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Autogenous transcriptional activation of a thiostrepton-induced gene in *Streptomyces lividans*

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Although the antibiotic thiostrepton is best known as an inhibitor of protein synthesis, it also, at extremely low concentrations ($<10^{-9}$ M), induces the expression of a regulon of unknown function in certain *Streptomyces* species. Here, we report the purification of a *Streptomyces lividans* thiostrepton-induced transcriptional activator protein, $TipA_L$, whose N-terminus is similar to a family of eubacterial regulatory proteins represented by MerR. $TipA_L$ was first purified from induced cultures of *S.lividans* as a factor which bound to and activated transcription from its own promoter. The *tipA*$_L$ gene was overexpressed in *Escherichia coli* and $TipA_L$ protein purified in a single step using a thiostrepton affinity column. Thiostrepton enhanced binding of $TipA_L$ to the promoter and catalysed specific transcription *in vitro*. $TipA_S$, a second gene product of the same open reading frame consisting of the C-terminal domain of $TipA_L$, is apparently translated using its own in-frame initiation site. Since it is produced in large molar excess relative to $TipA_L$ after induction and also binds thiostrepton, it may competitively modulate transcriptional activation.
Key words: autogenous regulation/*Streptomyces*/thiostrepton/transcription/translation

Introduction

Thiostrepton is a cyclic peptide produced as a secondary metabolite by *Streptomyces azureus* having at least two potent biological activities. It is most active as a diffusible inducer of gene expression in *Streptomyces lividans* 1326 (Murakami *et al.*, 1989) which is not known to produce thiostrepton-like compounds. The extraordinarily low non-lethal concentrations ($<10^{-9}$ M) at which thiostrepton induces transcription suggests that it might act as a bacterial signalling molecule (pheromone) to activate otherwise cryptic genes of unknown function. However, it is better known and characterized as an inhibitor of the prokaryotic ribosome which is responsible for its antibiotic activity (at concentrations of 2×10^{-7} M against *S.lividans*).

As a result of thiostrepton binding to the 50S ribosomal subunit ($K_D < 10^{-9}$ M) (Pestka *et al.*, 1976), aminoacyl-tRNA is prevented from entering the A-site and protein synthesis is irreversibly blocked (Cundliffe, 1971). Although it can be demonstrated *in vitro* that thiostrepton binds specifically to a region of base pairing extending for 58 nucleotides in the ribosomal RNA (Ryan *et al.*, 1991; Thompson and Cundliffe, 1991) but not to ribosomal proteins, both components are thought to play a role in forming a stable drug–ribosome complex. Resistance to the antibiotic in *S.azureus* is conferred by the action of a methylase which modifies a specific nucleotide within this sequence (Cundliffe, 1978; Thompson *et al.*, 1982a). The gene encoding the methylase, *tsr*, has been incorporated into most streptomycete cloning vectors including pIJ61 (Thompson *et al.*, 1982b) used in this study (Hopwood *et al.*, 1985) and is widely used as the primary selectable marker. Ribosomes isolated from *S.azureus* or *S.lividans* containing the cloned *tsr* gene do not detectably bind thiostrepton (Thompson *et al.*, 1982a). The fact that this secondary metabolite is a potent antibiotic and has a highly conserved region of the 23S rRNA as a target, reinforced the assumption that this was its only biological activity.

However, in light of the discovery that *S.lividans* 1326 responds to the drug by inducing synthesis of at least eight proteins (Murakami *et al.*, 1989; A.M.Puglia, J.Vohradsky and C.J.Thompson, unpublished), this view has had to be reconsidered. *Streptomyces lividans* 1326 is not known to contain thiostrepton resistance or biosynthetic genes. When one of these proteins was purified and the corresponding gene (*tipA*) was cloned and sequenced, it revealed no similarity to proteins in the databases. Transcription of the *tipA* gene was induced >200-fold by thiostrepton from a site located 345 bp upstream of the structural gene. Promoter probe experiments demonstrated that transcription could be induced from a 143 bp DNA fragment containing this initiation site by thiostrepton but not by structurally unrelated antibiotics.

Although these data suggested that induction involved a thiostrepton-specific receptor protein, they did not rule out a less specific response system. For example, at sublethal concentrations, a variety of antibiotics which act at the level of the ribosome can induce stress regulons such as the heat and cold shock genes in *Escherichia coli* (VanBogelen and Neidhardt, 1990). Alternatively, this hydrophobic peptide might also mimic some other general class of intracellular effectors such as denatured proteins, well known inducers of the heat shock stress response.

Here we describe experiments which identify a thiostrepton-specific recognition protein which is an autogenous transcriptional activator of the *tipA* gene.

Results

Purification of a protein required for thiostrepton-induced transcription

Gel retardation and run-off transcription assays (data not shown) were carried out using a fragment containing 143 bp of the *tipA* promoter region (*ptipA* fragment) and crude

extracts prepared from *S.lividans*(pIJ61) grown in the presence or absence of thiostrepton. Extracts prepared from induced, but not uninduced, *S.lividans* cultures were able to retard the migration of the fragment in acrylamide gels. *In vitro* run-off transcription assays using RNAP purified from *S.lividans* showed that extracts from induced cultures allowed the synthesis of a specific transcript whose size corresponded to transcription from *ptipA*; this activity was not found in uninduced cultures.

Subsequently, attempts were made to purify the thiostrepton-induced factor present in crude extracts of *S.lividans* which bound to *ptipA* and activated its transcription. Both RNAP and this factor co-purified after precipitation in PEI and extraction in 0.3 M $(NH_4)_2SO_4$. In order to address the question of whether this factor was part of the RNAP holoenzyme, material prepared in this way was applied to a heparin–Sepharose column and eluted in a gradient of NaCl. Fractions were assayed for their ability to retard the mobility of the *tipA* promoter fragment during electrophoresis, for RNAP activity, and for the ability to generate transcripts in the presence of *S.lividans* RNAP. A factor which retarded the *tipA* promoter fragment was separable from RNAP (Figure 1) and further purification of this factor, assayed by its ability to allow run-off transcription, was achieved by anion exchange (MonoQ), and finally by gel exclusion (Superose 12) chromatography. *In vitro* transcription experiments showed that the purified factor did not contain detectable RNAP activity (Figure 2, lane 1), but in the presence of purified RNAP, it strongly induced synthesis of a transcript (Figure 2, lane 2) not made by RNAP alone (Figure 2, lane 3). Transcriptional activation in these run-off assays (data not shown) was unaffected by the addition of a range of thiostrepton concentrations (10–150 μM; conditions defined in Materials and methods).

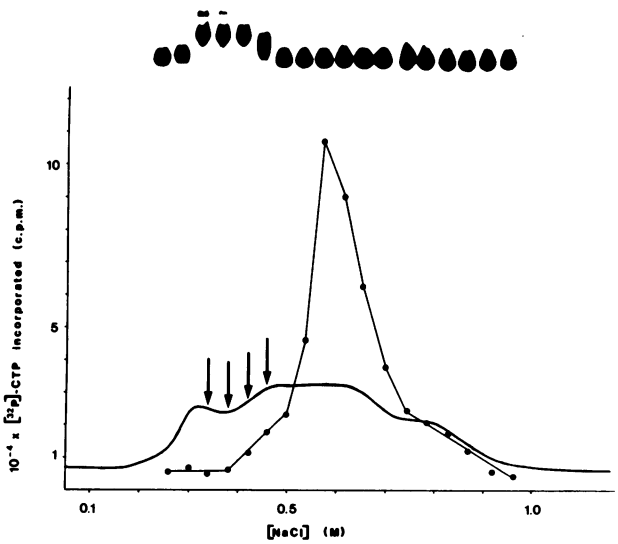

Fig. 1. Separation of a protein which binds the *tipA* promoter fragment from RNAP. Crude extracts prepared from *S.lividans* 1326(pIJ61) grown in the presence of thiostrepton (50 μg/ml) were applied to a heparin–Sepharose column. Proteins were eluted using a linear NaCl gradient and fractions were assayed for RNAP activity as well as their ability to retard the mobility of *tipA* promoter fragment (purified from pAK113 after *Hind*III–*Eco*RI digestion) during PAGE. The adsorbance at 280 nm (continuous line), RNAP activity (filled circles) and fractions containing retarding activity (downward arrows) are indicated. The mobility shift assay (see Materials and methods) for each fraction is shown above.

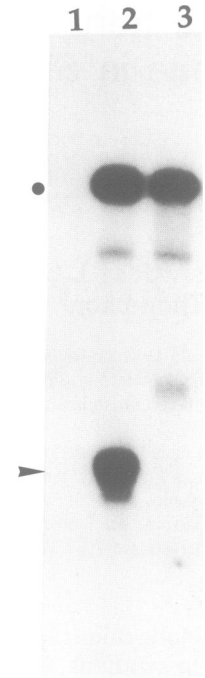

Fig. 2. *In vitro* transcription of the *tipA* promoter. Transcriptional run-off experiments in a reaction mixture (20 μl) which contained 0.3 pmol of the $ptipA_L$ fragment isolated from pAK113 digested with EcoRI and *Hind*III and 0.5 pmol each of RNAP and transcriptional activator protein ($TipA_L$) purified from *S.lividans* as well as buffer salts and nucleotides as defined in Materials and methods. Lane 1, transcriptional activator protein; lane 2, RNAP and transcriptional activator protein (see Figure 3); lane 3, RNAP. The arrow indicates the position of the $TipA_L$-specific 'run-off' transcript and the filled circle indicates the non-specific 'end to end' transcript.

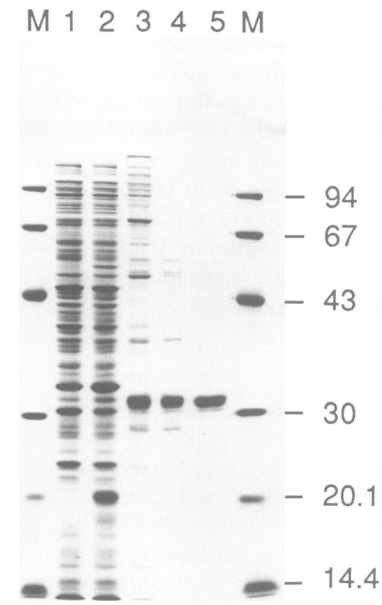

Fig. 3. Purification of a *ptipA* transcriptional activator protein from *S.lividans*. Samples representing progressive stages of the purification process were subjected to SDS–PAGE and stained with Coomassie Blue. Lane 1, crude extract of *S.lividans*(pIJ61) grown in the absence of thiostrepton; lane 2, crude extract of *S.lividans*(pIJ61) grown in the presence of thiostrepton; lanes 3, 4 and 5, samples obtained following passage of thiostrepton-induced extracts of *S.lividans*(pIJ61) through heparin–Sepharose, anion-exchange (Mono Q) and gel filtration (Superose 12) columns; lane M, molecular weight size markers.

Monitoring the steps of the purification by SDS−PAGE (Figure 3), showed that a 31 kDa protein was highly enriched by heparin−Sepharose and then further purified to >90% homogeneity in subsequent steps. During the Superose 12 gel exclusion chromotography step, the 31 kDa protein migrated with an apparent molecular weight of 62 kDa, suggesting that the native form of the protein is a dimer. The protein was finally isolated by two dimensional (2D) gel electrophoresis, electrophoretically transferred to an Immobilon membrane and its N-terminal sequence determined by Edman degradation (-YSVGQVAGF-AGVT-RTL).

The size of the *in vitro* transcript generated by this purified material indicated that its start site was indistinguishable from that predicted by *in vivo* transcript mapping (Murakami *et al.*, 1989). The direction of transcription, established using the template cleaved asymetrically, was the same as that found *in vivo*.

These experiments strongly implicated the 31 kDa protein purified from *S.lividans* as a transcriptional activator of *ptipA* and showed that its binding and transcriptional run-off activities were independent of the addition of thiostrepton to the reaction mix. However, since thiostrepton was needed to induce expression of this protein, it was considered likely that the drug was copurified (possibly as a ligand) with the protein.

Sequence analysis

Inspection of the *tipA* nucleotide sequence (Figure 4) immediately downstream of this transcriptional start site

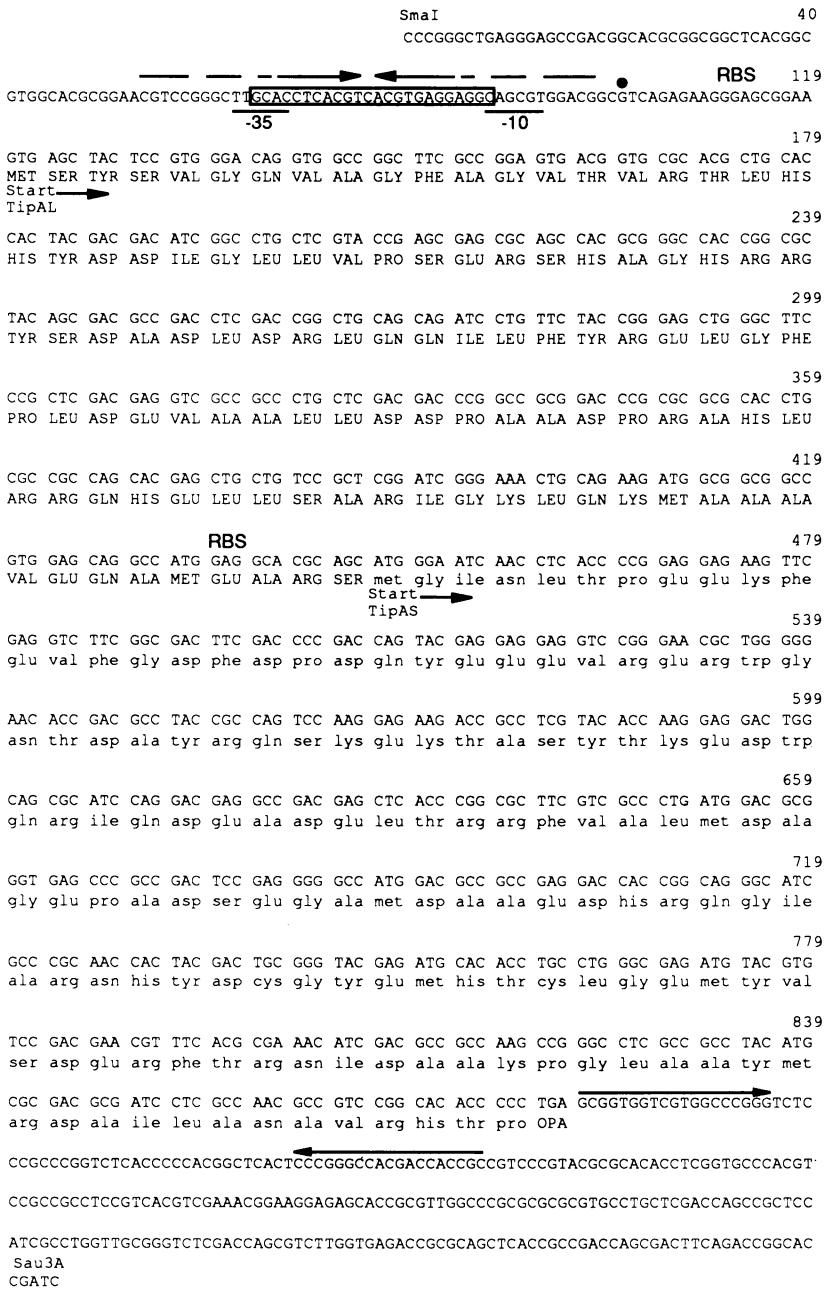

Fig. 4. Nucleotide sequence of *S.lividans* 1326 DNA containing the $tipA_L$–A_S gene complex. The transcription start point is indicated by a filled circle. As indicated in the figure, the $tipA_L$ translational start codon begins at position 120 and that for $tipA_S$ at position 446. The C-terminal amino acids, constituting $TipA_S$, are in lower case letters. Both gene products have been purified from *S.lividans* 1326 and their N-terminal sequences determined. Direct and inverted repeats before and after the coding sequences are shown by arrows. The boxed region corresponds to that protected from DNase digestion by $TipA_L$ (Figure 8). The presumed $tipA_L$ and $tipA_S$ ribosome binding sites are indicated by 'RBS'.

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                                <--------OOO--------->
TipAL.....MS--------Y-SVGQVAGFAGVTVRTLHHYDDIGLLVPSERSHAG-HRR-YSDADLDRLQQILFY-RELGFPLDEV
NolA.....MNRATPRRRRW-RIGELAEATGVTVRTLHHYEHTGLLAATERTE-GGH-RMYDRESGQRVHQIR-ALRELGFSLVEI
SoxR.....MEKKLPRIKALLTPGEVAKRSGVAVSALHFYESKGLI-TSIRNS-GNQRR-YKRDVLRYVAIIKIAQRI-GIPLATI
MerR.....---MENNLENL-TIGVFAKAAGVNVETIRFYQRKGLLLEPDKPY-GSIRR-YGEADVTRVRFVKSAQRL-GFSLDEI
Consensus..............G..A..*GV.V.*.*.Y...GL*....*...G..*R.Y.......*..*..*.R..G*.L.**
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Fig. 5. Alignment of $TipA_L$ (N-terminal 65 nucleotides) with MerR (Misra *et al.*, 1984), NolA (Sadowsky *et al.*, 1991) and SoxR (Amábile-Cuevas and Demple, 1991). The alignment was carried out using the Clustal V program (Higgins *et al.*, 1992) in which residues identical in all four proteins are indicated in the consensus sequence and residues similar in all four sequences are indicated by a ★. A block of 10 identical amino acids found within the NolA and $TipA_L$ helix−turn−helix regions (<-- OOO-->) is underlined.

revealed a ribosome binding site and an open reading frame (ORF) which encoded a protein having the N-terminal amino acid sequence of the *ptipA* transcriptional activator protein described above. In fact, the protein encoded by this ORF is an in-frame N-terminal extension of the previously described (Murakami *et al.*, 1989) TipA protein. These two overlapping genes will be referred to as $tipA_S$ (formerly *tipA*) and the extended ORF as $tipA_L$. While comparison of the $TipA_S$ sequence with the databases revealed no significant sequence similarity with proteins of known function, $TipA_L$ resembled MerR genes isolated from various Gram-negative as well as Gram-positive organisms, NolA from *Bradyrhizobium japonicum*, and SoxR from *E.coli* (Figure 5). The similarity between the four proteins is found only in the N-terminal 65 amino acids which include a helix−turn−helix motif (Figure 5). A block of 10 identical amino acids is found in the second, so called recognition, helix of this DNA binding motif in $TipA_L$ and NolA. Mutagenesis of MerR has revealed that the Hg(II) is recognized by its C-terminal region. This suggested that the C-terminal domain of $TipA_L$ might likewise be a ligand (thiostrepton) binding site.

$TipA_L$ and $TipA_S$ bind thiostrepton

In order to permit critical analysis of the activity of $TipA_L$, as a function of its interaction with thiostrepton and the promoter, the $tipA_L$ gene was cloned (see Materials and methods) and expressed in *E.coli* using the λP_L promoter under the control of the temperature sensitive repressor, cI857.

After 2 h of heat induction, a protein of 31 kDa, the approximate mass of the predicted $TipA_L$ gene product, was observed by SDS−PAGE (Figure 6A). The 31 kDa protein was tested for a possible thiostrepton binding function predicted by analogy to MerR. An *E.coli* crude cell extract containing $TipA_L$ was applied to a thiostrepton affinity column which was then extensively washed with 5 M NaCl without eluting the protein. The protein was released by denaturation in 7 M urea (Figure 6A) and could be refolded by removal of the urea by dialysis. The N-terminal sequence of the protein (-YSVGQVAG), obtained by Edman degradation, was identical to $TipA_L$.

Similarly, $TipA_S$ was produced in *E.coli* using the same expression vector (see Materials and methods). $TipA_S$ in crude cell extracts bound selectively to a thiostrepton column under the same conditions defined above for $TipA_L$. It could not be eluted from the column with 5 M NaCl but could be recovered (>90% pure) after denaturation in 7 M urea (Figure 6B).

DNA binding activity of $TipA_L$

$TipA_L$ generated in *E.coli* bound its promoter sequence with different affinities dependent on the presence or absence

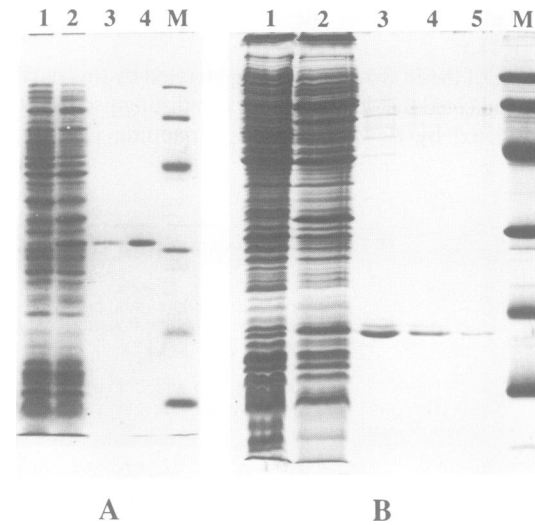

Fig. 6. Binding of $TipA_L$ and $TipA_S$ to thiostrepton. The $tipA_L$ or $tipA_S$ gene products were expressed under the control of the λP_L promoter in *E.coli* W3110c_I (pNB2 for $TipA_L$; pNB3 for $TipA_S$) containing a chromosomally encoded temperature-sensitive cI857 repressor. Following thermal induction, crude protein extracts were prepared as described in Materials and methods and applied to a thiostrepton affinity column. The column was washed with MB buffer containing 2 M NaCl and proteins eluted in 7 M urea for analysis. All samples were separated by SDS−PAGE and stained with Coomassie Blue. (**A**) W3110c_I(pNB2), (**B**) W3110c_I(pNB3). Lane 1, crude extracts of host grown at 30°C; lane 2, crude extracts after induction at 42°C; lanes 3−5, proteins eluted from a thiostrepton affinity column; lane 3A, 0.4 μg $TipA_L$; lane 4A, 2 μg $TipA_L$; lane 3B, 2 μg $TipA_S$; lane 4B, 0.3 μg $TipA_S$; lane 5B, 0.1 μg $TipA_S$. Lane M, molecular weight markers (kDa) 94, 67, 43, 30, 20 and 14.4.

of thiostrepton. The *ptipA* fragment was incubated with thiostrepton (60 μM) and various concentrations of $TipA_L$ ranging from 0.15 to 2.25 μM. Gel retardation assays (Figure 7) showed that the protein had an affinity for *ptipA* which was increased ~10-fold by the addition of thiostrepton. The dissociation constant of the $TipA_L$−thiostrepton complex from the promoter cannot be determined with precision from this protein which has been denatured in 7 M urea. Less than 50% of the renatured protein retains the ability to bind thiostrepton (C.J.Thompson, unpublished data). Furthermore, although we consider it to be unlikely, we cannot formally rule out the possibility that thiostrepton catalyses refolding of denatured $TipA_L$. Given these caveats, the data suggested a dissociation constant of $< 1 \times 10^{-7}$ M.

Location of the $TipA_L$ binding site

Footprinting experiments were carried out to localize the nucleotide sequences protected from DNase I digestion by $TipA_L$. The *ptipA* fragment was incubated with various amounts of $TipA_L$ in the presence or absence of

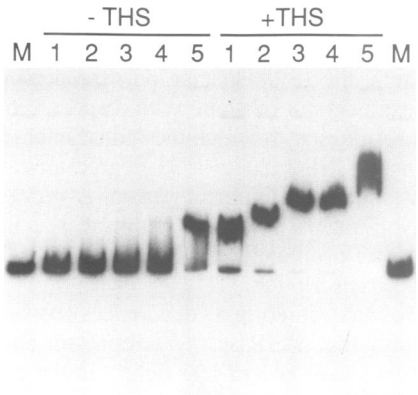

Fig. 7. Gel mobility shift assay using purified $TipA_L$. Radiolabelled *tipA*$_L$ promoter fragment (0.01 pmol) was incubated in 20 μl of reaction buffer [10 mM Tris pH 7.8, 10 mM $MgCl_2$, 150 mM NaCl, 2 mM DTT and 10% (v/v) glycerol] supplemented as indicated with 1.2 nmol thiostrepton (THS) and various amounts of $TipA_L$: lanes 1, 3 pmol; lanes 2, 7.5 pmol; lanes 3, 15 pmol; lanes 4, 22.5 pmol; lanes 5, 45 pmol.

thiostrepton. While thiostrepton alone did not protect any part of the sequence from DNase I digestion, $TipA_L$ protected 24 bp located between 13 and 36 bp upstream of the transcriptional start site within a palindromic sequence (Figure 8). The protection pattern was not altered by thiostrepton but, significantly, the affinity of $TipA_L$ for the promoter was enhanced (as in the case of the gel retardation experiments).

In vitro transcription

$TipA_L$ purified from *E.coli* as described above also catalysed run-off transcription from *ptipA*. Numerous experiments showed that neither *S.lividans* RNAP nor $TipA_L$ with or without the addition of thiostrepton allowed transcription from *ptipA*. In the experiment shown in Figure 9, the combination of $TipA_L$ and RNAP was inactive until thiostrepton was added. Other experiments (data not shown) showed that 10-fold increased concentrations of $TipA_L$ also allowed transcription from the same site in the absence of thiostrepton. Run-off transcripts dependent on $TipA_L$ produced in *E.coli* or *S.lividans* comigrated (data not shown) and corresponded in size to the *in vivo* initiation site previously identified from S1 and primer extension mapping experiments (Murakami *et al.*, 1989).

Discussion

$TipA_L$ is an autogenously regulated bacterial transcriptional activator. It is clear that a negative regulatory system must intervene to avoid uncontrolled amplification of its expression. Studies reported here give a first insight into how activation takes place and suggest how a self-contained negative regulatory mechanism might operate. In addition, the observation that $TipA_L$ specifically binds thiostrepton to activate transcription establishes that biological systems exist in nature capable of responding to this secondary metabolite.

Mechanism of $TipA_L$-mediated transcriptional regulation

Transcriptional activation by $TipA_L$ shares some unusual features with MerR, a protein which regulates the *merT* and

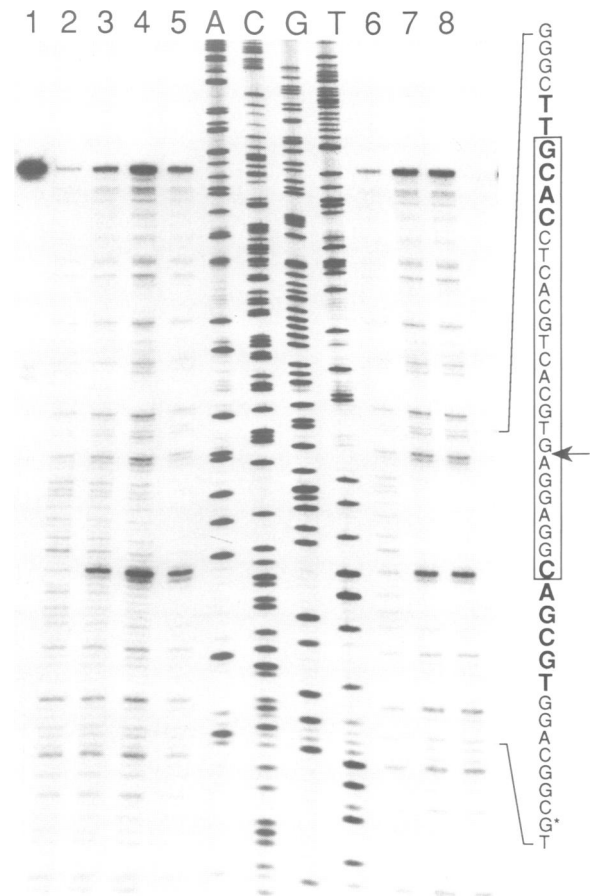

Fig. 8. Localization of the $TipA_L$ binding site by DNase I footprinting. The *tipA*$_L$−*A*$_S$ promoter was isolated from pAK113 as an *Eco*RI−*Pst*I fragment and radiolabelled by filling in the *Eco*RI-generated terminus with [α-^{32}P]dATP. DNA was then incubated with various amounts of $TipA_L$ in the presence and absence of thiostrepton (60 μM). All reaction mixes (50 μl) contained 0.3 pmol radiolabelled DNA, in addition to, lane 1, 30 pmol $TipA_L$ (no DNase control); lane 2, no addition; lane 3, 30 pmol $TipA_L$; lane 4, 60 pmol $TipA_L$; lane 5, 120 pmol $TipA_L$; lane 6, 3 pmol $TipA_L$ and thiostrepton; lane 7, 30 pmol $TipA_L$ and thiostrepton; lane 8, 45 pmol $TipA_L$ and thiostrepton. Samples in lanes 2−8 were treated with 5 ng DNase I for 1 min at room temperature. Lanes A, C, G and T are dideoxy sequence reaction as markers, the primer for which coincides with the end of the ^{32}P-radiolabelled promoter fragment. The sequence complementary to that read from the sequencing reactions is represented on the right (however, note that the DNase hypersensitivities of the two strands are not necessarily identical). The transcription start point is indicated by an asterisk, the −10 and −35 sequences are in bold letters, the protected bases are boxed and a DNase I hypersensitve bond is indicated with an arrow.

merR mercury resistance operons in a wide variety of eubacteria (Summers, 1992). While $TipA_L$-mediated transcriptional activation can presently best be understood in relation to this system, our data also revealed important features which are different from those observed for MerR and thus expand our understanding of this family of regulatory proteins.

Between the −10 and −35 hexamer motifs of promoters controlled by $TipA_L$ and MerR are found exceptionally long, 19 bp (rather than 17 bp) spacers containing inverted repeat sequences. Both proteins exist in solution as dimers and bind to regions of dyad symmetry (O'Halloran and Walsh, 1987). Both bind in the presence or absence of

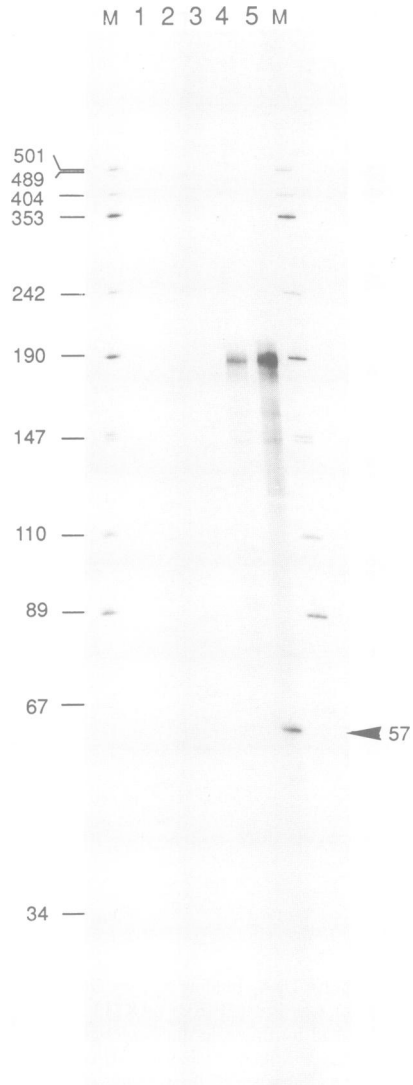

Fig. 9. *In vitro* transcription catalysed by $TipA_L$ produced in *E.coli*. Purified $TipA_L$ was assayed for its ability to activate run-off transcription (arrow) in the presence of *S.lividans* 1326 RNAP. In the absence of thiostrepton, $TipA_L$ appeared to stimulate the end-to-end transcription (filled circle) characteristic of such assays. All samples contained the *tipA* promoter fragment (0.3 pmol of the *Eco*RI–*Hin*dIII fragment from pAK113) as template. Lane 1, *S.lividans* 1326 RNAP; lane 2, *S.lividans* 1326 RNAP and 60 μM thiostrepton; lane 3, 0.3 pmol $TipA_L$; lane 4, *S.lividans* 1326 RNAP and 0.3 pmol $TipA_L$; lane 5, *S.lividans* 1326 RNAP, 0.3 pmol $TipA_L$ and 60 μM thiostrepton; lanes M, molecular weight size markers (*Hpa*II-digested pUC18).

inducer (O'Halloran and Walsh, 1987) and activate transcription in combination with the ligand (Summers, 1992). This is unusual with respect to other prokaryotic transcriptional activators which interact with sequences within or upstream of the −35 hexamer and do not have an inactive promoter-bound form (Collado-Vides *et al.*, 1991).

These similarities predict that $TipA_L$ will share other features with MerR which plays multiple roles in regulating the *merT* promoter. MerR facilitates binding of RNAP *in vivo* to allow formation of a transcriptionally repressed RNAP–MerR–promoter complex (Heltzel *et al.*, 1990) primed for rapid induction. *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 presumed −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 degree unwinding and thus allows functional alignment (Ansari *et al.*, 1992). We are currently testing the prediction that decreasing the spacing between the *tipA* promoter hexamers to 17 bp will result in constitutive transcription.

There are also important differences between these 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 ligand both destabilizes the MerR–DNA complex and converts MerR from a repressor into an activator. In contrast, $TipA_L$ can bind to its target site and activate transcription whether thiostrepton is present or not. Its affinity for the site is increased by an order of magnitude when the ligand is present.

There is also a fundamental difference in how $TipA_L$ and MerR regulate the promoters controlling their own expression. Whereas MerR acts as a feedback repressor of *pmerR*, our *in vitro* experiments have not revealed this to be the case for the transcriptional activator $TipA_L$ which instead promotes its own expression. This implies the intervention of a negative regulatory mechanism to avoid uncontrolled amplification of *ptipA* transcription mediated by the $TipA_L$–thiostrepton complex.

The $TipA_L$ and MerR proteins are characterized by their N-terminal DNA binding motifs and C-terminal ligand binding domains. In the case of TipA, an alternative translational initiation site apparently allows production of the substrate binding domain, $TipA_S$, in large molar excess to $TipA_L$. Since $TipA_S$ may inhibit $TipA_L$-mediated transcription by sequestering thiostrepton, the ratio of these two gene products is a critical parameter for determining levels of transcription.

$TipA_S$ and $TipA_L$ represent alternative translational products of the same open reading frame

Among thousands of prokaryotic genes that have been characterized, there are few cases comparable with *tipA* where ribosomes initiate translation of one continuous open reading frame at two sites (Bläsi *et al.*, 1989; Kofoid and Parkinson, 1991; Nivinskas *et al.*, 1992). In these reports, the efficiency of the upstream site seems to be limited by mRNA secondary structures which overlap translational initiation sites. The stability of such regulatory structures, which can be as little as −6.3 kcal/mol in the case of the λ S gene product (Bläsi *et al.*, 1989), are related to their inhibitory effect on translational initiation (Bläsi *et al.*, 1989; Hall *et al.*, 1982). Inefficient loading of ribosomes at the upstream sequence allows more efficient initiation at the downstream site (Bläsi *et al.*, 1989; Kofoid and Parkinson, 1991).

The codon for the initial methionine of $TipA_S$ is preceded by a canonical ribosome binding site and it is therefore probable that the protein is the result of an authentic translational initiation event rather than a product of $TipA_L$ degradation. The yield of $TipA_s$ relative to $TipA_L$ in cultures grown in the presence of thiostrepton is >20:1; using 2D gel electrophoresis of radiolabelled proteins, we

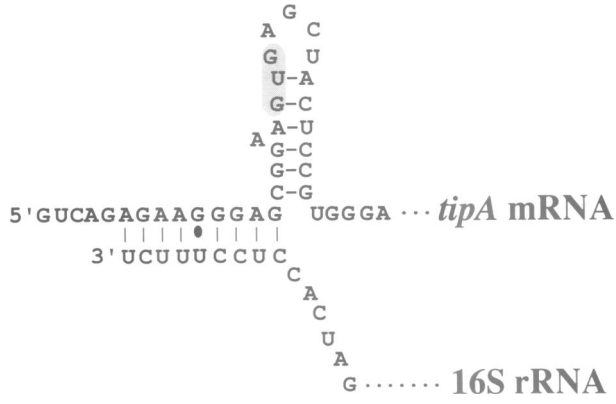

Fig. 10. Potential secondary structure ($\Delta G = -7.0$ kcal/mol) (Tinoco *et al.*, 1973) in the translational initiation region of $tipA_L$. The GUG translational start site is shaded, the presumed ribosome binding site is shown paired to the sequence of the 3′ terminus of the 16S rRNA sequence of *S.lividans* (Bibb and Cohen, 1982). A/U and G/C base pairing is indicated by lines; a G/U base pair is indicated by the solid oval.

have not been able to detect synthesis of either gene product in cultures grown without thiostrepton (A.M.Puglia, J.Vohradsky and C.J.Thompson, unpublished data). The fact that S1 and Northern blot analyses (Murakami *et al.*, 1989) did not reveal a major species of truncated mRNA does not support the hypothesis that this ratio can be explained by processing of the transcript to remove the $tipA_L$ ribosome binding site (Figure 10) and thereby direct translation downstream at $tipA_S$. Instead, this ratio may be determined by ribosome binding sites with different efficiencies for translational initiation. This could be inalterably programmed by their primary sequence. Alternatively, ribosome binding sites sequestered within a secondary structure which could be stabilized or destabilized would offer the possibility of regulating the ratio of the two gene products. An inverted repeat sequence overlaps the $tipA_L$ (-7.0 kcal/mol) but not the $tipA_S$ translational initiation sites (Figure 10).

What is the biological role of the thiostrepton-induced regulon?

We were initially interested in knowing how exposure to extremely low concentrations of thiostrepton induces gene expression in *Streptomyces* in order to gain a better understanding of the biological function of thiostrepton and the regulon it controls. Analysis of the predicted $TipA_L$ amino acid sequence showed that it is a member of a family of transcriptional activator proteins. These proteins are characterized by a conserved N-terminal motif which interacts with promoter sequences, coupled to various C-terminal domains which are specific for diverse substrates including Hg(II) (MerR), genistein (NolA) or an unknown mediator of the superoxide response (SoxR).

In the early stages of these studies, we considered the possibility that thiostrepton induced gene expression by mimicking one of the above mentioned compounds or evoking a non-specific stress response. *In vivo* promoter probing experiments (carried out as described by Murakami *et al.*, 1989) revealed no other antibiotic (outside of the thiostrepton family), heavy metal (including mercury, silver, cobalt, zinc, manganese, copper or antimony), flavinoid (genistein), or superoxide regulon inducers (paraquat, H_2O_2) which activated *ptipA* in *S.lividans* (C.J.Thompson, unpublished data). In principle, *tsr*-directed methylation prevents interaction of thiostrepton with the ribosomes (Thompson *et al.*, 1982a); however, even a small number of non-methylated sensitive ribosomes could disrupt translation on polysomes and lead to a stress response. Biochemical data reported here demonstrate that, whatever its biological function, thiostrepton can activate expression by direct interaction with a specific receptor protein, $TipA_L$. This raises questions about the function of the regulon having thiostrepton-like peptides as inducers.

In addition to $TipA_L$ and $TipA_S$, there are at least six other thiostrepton-induced proteins which can be visualized by 2D gel electrophoresis (A.M.Puglia, J.Vohradsky and C.J.Thompson, unpublished data). It has also been observed that thiostrepton induces resistance to the unrelated antibiotics daunorubicin (Guilfoile and Hutchinson, 1991), sparsomycin (T.Katoh and C.J.Thompson, unpublished observations) and tetranactin (Plater and Robinson, 1992) in *S.lividans*. In addition to $TipA_L$ and $TipA_S$, there is at least one thiostrepton-induced protein whose synthesis is dramatically reduced in a $tipA_L$ mutant which we have recently constructed (T.Katoh, A.M.Puglia and C.J.Thompson, unpublished). Corresponding genes are probably activated by $TipA_L$ binding to similar recognition sequences in at least one other promoter and activating transcription upon exposure to thiostrepton.

We cannot rule out the possibility that *tipA* proteins interact with a peptide biosynthetic pathway which is not yet identified in *S.lividans*. Indeed, relatively recently a small diffusible peptide of ~18 amino acids (SapB) synthesized by *S.lividans* and characterized in a closely related strain, *Streptomyces coelicolor*, has been shown to play a key role in the developmental cycle of these organisms (Willey *et al.*, 1991). SapB as well as another chemically uncharacterized diffusible factor are needed for the erection of aerial mycelium leading to sporulation in *S.coelicolor* (Willey *et al.*, 1991). One explanation presented by Willey *et al.* was that SapB might be a pheromone-like activator of differentiation (for a review, see Gräfe, 1989) known to be synthesized by many different actinomycetes. These include A Factor, B Factor, Factor C, L-Factor and the antibiotic pamamycin. It remains to be seen whether thiostrepton might play a similar role in some form of intercellular communication.

Materials and methods

Thiostrepton

Thiostrepton (molecular mass of 1665 Daltons) was provided by Squibb.

Bacterial strains, plasmids and growth conditions

Streptomyces lividans prototrophic strain 1326 (Lomovskaya *et al.*, 1972) was grown on NE agar plates (Murakami *et al.*, 1986) or YEME liquid medium containing 34% sucrose (Hopwood *et al.*, 1985). Resistance to thiostrepton was provided by *tsr* cloned in the streptomycete cloning vector pIJ61 (Thompson *et al.*, 1982b).

Escherichia coli W3110 containing the cI857 bacteriophage λ repressor (W3110cI) (Campbell *et al.*, 1978) was grown in LB-medium containing ampicillin (100 μg/ml) when appropriate.

Plasmid pAK113 (Murakami *et al.*, 1989) contained the *S.lividans* 1326 *tipA* promoter cloned as a 143 bp *SmaI*–*NaeI* fragment into the *SmaI* site of pUC13 (Yanisch-Perron *et al.*, 1985).

DNA techniques

Restriction endonucleases and DNA modifying enzymes were purchased from Boehringer (Mannheim, Germany) or Pharmacia (Uppsala, Sweden)

and used as recommended by the supplier. DNA was isolated and manipulated as described in Maniatis *et al.* (1982) for *E.coli*, and as in Hopwood *et al.* (1985) for *S.lividans*. DNA fragments were isolated from low melting point agarose according to the method of Langridge *et al.* (1980), and subsequently labelled with [α-^{32}P]dATP and Klenow fragment of DNA polymerase I or Sequenase (US Biochemical Corp.), according to the manufacturer's instructions.

Purification of RNA polymerase

RNA polymerase (RNAP) was assayed and purified using procedures based on those described by Buttner *et al.* (1988). In order to obtain dispersed cultures, *S.lividans* 1326 was pre-grown in liquid seed medium (Ogawa *et al.*, 1983) for 72 h at 30°C, diluted 1/50 into YEME medium supplemented with 34% sucrose and grown for 40 h. Mycelia (30 g wet weight) were washed in buffer MA [10 mM Tris−HCl, pH 8.0; 10 mM $MgCl_2$; 0.1 mM Na_2EDTA; 0.3 mM dithiothreitol (DTT)] and lysed by sonication in 100 ml of the same buffer. Cell debris was removed by centrifugation (40 000 *g*, 10 min, 4°C). Polyethylenimine (PEI, Polymin P, Sigma) neutralized to pH 7.8 was slowly added to a final concentration of 0.15%, the mixture was incubated on ice for 20 min, and the resulting precipitate was collected by centrifugation. The pellet was extracted in 0.3 M $(NH_4)_2SO_4$ (2 × 25 ml) and the supernatant collected following centrifugation. Ammonium sulfate was slowly added to 75% saturation, the mixture was incubated on ice for 30 min, and the precipitate was collected by centrifugation. The pellet was resuspended by the addition of 5 ml of MB (10 mM Tris−HCl, pH 8.0; 10 mM $MgCl_2$; 50 mM NaCl; 0.1 mM Na_2EDTA; 1 mM DTT; 10% glycerol) and this protein solution was dialysed against 1 l of MB for 14 h. The extract was then clarified by centrifugation (12 000 *g*, 4°C), concentrated by ultrafiltration (Centricon-10 filter, supplied by Amicon), and filtered through 0.22 μm Millex-GS membranes (Millipore) before applying to a heparin−Sepharose CL-6B column (Pharmacia). The column was thoroughly washed with MB buffer and RNAP was then eluted using a gradient of 50 mM−1 M NaCl. Active fractions were desalted by ultrafiltration (Centricon-10, Amicon) and loaded on to an anion-exchange column (Mono Q HR 5/5 Pharmacia) which was then washed with MB buffer. RNAP activity was eluted in a single protein peak (as measured by optical density at 280 nm) using a gradient of 0.05−1 M NaCl. Active fractions from this column were concentrated and RNAP further purified on a gel exclusion column (Superose 6 HR 10/30, Pharmacia).

Purification of proteins

Purification of $TipA_L$ from S.lividans. Crude extracts were prepared from *S.lividans* 1326(pIJ61), grown in the presence of thiostrepton (50 μg/ml). $TipA_L$ activity in these extracts and throughout the purification was monitored by its ability to bind to and activate transcription from a DNA fragment containing the $tipA_L$ promoter using gel retardation and *in vitro* transcription assays as described below. The purification protocol was similar to that used for RNAP. $TipA_L$ could be separated from RNAP using a heparin−Sepharose CL-6B column. The protein was subsequently eluted from an anion-exchange column (Mono Q HR 5/5, Pharmacia) using a linear salt gradient (50 mM−1 M). $TipA_L$ was finally purified on a gel filtration (Superose 12 HR 10/30, Pharmacia) column equilibrated in MB buffer.

Purification of $TipA_L$ from E.coli. The $tipA_L$ gene was amplified from pAK109 (a *Kpn*I fragment containing the *tipA* gene subcloned from λAK1 into the *Kpn*I site of pUC19; Murakami *et al.*, 1989) using the polymerase chain reaction (PCR) employing oligonucleotide primers with restriction endonuclease sites engineered at their ends:

Oligonucleotide 1, CCCGGTACCCATATGAGCTACTCCGTGGGAC-AGGTGG;
Oligonucleotide 2, CCCGGATCCGGTGCCGGTCTGAAGTCGCTGGT-CGGCGGTGA.

The fidelity of the PCR reaction was confirmed by sequencing the cloned $tipA_L$ coding region by the Sanger method (Sanger *et al.*, 1977). The fragment was cloned as an *Nde*I−*Sma*I fragment into an expression vector (pIP998) modified from pL-RCV (Lokker *et al.*, 1991). In this plasmid, pNB2, $tipA_L$ transcription was directed by the bacteriophage λ pL promoter controlled by the temperature-sensitive cI857 repressor in the chromosome of W3110 (Lokker *et al.*, 1991) (W3110/cI).

A plasmid for overexpression of $TipA_S$, pNB3, was derived from pNB2 by replacing the sequence encoding the N-terminus of $TipA_L$ with the corresponding $TipA_S$ sequence. A fragment containing the N-terminal of $TipA_S$ with an engineered *Nde*I site overlapping with the ATG start site was obtained from pAK109 by PCR employing the following oligonucleotide primers:

Oligonucleotide 3, GCCCATATGGGAATCAACCTCACCCCG;
Oligonucleotide 4, GGCGGCGTCCATGGCCCC.

This fragment was cloned into pNB2 after cleavage of both vector and fragment with *Nde*I and *Sac*I to generate pNB3. The fidelity of the PCR reaction was confirmed by sequencing the cloned $tipA_S$ coding region (Sanger *et al.*, 1977).

$W3110c_I$(pNB2) or $W3110c_I$(pNB3) were incubated in LB liquid medium for 16 h at 30°C and 2.5 ml of this culture was used to inoculate each of 10 flasks (250 ml) containing 12.5 ml LB liquid medium prewarmed to 42°C. Incubation at 42°C was continued for a further 2 h and the cells were collected by centrifugation, washed in buffer MB, and disrupted by sonication in the same buffer. Cell debris was removed by centrifugation (20 000 *g*) and the supernatant was loaded on to a 5 ml thiostrepton affinity column prepared as described below. $TipA_L$ or $TipA_S$ was retained on the column after extensive washing with MB buffer containing 5 M NaCl and eluted by the addition of 7 M urea. $TipA_L$ was then dialysed twice for 6 h against 10^3 vols of MB buffer, aliquoted and stored at −80°C.

Protein analyses

Protein concentrations were assayed using the Bio-Rad protein assay kit. SDS−PAGE was carried out according to Laemmli (1970) and 2D gel electrophoresis according to Holt *et al.* (1992). N-terminal protein sequences were obtained by Edmann degradation after electrophoretic transfer of proteins from SDS−PAGE or 2D gels to Immobilon-P filters (Millipore; Holt *et al.*, 1992). Molecular weight standards were obtained from Pharamacia (phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa and α-lactalbumin, 14.4 kDa).

Preparation of a thiostrepton affinity column

Thiostrepton was covalently bound to Affi-Gel 10 (Bio-Rad), a matrix which reacts with primary amine groups. Thiostrepton contains only one such group located on an 'arm' extending from the main body of the molecule. The gel matrix was washed with 3 vols of dimethyl sulfoxide (DMSO) and then resuspended in the same volume of DMSO. Thiostrepton was then added such that the molar ratio of active ester to antibiotic was ~1:1. Following incubation at 20°C for 16 h, ethanolamine was added to block any remaining unreacted ester groups and incubation was continued for 5 h. The resulting gel matrix was washed extensively first with DMSO and then with buffer MB.

Gel retardation assays

Protein solutions were pre-incubated at 30°C for 20 min in the absence or presence of thiostrepton (50 μg/ml) in a buffer containing 10 mM Tris (pH 7.8), 10 mM $MgCl_2$, 150 mM NaCl, 2 mM DTT and 10% (v/v) glycerol. The *Eco*RI−*Hin*dIII fragment of pAK113 (0.01 pmol), containing the *tipA* promoter radiolabelled by filling in the ends using [α-^{32}P]dATP, was added and the incubation continued for a further 20 min. When crude cell extracts were used, 1 μg of salmon sperm DNA was included in the incubation mix to minimize non-specific protein binding to the *tipA* promoter fragment. Protein−DNA complexes were separated by electrophoresis on 5% polyacrylamide (38:2 acrylamide/bis), TBE (100 mM Tris-borate pH 8.3, 1 mM EDTA) gels. These were dried and the promoter fragment visualized by autoradiography.

In vitro run-off transcription

The conditions for *in vitro* transcription were as described by Buttner *et al.* (1988) using the *Hin*dIII−*Eco*RI fragment of pAK113 containing the *tipA* promoter as template. Activator protein preparations were pre-incubated with or without thiostrepton (60 μM) for 15 min at 30°C in buffer containing 7.5 mM Tris−Cl (pH 8.0); 10 mM $MgCl_2$; 0.1 mM EDTA; 1 mM KPO_4 (pH 8.0) 0.1 mM DTT; 10% glycerol; 1 mM each of ATP, GTP, UTP and 10 mCi [α-^{32}P]CTP (3000 Ci/mmol). Template was added and the incubation continued for 2 min before the reaction was initiated by the addition of RNAP. Re-initiation by the polymerase was prevented by the addition of heparin (3 μg/ml final concentration) and incorporation of [α-^{32}P]CTP was chased by the addition of unlabelled CTP (1.5 mM final concentration). Samples were precipitated with isopropanol, collected by centrifugation, resuspended in formamide sample buffer, separated on a 6% polyacrylamide sequencing gel and visualized by autoradiography.

DNase I Footprinting

Various amounts of $TipA_L$ were incubated with the radiolabelled *Eco*RI−*Pst*I fragment of pAK113 (0.3 pmol), containing the $tipA_L$ promoter, in the presence or absence of thiostrepton (60 μM) at room temperature for 10 min in MB buffer. Samples were then diluted 2-fold with DNase I buffer (10 mM HEPES−HCl, pH 7.8), 5 mM $MgCl_2$,

1 mM $CaCl_2$ and 15 mM NaCl) and treated with 5 ng DNase I for 1 min at the same temperature. These were precipitated with ethanol in the presence of 0.3 M sodium acetate, resuspended in 95% formamide containing 20 mM EDTA and loaded on a 6% polyacrylamide sequencing gel. The gel was fixed in 10% acetic acid: 10% methanol, dried and radioactive bands were visualized by autoradiography.

Protein alignments and database searches

Programs FASTA (Pearson and Lipman, 1988) and BLAST (Altschul *et al.*, 1990) were used to scan the databases GenPept (Release 72) and NBRF/PIR (Release 31). CLUSTAL V (Higgins *et al.*, 1992) was used for multiple protein alignments.

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