteriol* **174**, 3097–3101.
- Sun, J. H., Kelemen, G. H., Fernandez-Abalos, J. M. & Bibb, M. J. (1999).** Green fluorescent protein as a reporter for spatial and temporal gene expression in *Streptomyces coelicolor* A3(2). *Microbiology* **145**, 2221–2227.
- Takano, E., White, J., Thompson, C. J. & Bibb, M. J. (1995).** Construction of thiostrepton-inducible, high-copy-number expression vectors for use in *Streptomyces* spp. *Gene* **166**, 133–137.
- Thompson, C. J., Ward, J. M. & Hopwood, D. A. (1982).** Cloning of antibiotic-resistance and nutritional genes in streptomycetes. *J Bacteriol* **151**, 668–677.
- Ward, J. M., Jansen, G. R., Kieser, T., Bibb, M. J., Buttner, M. J. & Bibb, M. J. (1986).** Construction and characterisation of a series of multi-copy promoter-probe plasmid vectors for *Streptomyces* using the aminoglycoside phosphotransferase gene from Tn5 as indicator. *Mol Gen Genet* **203**, 468–478.
- Yanisch-Perron, C., Vieira, J. & Messing, J. (1985).** Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.
- Yu, T. W. & Hopwood, D. A. (1995).** Ectopic expression of the *Streptomyces coelicolor* *whiE* genes for polyketide spore pigment synthesis and their interaction with the *act* genes for actinorhodin biosynthesis. *Microbiology* **141**, 2779–2791.

Received 21 June 2001; revised 12 September 2001; accepted 17 September 2001.