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Broad Spectrum Thiopeptide Recognition Specificity of the *Streptomyces lividans* TipAL Protein and Its Role in Regulating Gene Expression*

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Microbial metabolites isolated in screening programs for their ability to activate transcription of the *tipA* promoter (*ptipA*) in *Streptomyces lividans* define a class of cyclic thiopeptide antibiotics having dehydroalanine side chains ("tails"). Here we show that such compounds of heterogeneous primary structure (representatives tested: thiostrepton, nosiheptide, berninamycin, promothiocin) are all recognized by TipAS and TipAL, two in-frame translation products of the *tipA* gene. The N-terminal helix-turn-helix DNA binding motif of TipAL is homologous to the MerR family of transcriptional activators, while the C terminus forms a novel ligand-binding domain. *ptipA* inducers formed irreversible complexes *in vitro* and *in vivo* (presumably covalent) with TipAS by reacting with the second of the two C-terminal cysteine residues. Promothiocin and thiostrepton derivatives in which the dehydroalanine side chains were removed lost the ability to modify TipAS. They were able to induce expression of *ptipA* as well as the *tipA* gene, although with reduced activity. Thus, TipA required the thiopeptide ring structure for recognition, while the tail served either as a dispensable part of the recognition domain and/or locked thiopeptides onto TipA proteins, thus leading to an irreversible transcriptional activation. Construction and analysis of a disruption mutant showed that *tipA* was autogenously regulated and conferred thiopeptide resistance. Thiostrepton induced the synthesis of other proteins, some of which did not require *tipA*.

Directed searches for microbial secondary metabolites that inhibit bacterial growth led to the discovery of antibiotics and thus gave rise to the traditional interpretation that their only biological relevance is to inhibit growth of competing organisms. Nevertheless, antibiotics often have alternative molecular targets and, like other secondary metabolites, elicit numerous "unexpected" effects on microbial differentiation (1–4) and mammalian cell function (1). Here we describe how a single transcriptional activator can interact with diverse thiopeptide antibiotics to elicit autogenous expression of its own promoter as well as a modulon in *Streptomyces lividans* (SL).[1]

Thiopeptides are a family of antibiotics composed of a ring structure containing highly modified amino acids and a linear peptide containing dehydroalanines extending from the ring at a pyridyl group ("tail") (Fig. 1). They were first discovered as antibiotics synthesized by diverse bacteria including *Streptomyces*, *Bacillus*, and *Micrococcus*. These compounds later proved to be effective growth promotants for domestic animals (2–4), an effect whose biological basis is not clear. Thiostrepton, whose antibiotic activity is best understood, acts by binding tightly to the procaryotic ribosome and thus inhibiting translation (5–8). In a thiostrepton-producing organism, *Streptomyces azureus*, methylation of a specific nucleotide in the 23 S rRNA can provide resistance. Such methylated ribosomes do not bind and are therefore not sensitive to thiostrepton (9). The gene encoding this methylase (*tsr*) was originally cloned as an antibiotic resistance determinant (10) and has been incorporated into most *Streptomyces* cloning vectors (11–13).

Routine use of thiostrepton to select for *tsr*-containing vectors in *S. lividans* revealed several unexpected biological activities. Thiostrepton made *S. lividans* more resistant to a variety of structurally heterogeneous antibiotics (Ref. 14, and references therein) and caused accumulation of thiostrepton-induced proteins (Tip). Two of these proteins, TipAL and TipAS, proved to be alternate in-frame translation products of the same gene (15) (*tipA*, Fig. 2). At its N terminus, TipAL contained a conserved helix-turn-helix DNA binding motif that defined it as a member of the MerR family of transcriptional activators. The non-homologous C-terminal domains of these genes interact with different low molecular weight compounds: MerR with mercuric ion (16, 17), BmrR and BltR with rhodamine 6G and tetraphenylphosphonium chloride (18), and SoxR with an unknown compound (19) reflecting superoxide anion toxicity (20). A structural basis for multidrug recognition has recently been revealed by the crystal structure of the binding domain of BmrR complexed with tetraphenylphosphonium chloride (21). After binding the respective regulators, these

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[1] The abbreviations used are: SL, *Streptomyces lividans*; KT, *Streptomyces lividans* containing a disrupted *tipAL* gene; *J*, spin-spin coupling constant; s, singlet; d, doublet; q, quartet; m, multiplet; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; *m*/*z*, mass to charge ratio; ESI-MS, electrospray ionization mass spectrometry; HRFAB-MS, high resolution fast atom bombardment mass spectrometry; hygR, resistant to hygromycin; vioS, sensitive to viomycin; kb, kilobase pair(s); Tricine, *N*-tris(hydroxymethyl)methylglycine.

proteins activate transcription of corresponding regulons that confer resistance to mercury (MerR), superoxide stress (SoxR), or diverse antibiotics and antiseptics (SoxR, BmrR, and BltR). The corresponding C-terminal region of TipAL, lacking the helix-turn-helix motif, is translated independently in vast molar excess as TipAS. Both TipAS and TipAL can covalently bind thiostrepton (22). A reaction between a dehydroalanine residue in thiostrepton and one of two C-terminal cysteines (Fig. 2) creates an antibiotic-TipAL complex (23) that activates transcription of a monocistronic mRNA from the *tipA* promoter (*ptipA*) (22).

The *ptipA* has proven to be a valuable tool that has led to the discovery of new antibiotics. When actinomycete metabolite libraries, a traditional source of molecular diversity, were empirically screened for *ptipA*-inducing activities, 15 compounds were identified (24–27). While they had quite different chemical structures, all were thiopeptides having antibiotic activity.

Since the TipA proteins are found in *Streptomyces* strains not known to have the thiopeptide biosynthetic genes, it is unclear what metabolic signals are the natural inducers of *tipA*. Here we investigate the TipAL regulon and thiopeptide structural motifs that are important for TipA interactions.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Compounds used in these studies included (sources indicated in parentheses): thiostrepton A (hereafter referred to as thiostrepton; Squibb); nosiheptide and pristinamycin II (Rhône Poulenc); thiostrepton B (Eric Cundliffe, University of Leicester); viomycin (Pfizer); berninamycin A and B (Upjohn Chemicals); GE 2270A (Bioserach Italia); nisin A (Harry Rollema and Oscar Kuipers, Netherlands Institute for Dairy Research); diketopiperazines (Matthew Holden and Gordon Roberts, University of Nottingham); and farnesol, shikimic acid, and cerulenin (Fluka); other thiopeptides have been purified as described previously (32–35). L-[^{35}S]Methionine protein labeling mix (7.9 mCi/ml L-[^{35}S]methionine and 2.4 mCi/ml L-[^{35}S]cysteine) was from NEN Life Science Products. Me_2SO was purified by passing through dry alumina and dry silica columns.

Promothiocins MO and MN were isolated from the methanolysate of promothiocin B. Dried amberlite 15 (1 g) was added to a solution of promothiocin B (90 mg) in 3 ml of methanol. This mixture was refluxed for 24 h under N_2 and then dried under reduced pressure. The residue, dissolved in 2 ml of chloroform/methanol (5:1), was applied to preparative silica TLC plates and developed with chloroform/methanol (15:1). The two spots with R_F values of 0.59 and 0.51 corresponded to promothiocin MO and MN, respectively. Both spots were scraped off the plates and then purified further by preparative HPLC (YMC-packed C18 column, 20 × 250 mm, with a flow rate of 18 ml/min). The HPLC column was run with a gradient of acetonitrile and water to yield the purified promothiocins (32 mg of MO eluted at 38% acetonitrile; 21 mg of MN eluted at 33% acetonitrile).

Promothiocin MO was a white crystalline powder. The HRFAB-MS spectrum had a MH^+ species with m/z of 762.2175, which corresponded to a molecular formula $C_{34}H_{35}N_9O_8S_2$ (theoretical 762.2128 Da). ^{1}H NMR of promothiocin MO in $CDCl_3/CD_3OD$ (9:1) had resonance peaks at 8.55 ppm (1H, d, J = 7.9 Hz), 8.20 ppm (1H, d, J = 8.2 Hz), 8.07 ppm (1H, s), 8.01 ppm (1H, s), 5.38 ppm (1H, q, J = 7.0 Hz), 5.30 ppm (1H, q, J = 7.0 Hz), 4.70 ppm (1H, d, J = 16.2 Hz), 4.35 ppm (1H, d, J = 6.4 Hz), 4.12 ppm (1H, d, J = 16.2 Hz), 4.00 ppm (3H, s), 2.53 ppm (3H, s), 2.51 ppm (3H, s), 2.22 ppm (1H, m), 1.62 ppm (3H, d, J = 7.0 Hz), 1.54 ppm (3H, d, J = 7.0 Hz), 1.54 ppm (3H, d, J = 7.0 Hz), and 1.01 ppm (6H, d, J = 6.7 Hz).

Promothiocin MN was a white crystalline powder. The HRFAB-MS spectrum had a MH^+ species with m/z of 747.2086, which corresponded to a molecular formula $C_{33}H_{34}N_{10}O_7S_2$ (theoretical 747.2132 Da). ^{1}H NMR of promothiocin MN in $CDCl_3/CD_3OD$ (9:1) had resonance peaks at 8.44 ppm (1H, d, J = 8.2 Hz), 8.21 ppm (1H, d, J = 8.2 Hz), 8.07 ppm (1H, s), 8.03 ppm (1H, s), 5.39 ppm (1H, q, J = 7.0 Hz), 5.34 ppm (1H, q, J = 7.0 Hz), 4.65 ppm (1H, d, J = 16.2 Hz), 4.37 ppm (1H, d, J = 6.1 Hz), 4.15 ppm (1H, d, J = 16.2 Hz), 2.52 ppm (3H, s), 2.39 ppm (3H, s), 2.24 ppm (1H, m), 1.62 ppm (3H, d, J = 6.7 Hz), 1.60 ppm (3H, d, J = 7.0 Hz), 1.01 ppm (3H, d, J = 6.7 Hz), and 1.00 ppm (3H, d, J = 7.0 Hz).

Bacterial Strains and Plasmids—*Escherichia coli* TG1 was used as a host for pUC19 and its derivatives. *E. coli* S17-1 was used as a donor strain for conjugation to *S. lividans*. pPS24 was the source of the viomycin resistance gene (28). TipA studies were done using SL carrying pAK114 (SL/pAK114) (15).

Growth Conditions—*S. lividans* spores produced on MS plates (1% agar containing 2% mannitol and 2% soybean meal) were filtered through cotton, and stored in 20% glycerol at −20 °C. Liquid cultures of SL/pIJ486 were grown in YEME medium (29). *E. coli* strains were grown at 37 °C in Lysogeny broth using 100 mg/liter ampicillin or 50 mg/liter kanamycin for selection (30). Nutrient agar was made from nutrient broth (plus 15 g/liter agar) obtained from Eiken Chemical Co., Tokyo, Japan.

Thiostrepton Sensitivity Assay—Thiostrepton sensitivity disc assays were performed using *S. lividans* strains spread on NE agar (31). Measured amounts of thiopeptide (in Me_2SO) were applied on a paper disc, and the culture was incubated overnight at 30 °C. Alternatively, the minimal amount of antibiotic required to inhibit colony formation on NE agar was determined.

In Vivo tipA Promoter Induction Assays—The *tipA* promoter was cloned into pIJ486 in which a kanamycin resistance gene served as the reporter of promoter activity (15). This plasmid was used either in a disc (15) or plate assay. In the plate assay, SL/pAK114 spores were spread on nutrient agar supplemented with 10 μg/ml kanamycin and a series of 2-fold diluted thiopeptide compounds. The plate was scored for growth after 36-h of incubation at 30 °C.

Preparation of Purified TipA Proteins—*E. coli* cultures containing *tipAL* or *tipAS* expression vectors were grown to late exponential phase in a 25-liter fermentor at 30 °C. Expression of *tipA* genes was induced by the addition of 1 mM isopropylthiogalactoside for 3 h at 37 °C. The cells were collected by centrifugation at 10,000 × *g* for 30 min and stored at −20 °C. The cell paste was suspended in the lysis buffer containing 400 mg/liter lysozyme and 10 mg/liter deoxyribonuclease I and stirred gently overnight (this and all subsequent steps were carried out at 4 °C). Cell debris was removed by centrifugation at 22,000 × *g* for 30 min to yield a crude extract that was fractionated by adding solid ammonium sulfate to reach 45% w/v saturation and then centrifuged at 22,000 × *g* for 15 min. Additional ammonium sulfate powder was added to this supernatant to reach 90% w/v saturation. The pellet was resuspended in three volumes of buffer (all subsequent steps were carried out in MA buffer: 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, and 5 mM dithiothreitol) and then dialyzed. After centrifugation, the dialysate was applied to a DEAE-Sepharose column and eluted using a gradient of 0–0.2 M NaCl. TipA-containing fractions were collected, dialyzed, applied to a Q-Sepharose column (Amersham Pharmacia Biotech), and eluted using a gradient of 0–0.5 M NaCl. TipA-containing fractions were collected, dialyzed, applied to a Source-Q column, and eluted in a gradient of 0–0.5 M NaCl. The fractions were collected and concentrated with an Amicon Ultrafiltration cell with a YM10 membrane. The concentrated solution was loaded on a Superose 12 HR 10/30 column (Amersham Pharmacia Biotech) and eluted isocratically. The fractions containing the TipA protein were concentrated with Centricon 10 filters (Amicon).

Protein-Antibiotic Reactions—Before reaction with thiostrepton or other antibiotics, TipAS or TipAL (10 μg in 15 μl of 50 mM Tris, pH 8.0, 1 mM EDTA) was reduced by the addition of sodium cyanoborohydride (2 μl of a freshly made 1 mM aqueous stock). After 5 min at room temperature, excess sodium cyanoborohydride was inactivated by adding 1 μl of acetone. Thiopeptides (10 μl of a 50 mg/liter purified Me_2SO solution) were then added and the reaction was incubated at room temperature for 1 h. Protein products were desalted using a C4 HPLC column eluting with a gradient of 100% water to 75% acetonitrile/25% water. The proteins were subjected to SDS-PAGE or digested with V8 protease in 50 mM $NaPO_4$, pH 7.0, at 37 °C for 24 h and analyzed with ESI-MS.

Electrophoretic Analyses—Mycelium was collected by centrifugation at 10,000 × *g* for 30 min, and then washed with lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 50 mg/liter benzamidine, 10 mg/liter leupeptin, 20 mM phenylmethanesulfonyl fluoride, and 5 mM dithiothreitol) at 4 °C. Cells were resuspended in lysis buffer, glass beads (0.1 mm diameter) were added to form a 30% v/v solution, and the tube was cooled on ice for 10 min. The mycelia in this slurry were sheared twice in a Bio-spec Products Minibead beater run at 3000 rpm for 1 min. The crude extract was centrifuged using a tabletop centrifuge at 14,000 rpm for 5 min. Samples of the crude extract were boiled for 3 min in a SDS-PAGE lysis buffer containing 20% w/v glycerol, 6% SDS, 10% 2-mercaptoethanol, and 0.05% bromphenol blue in 0.25 M Tris, pH 6.8. SDS-PAGE was carried out according to Laemmli (32) or Schägger and von Jagow (33). Gels were stained with Coomassie Blue (0.1% in 10% acetic acid), destained in 40% ethanol/10% acetic acid, and equilibrated in 10% ethanol/3% glycerol. After

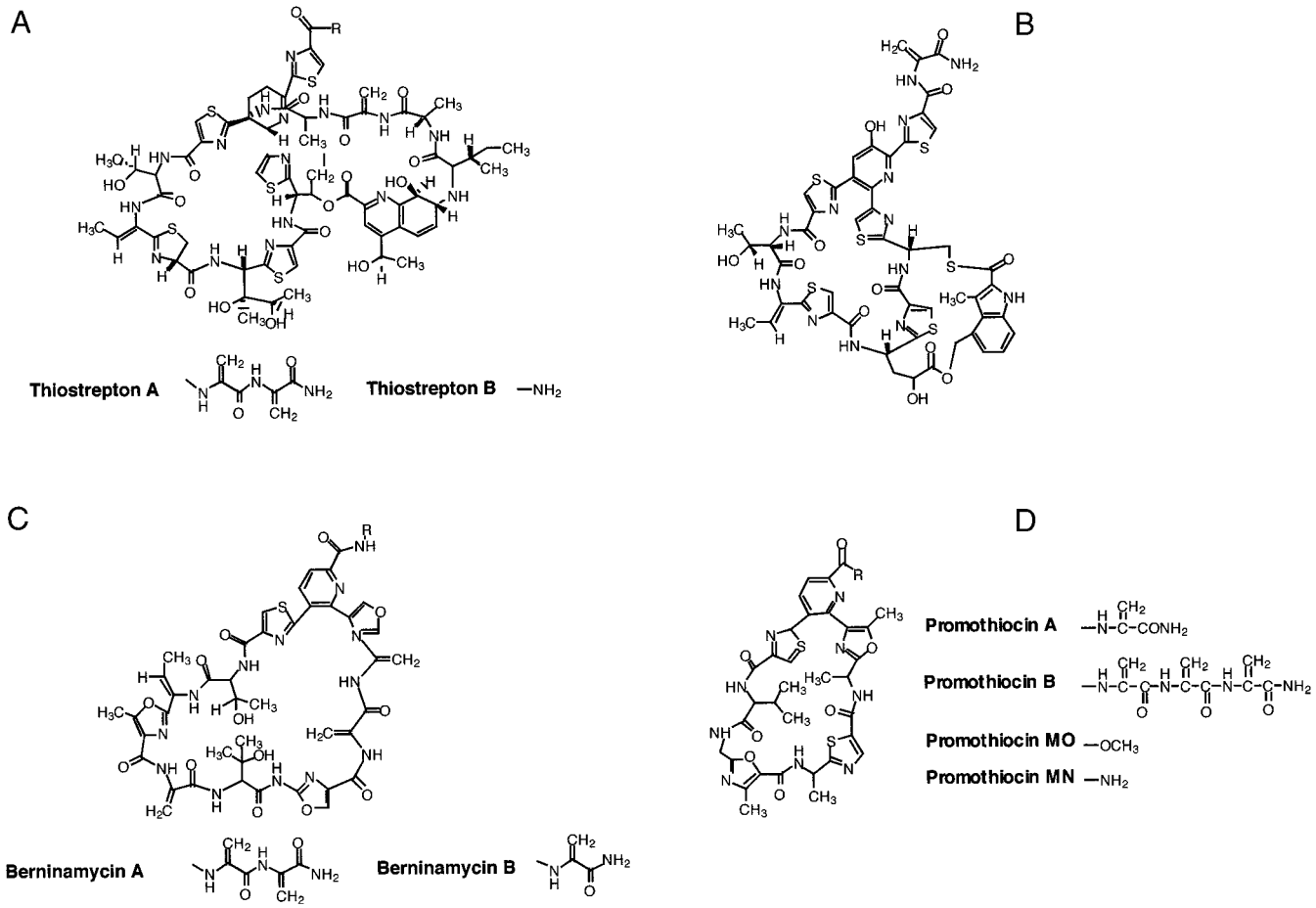

FIG. 1. **Chemical structures of thiopeptide inducers of *ptipA*.** Inducers of *ptipA* expression include: *A*, thiostrepton (1664 Da); *B*, nosiheptide (1222 Da); *C*, berninamycin (A, 1147 Da; B, 1363 Da); and *D*, promothiocin (A, 800 Da; MO, 762 Da; MN, 747 Da). These antibiotics are characterized by a pyridine or reduced pyridyl moiety with a macrocycle-containing thiazoles or oxazoles. The tail of these structures corresponds to a linear peptide extension from the pyridyl ring on the macrocycle.

drying, radioactive spots were recorded on Fuji AIF-RX medical x-ray film. Two-dimensional gel analyses were carried out as described by Puglia *et al.* (34).

RESULTS

Construction of a Strain (KT) Having a Disrupted tipAL Gene—The *tipA* gene was disrupted to determine whether it was required for thiopeptide-induced changes in gene expression. The hygromycin resistance gene (*hyg*) was first inserted into the 5′ region of the monocistronic *tipA* gene cloned in *E. coli* plasmid pAK315 (construction described in Fig. 3). pAK315 was then transferred from *E. coli* S17-1 to *S. lividans* by conjugation (28). Since this plasmid cannot replicate in *S. lividans*, it could only be maintained by integration into the chromosome. Hygromycin-resistant and viomycin-sensitive (hygR/vioS) mutants would result from a double crossover event disrupting the *tipA* gene.

Total DNAs from SL and such hygR/vioS *tipAL* disruption candidates were digested with *Sac*I or *Kpn*I and probed with the 1.2-kb pAK108 *Sac*I fragment which included the site of *hyg* insertion (blots not shown). In the parental strain, this probe hybridized to the 1.2-kb *Sac*I fragment or a 3.5-kb *Kpn*I fragment. These fragments were not present in the *tipA::hygR* disruption strain (KT); instead, the probe hybridized to 1.1- and 1.5-kb *Sac*I and 5.1-kb *Kpn*I fragments corresponding in size to that predicted for the *hyg*-disrupted *tipA* locus.

Inactivation of the tipA Gene Resulted in Sensitivity to Thiostrepton—KT was compared with SL to determine whether disruption of *tipA* altered resistance to thiostrepton. An antibiotic disc sensitivity test revealed that about 5 times less thiostrepton was needed to obtain the same zones of inhibition on a KT lawn. This was further confirmed by streaking SL and KT out on the same medium containing increasing amounts of thiostrepton. Whereas the minimal inhibitory concentration for SL colony formation was 1 μg/ml, KT colony formation was inhibited by less than 0.2 μg/ml.

Thiopeptide-induced ptipA Expression Was Dependent on tipA—The transcriptional activity of *ptipA* was monitored by cloning it into a *Streptomyces* promoter probe vector (pIJ486) so that it controlled a kanamycin resistance reporter gene (pAK114) (15). Induction of *ptipA* could be visualized with a sensitive disc assay. Agar plates containing kanamycin were overlaid with spores of the indicator strain SL/114 or KT/114. When discs containing thiostrepton were applied to the surface of the plate, induction of *ptipA* was indicated by a zone of kanamycin-resistant growth whose diameter was dependent on both the amount of kanamycin in the plate and the amount of thiopeptide inducer in the disc (15). In the absence of inducers, no kanamycin-resistant growth was observed on plates containing greater than 5 mg/liter kanamycin. Whereas this very sensitive assay was able to detect less than 2 ng of thiostrepton using the SL/pAK114 indicator strain, 1 mg elicited no response in the *tipA* mutant KT/pAK114 (lacking the intact *tipAL* gene) or SL/pIJ486 (lacking *ptipA*). These results showed that the reporter system had a strict requirement for both the *ptipA* and a functional *tipA* gene.

TipAL Controlled a Subset of the Thiostrepton-induced Proteins—Exponentially growing (15 h) or stationary phase cultures (40 h) of SL/pIJ486 and KT/pIJ486 were induced with

TABLE I
The structure of thiopeptide dehydroalanine tails relates to their ptipA induction activity

Structures of tail	Compound	MinC[a]
		ng/ml
Three deala[b] residues and C-terminal amide	Promothiocin B	0.6 (0.63 nM)
Two deala residues and C-terminal amide	Geninthiocin	1.2 (1.0 nM)
	Berninamycin A	1.2 (1.0 nM)
	Thiostrepton A	2.4 (1.4 nM)
One deala residue and C-terminal amide	Promothiocin A	20 (24 nM)
	Thioactin	40 (38 nM)
Four deala residues and C-terminal carboxylic acid	Promoinducin	4 (30 nM)
	Thiotipin	80 (32 nM)
	Thioxamycin	80 (63 nM)
	A10255G	80 (66 nM)
No deala tail	Promothiocin MO	2500 (3.3 μM)
	Promothiocin MN	2500 (3.3 μM)
	Thiostrepton B	10[c] (67 nM)
	Cyclothiazomycin	>1000 (>0.7 μM)
	GE2270A	>1000 (>1 μM)
	Amythiamicin A	>1000 (>0.8 μM)

[a] Minimum induction concentrations (MinCs) were determined by agar dilution (see "Experimental Procedures"). Similar results were obtained using the disc assay (15).
[b] Dehydroalanine.
[c] Estimated using the disc assay (15).

thiostrepton (2 mg/liter) and labeled for 1 h with [^{35}S]methionine. The changes in protein expression patterns that occur as a function of *tipA* and thiostrepton as visualized by SDS-PAGE were complex (Fig. 4). Although the thiostrepton response was noticeably different in exponential as compared with stationary phase cultures, this was not studied in detail. The exponential culture was more critically defined by automated analysis of two-dimensional PAGE patterns (gels not shown). Several thiostrepton-induced SDS-PAGE protein bands were observed in SL/pIJ486 (Fig. 4, compare exponential growth (*lanes 1* and *2*) or stationary phase (*lanes 5* and *6*)). Seventeen thiostrepton-induced two-dimensional PAGE protein spots were observed. Several of these bands, including TipAS, were not found in KT (compare exponential growth (*lanes 2* and *4*) or stationary phase (*lanes 6* and *8*)); these corresponded to eight two-dimensional PAGE protein spots. Finally, these analyses also revealed thiostrepton-repressed SDS-PAGE bands in both SL and KT; these corresponded to four two-dimensional PAGE spots in SL and two in KT.

TipA Proteins Reacted in Vitro with Thiopeptides Having Dehydroalanine-containing Tails but Different Ring Structures—TipAL and TipAS proteins were produced using an *E. coli* expression system. An *Nde*I/*Hin*dIII fragment of pNB2–5A1 containing *tipAS* was cloned into pDS8 (pMF101); an *Nde*I/*Hin*dIII fragment of pNB2-AL containing *tipAL* was cloned into pDS8 (pMF102) (22, 35–37). *E. coli* strains containing these constructions were able to produce large amounts of TipA proteins in a soluble form. These proteins were purified (see "Experimental Procedures") for *in vitro* studies.

Purified TipAS was mixed with thiostrepton, promothiocin A, promothiocin B, nosiheptide, or berninamycin (A and B) (Fig. 1) and then analyzed by SDS-PAGE (promothiocin B and thiostrepton are presented in Fig. 5*A*). All of these compounds formed very stable complexes which could not be disrupted in 1% SDS/1% 2-mercaptoethanol (boiling), 8 M urea, or 6 M guanidine hydrochloride. Their apparent molecular masses corresponded roughly to uncomplexed TipAS (~17 kDa) or TipAS bound to one molecule of thiopeptide (~18–19 kDa). ESI-MS experiments of TipAS or TipAL mixed with nosiheptide or promothiocin A confirmed that the TipA/thiopeptide stoichiometry was equimolar.[2]

[2] T. Holt and P. Griffin, unpublished results.

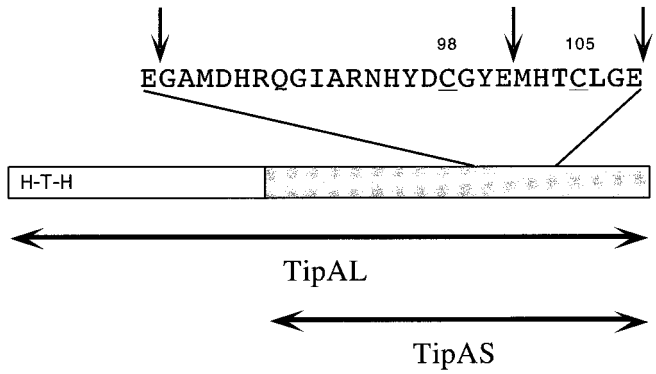

FIG. 2. **Primary structure of TipAS and TipAL proteins.** The N-terminal domain of TipAL has sequence similarity to other transcriptional regulators of the *merR* family (*soxR*, *bmrR*, *bltR*, and *ywnD* identified in the *Bacillus subtilis* genome sequencing project; Ref. 59) with putative helix-turn-helix motifs (*H-T-H*). TipAS and TipAL share a C-terminal domain containing the only two cysteines (Cys-98 and Cys-105). The fact that V8 peptidase cleaved (*arrows*) at a glutamate residue between the two cysteines allowed mapping of the modified residue to Cys-105 (Fig. 6).

Previous studies using thiostrepton had shown these complexes were generated by a covalent reaction between a dehydroalanine residue in thiostrepton and one of the two cysteine residues in the TipA proteins (23). Thiostrepton has one dehydroalanine residue in the ring and two in the tail (Fig. 1). In order to map the reactive dehydroalanine residues to the ring or tail, a tailless derivative of thiostrepton (thiostrepton B, Fig. 1) was tested. Thiostrepton (Fig. 5*A*, *lane 2*) but not thiostrepton B (Fig. 5*A*, *lane 4*) altered the mobility of TipAS (Fig. 5*A*, *lane 7*) on SDS-PAGE gels.

To confirm the generality of this observation, tailless promothiocin derivatives MN (tail replaced by an amide group) and MO (tail replaced by an *O*-methyl group) were prepared from promothiocin B (Fig. 1) and purified to greater than 99.9% homogeneity (see "Experimental Procedures"). Promothiocin B was chosen as representative thiopeptide since it was composed of a simple ring structure having no dehydro amino acids and a tail containing three dehydroalanines (Fig. 1). TipAS formed a higher molecular mass complex with promothiocin B (Fig. 5*A*, *lane 3*) in a reaction presumably analogous to that with thiostrepton. This effect was dependent on the tail structure. Pro-

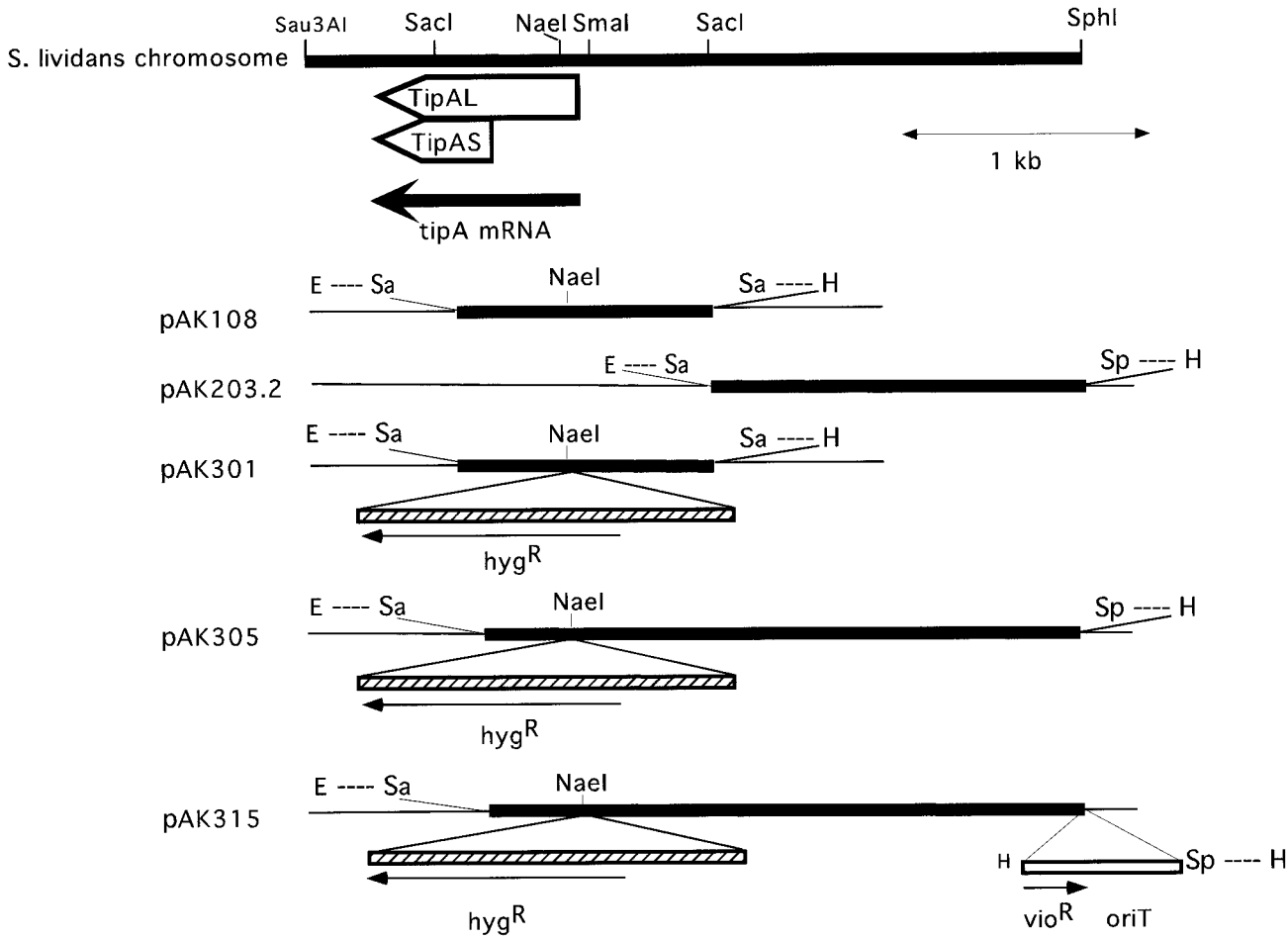

FIG. 3. **Construction of plasmids used for *tipA* gene disruption.** A 1.2-kb *Sac*I and the adjacent 1.8-kb *Sac*I-SphI fragment of *S. lividans* genomic DNA were cloned from λ clone AK1 (15) into pUC19 to generate pAK108 and pAK203.2, respectively. The hygromycin resistance gene (*hygR*) together with its promoter and terminators (*cross-hatched*; a 1.6-kb *Bam*HI fragment from pMZ19.16 (60) filled in using DNA polymerase I (60)) was subcloned into the pAK108 *Nae*I site (pAK301). pAK301 was partially digested with *Sac*I and completely with *Hin*dIII and ligated to the 1.6-kb pAK203.2 *Sac*I/*Hin*dIII fragment; pAK305). pAK305 was digested with *Hin*dIII and ligated to a cassette containing the RK2 origin of transfer (*oriT*) and viomycin resistance gene (*vioR*; pAK315). *E. coli* S17-1 was transformed with pAK315 and used as a donor strain for conjugation to *S. lividans* (28). *E*, *Eco*RI; *H*, *Hin*dIII; *Sa*, *Sac*I; *Sp*, *Sph*I.

mothiocin derivatives MN (Fig. 5*A*, *lane 5*) and MO (Fig. 5*A*, *lane 6*) were nonreactive with TipAS.

TipA Proteins Reacted in Vivo with Thiopeptide Antibiotics Having Dehydroalanine-containing Tails but Different Ring Structures—SDS-PAGE analyses of pulse-radiolabeled cultures demonstrated that various thiopeptides induced Tip proteins (Fig. 5*B*). TipAS-thiopeptide complexes, indistinguishable in size from those produced *in vitro* (Fig. 5*A*), were observed after induction using compounds having various ring structures including nosiheptide, berninamycin A and B, and promothiocins A and B (data not shown).

In order to test the requirement for the tail *in vivo*, thiostrepton- and promothiocin-induced proteins were compared with proteins induced by their tailless derivatives (Fig. 5). Tailless derivatives thiostrepton B, promothiocin MO, and promothiocin MN induced the synthesis of TipAS protein but not TipAS-antibiotic complexes. The absence of a TipAS complex after induction with thiostrepton B indicated that the dehydroalanine and dehydrobutyrine residues in the cyclic peptide domain did not participate in covalent TipAS complex formation. TipA proteins were not seen after the addition of any of these antibiotics to KT (data not shown).

Mapping the Reactive Cysteine in TipAS—When TipAS or TipAL were pretreated with *N*-ethylmaleimide, they did not form protein complexes with promothiocin (A and B), berninamycin (A and B), or nosiheptide that could be observed by SDS-PAGE (data not shown). This confirmed that at least one of the cysteines in the TipA proteins was necessary for covalent binding.

V8 protease digestion of TipAS-thiostrepton complexes defined which cysteine residue bound to thiostrepton. Cleavage at the glutamic acid between the only two cysteines in TipAS generated unique peptide fragments from both partial and complete digests. HPLC fractions of the protease digests of TipAS with TipAS-thiostrepton complex were analyzed with ESI-MS. Only the double and triple charged peptide fragment containing the second cysteine residue (Cys-105 in TipAS) had an additional molecular mass which corresponded to that of thiostrepton (Fig. 6*A*). The experimental mass to charge ratios (1197.4, 1228.4) corresponded well with the theoretical values (1197.9, 1228.7) of a peptide fragment containing Cys-105 reacted with one molecule of thiostrepton. The fragment containing Cys-98 had a mass corresponding to the peptide with its second cysteine unaltered (Fig. 6*B*). It had an experimental mass to charge ratio of (646.1, 966.8) which matched well with the corresponding theoretical values (967.4, 645.2). No peaks corresponding to thiostrepton-reacted Cys-105 peptide were found.

TipAL Ligand Specificity—Most cyclic thiopeptide antibiotics induce *ptip*A (15, 26, 27). Visual comparison of these structures (Fig. 1) suggested common motifs that could be important for induction efficiency: dehydroalanine or dehydrobutyrine

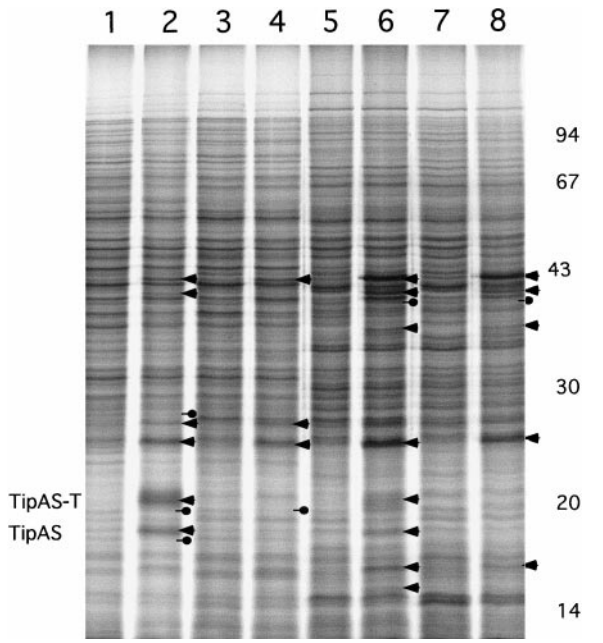

FIG. 4. **Thiostrepton- and *tipA*-dependent proteins defined by SDS-PAGE.** SL/pIJ486 and KT/pIJ486 cultures were grown in YEME medium for 15 h (*lanes 1–4*) or 40 h (*lanes 5–8*), and then induced with thiostrepton in Me_2SO (3 μg/ml) for 1 h in the presence of [^{35}S]methionine/cysteine (100 μCi/ml). Radiolabeled proteins were separated by SDS-PAGE (32, 33) and recorded on x-ray film. *Lanes 1* and *5*, SL, uninduced; *lanes 2* and *6*, SL induced with thiostrepton; *lanes 3* and *7*, KT uninduced; *lanes 4* and *8*, KT induced with thiostrepton. *Arrowheads* mark thiostrepton-induced bands. *Filled circles* mark thiostrepton-repressed bands.

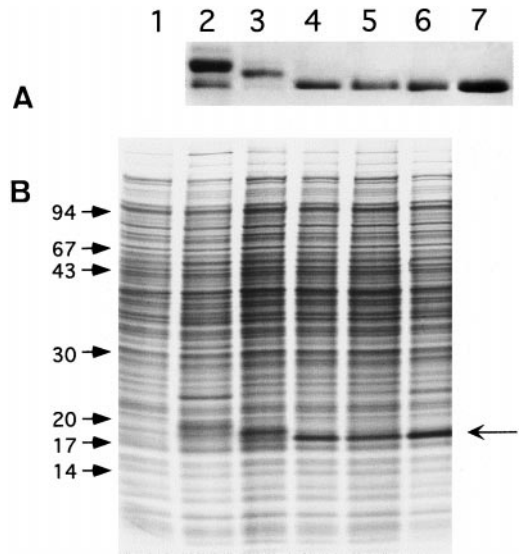

FIG. 5. **TipA-antibiotic covalent complex formation *in vivo* and *in vitro*.** Discontinuous Tricine-SDS-PAGE (33) was used to demonstrate the increase in mass of TipAS after reactions with thiopeptides *in vitro* (*A*) or *in vivo* (*B*). TipAS (*arrow* on *right* side of lower gel) migrated at ~17 kDa with respect to molecular mass markers. *A*, TipAS (60 pmol) (*lane 7*) was reduced with sodium borohydrate and treated with 3 nmol of thiopeptides: thiostrepton (*lane 2*), promothiocin B (*lane 3*), or their tailless derivatives thiostrepton B (*lane 4*), promothiocin MN (*lane 5*), or promothiocin MO (*lane 6*). Thiopeptides containing dehydroalanine tails increased the apparent molecular mass of TipAS from 17 to ~18–20 kDa. *B*, cultures of SL/pIJ486 were grown in YEME medium for 24 h at 30 °C. Two ml of this culture were transferred into a 15-ml Falcon tube at 30 °C, induced with different thiopeptides (25 mg/liter) for 5 min, and labeled with L-[^{35}S]methionine/cysteine (700 μCi) for 1 h. Radiolabeled proteins were separated by SDS-PAGE (33) and recorded on x-ray film. Lanes correspond to samples labeled after the addition of thiopeptides as in *A* above.

residues, quinaldic acid, thiazole, and pyridyl moieties. To screen compounds that might define the activity of these groups, plate or disc assays were adopted. Both were based on a reporter gene construction in which *ptipA* controlled the expression of the kanamycin resistance gene (pAK114; see "Experimental Procedures").

The minimal amount of various thiopeptides needed to allow *ptipA*-dependent kanamycin resistant growth (MinC) was semiquantified and thereby compared using the plate assay (Table I). MinC was defined as the minimal amount of thiopeptide needed to allow growth of SL/pAK114 on plates containing 10 mg/liter kanamycin. Thirteen of the 16 thiopeptides tested were active; however, the activity of different compounds varied considerably.

The MinC of the thiopeptides related to the number of dehydroalanine residues in the tail. The most active, promothiocin B (MinC = 0.6 ng/ml), has three dehydroalanine residues. Compounds with two (MinC = 1.2–2.4 ng) or one (MinC = 20–40 ng) dehydroalanines had progressively less activity. Thiopeptides whose tails had carboxyl termini were even less active (MinC = 40–80 ng), even though they contained four dehydroalanine residues. Compounds without dehydroalanine tails had reduced activity. In the case of thiostrepton, its activity was minimally reduced (about 3-fold) in its tailless analog, thiostrepton B. Chemical elimination of the promothiocin tail severely reduced its activity (more than 1000-fold) regardless of its remaining C-terminal group (amide in MN or carboxyl in MO).

Compounds that contained reactive vinyl groups (23) analogous to dehydroalanine were screened for *ptipA* induction using the disc assay. Acrylamide, cerulenin, fusidic acid, farnesol, *N*-ethylmaleimide, novobiocin, pristinamycin II, shikimic acid, and viomycin did not induce kanamycin-resistant growth. Nisin A, an antibiotic having several dehydroalanines, also did not induce kanamycin resistance.

In addition, analogs of residues in the ring structure including quinaldic acid (nalidixic acid, menadione), a pyridyl group (diketopiperazines, pyridine, pyralinamide, and aniline), or thiazole (thiazolidine carboxylic acid) did not induce *ptipA*.

DISCUSSION

The *tipA* gene was initially viewed as a simple, thiostrepton-induced activator of its own transcription (22). Here we show that *tipA* serves as a multipeptide sensor and antibiotic resistance gene. Furthermore, we demonstrate that thiostrepton, in addition to being a ribosome-specific antibiotic, has other biological effects involving induction of both *tipA* dependent and independent modulons.

The observation that all thiopeptides which induce *ptipA* irreversibly altered the size of the TipA proteins not only facilitated detection of drug/protein interaction but also may have functional implications. Covalent interaction of these antibiotics with TipAL apparently increased their potency as activators of the *tipA* promoter. However, SDS-PAGE studies showed that compounds unable to covalently bind to TipAS (thiostrepton B, promothiocin MO and MN) were nevertheless able to induce the promoter as well as synthesis of unmodified TipAS. This provided evidence that covalent TipA-antibiotic complex formation was not necessary for thiopeptide-induced expression *in vivo*. Previous *in vitro* studies using a thiostrepton-binding column showed that TipA proteins were able to specifically and reversible associate with this ligand (22) (in these experiments, dithiothreitol in the buffer would have covalently reacted with thiostrepton, and thus irreversibly blocked the dehydroalanine residues). Together, these results showed that covalent attachment via the dehydroalanine tail was not required for stable

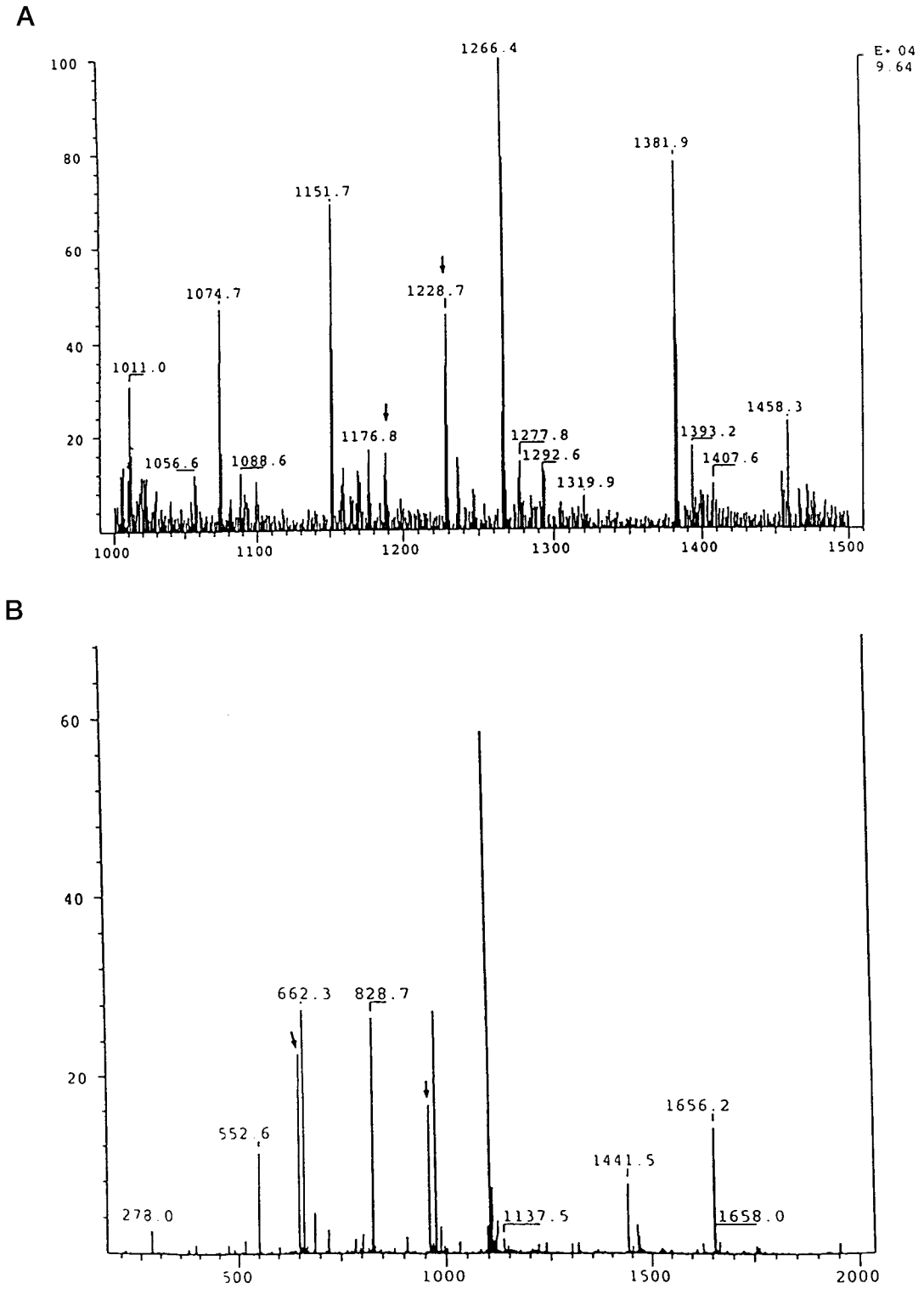

FIG. 6. **Electrospray ionization mass spectrometry of TipAS-thiostrepton peptides.** TipAS-thiostrepton complex was digested with V8 protease. Peptides were separated by C8 HPLC and analyzed by ESI-MS focusing on peptides containing either Cys-98 or Cys-105. A peptide fragment containing unaltered Cys-105 was not found. However, a fragment was identified having a mass to charge ratio (1197.4, 1228.4) that corresponded well with double and triple charged species of the Cys-105 peptide fragment reacted with one molecule of thiostrepton (mass to charge ratio values (1197.9, 1228.7) (*A* marked with *arrows*). A fragment was identified as the Cys-98 peptide by its charge ratio (observed, 646.1, 966.8; theoretical, 645.2, 967.4) (*B* marked with *arrows*). Peaks corresponding to a thiostrepton-modified Cys-98 peptide were not detected.

interaction *in vitro* or *in vivo*, and that the ring contained a TipA recognition structure.

Comparisons of the ring structures of active peptides (such as berninamycin, geninthiocin, promothiocins, nosiheptide, promoinducin, thiotipins, thioactin, thiostrepton, thioxamycin; representatives are shown in Fig. 1) with those which were not active (Fig. 7, amythiamicins, cyclothiazomycins, and GE 2270A), suggested a minimal motif for TipAL recognition involving thiazole and pyridyl groups. Active compounds had a pyridyl group with attached thiazoles or oxazoles at the 4- or 5-positions which in turn were extended by linear peptides. While inactive compounds (amythiamicin, cyclothiazomycin

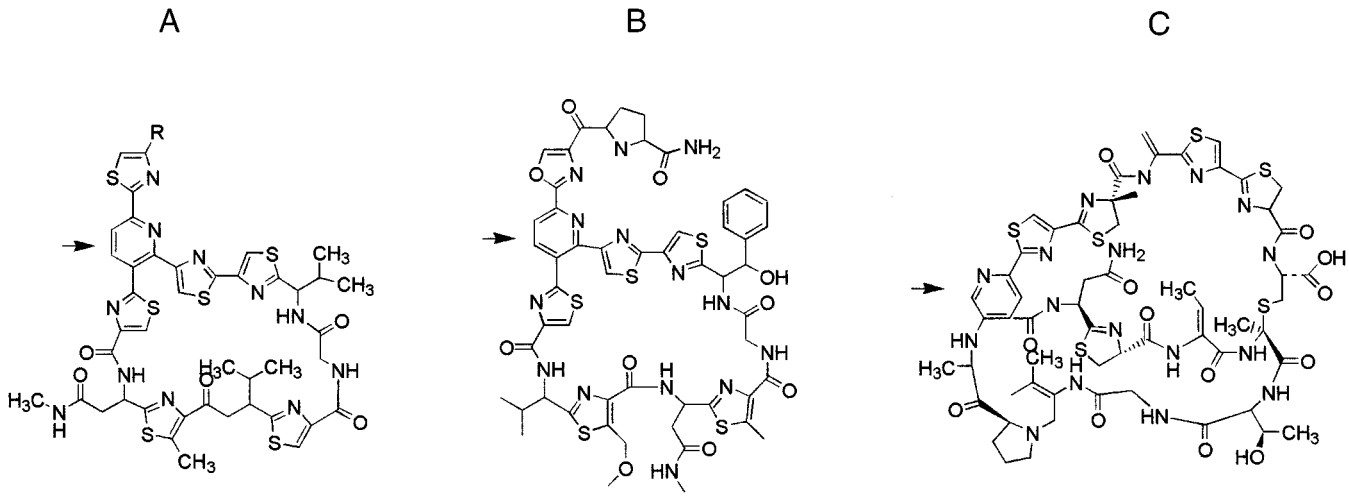

FIG. 7. **Chemical structures of thiopeptide noninducers of *ptipA*.** Noninducers of *ptipA* expression (*A*, amythiamicin A; *B*, GE2270A; *C*, cyclothiazomycin, amythiamicin A, GE2270A, and cyclothiazomycin) have pyridyl groups (marked with *horizontal arrowheads*) but lack linear peptide extensions to vicinal oxazoles or thiazoles. Cyclothiazomycin have pyridyl substitutions that are different from the thiostrepton family.

and GE 2270A) have thiazoles or oxazoles similarly bound to the pyridyl ring, they are extended by a bis-thiazole group rather than linear peptides. In addition, cyclothiazomycin is substituted at the 1-, 2-, and 5-positions of the pyridyl ring. These observations suggested a requirement for the pyridyl ring substitutions and the proximal thiazoles or oxazoles.

In a cyclic peptide, internal ring structures as well as dehydro amino acids can provide conformational constraint on the peptide backbone and restrict the orientation of the adjacent amino acid side chains (38). Thiostrepton, which contains two dehydro amino acids in its double ring, is not highly dependent on its dehydroalanine tail to provide activity *in vivo* (*i.e.* thiostrepton B is only slightly less active than thiostrepton itself). On the other hand, promothiocin does not have dehydro amino acids in its single ring structure and is more than 1000 times more active than its tailless derivatives (MO and MN). This suggests that weaker inductions by such compounds may reflect a lower affinity constant rather than differences in their effect on TipAL conformation. Covalent binding probably locks TipAL into an active form, thereby serving as an irreversible switch.

The mechanism of transcriptional activation employed by well characterized proteins similar to TipAL, MerR, and SoxR involves changes in the oxidation state of cysteine residues located near their C termini. MerR activation is dependent on a protein dimer-Hg complex involving three cysteines; SoxR activation results from oxidation of an iron sulfur complex (2Fe/2S) involving two cysteine residues/protein. TipAL contains two C-terminal cysteine residues involved in activation. Thiostrepton, and presumably other active thiopeptides, react covalently with one of these cysteine residues. These two cysteine residues might form a reversible disulfide bond or iron sulfur cluster that reacts to redox conditions, and thus serve as a sensor. Modification of this structure by dehydroalanine and introduction of a bulky ring structure might irreversibly lock the protein in the transcriptionally active form.

Thus, our studies show that thiopeptides employ two different functional groups to elicit their effect on the TipAL protein. These include specific protein recognition structures (a portion of the ring) and a chain of thiol-reactive groups that form the tail. The fact that two unusual functional groups are combined in these highly active thiopeptides and that they react primarily with a specific protein suggests a unified biological function, *i.e.* transcriptional activation of *ptipA*. It is appealing to think that active thiopeptides are not only antibiotics but may serve as, or perhaps mimic, a physiological or developmental signal (22).

The *tipA* serves as an autogenously controlled antibiotic resistance system. When the *tipA* gene was disrupted, the strain became more sensitive to thiostrepton, berninamycin A, and nosiheptide. In response to thiostrepton, TipAL activates synthesis of a single mRNA that includes TipAS (15), an antibiotic-inactivating protein that apparently is much more efficiently translated. TipAS sequestration of thiostrepton would eventually limit activation of the TipAL-dependent promoter and thereby provide a self-contained low-level antibiotic resistance system.

Higher concentrations of thiostrepton that could be resisted by strains containing the *tsr* gene elicited a *tipAL*-independent effect on gene expression as observed by SDS- or two-dimensional PAGE. Synthesis of many proteins changed after induction of the *tipA* defective strain indicating involvement of another stress response system. Dehydro amino acids of thiostrepton are highly reactive toward low molecular weight thiols that maintain the cytoplasmic redox potential. In Actinomycetes, these include ovothiol A, ergothionine, and U17 present in the cytoplasm at millimolar concentrations (39–42). It seems unlikely that thiostrepton levels routinely used in labeling experiments or for selection of *tsr*-containing vectors (~10 μM) could reduce the concentrations of these compounds stoichiometrically and thereby disrupt overall cellular thiol balance. Amplification might be achieved by reactions between dehydroalanine and thiol-dependent redox regulatory compounds or proteins (other members of the *tipA*/*soxR* family have been uncovered by the *Streptomyces coelicolor* genome sequencing project). Disruption of redox balance would compromise systems for protein folding (43) and scavenging oxygen free radicals (44). Effects on metabolic flux might be effected through modulation of certain redox dependent enzymes (45, 46) or reduction in NADPH or NADH (47) pools.

Decreases in the redox potential are known to elicit increased resistance to unrelated antibiotics and redox cycling agents. Thiostrepton induces resistance to antibiotics having different cellular targets including daunorubicin (48, 49), sparsomycin (50–52), tetranactin (53), and GE2270A (54, 55). Interestingly, daunorubicin, best known to interact with DNA, and sparsomycin, a ribosome inhibitor, disrupt the redox balance of the cell (56–58). Characteristics of the TipA proteins reported here, as well as its homology to MerR, SoxR, BmrR, and BltR, suggest that it may directly regulate resistance to

stress induced by heavy metals, antibiotics, or redox, a concept we are now exploring.

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Broad Spectrum Thiopeptide Recognition Specificity of the*Streptomyces lividans* TipAL Protein and Its Role in Regulating Gene Expression

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