A Transcriptional Regulator of a Pristinamycin Resistance Gene in Streptomyces coelicolor*

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Pip is a pristinamycin-induced transcriptional regulator protein detected in many Streptomyces species by its ability to specifically bind sequence motifs within the promoter of a Streptomyces pristinaespiralis multidrug resistance gene (ptr). To investigate the possible role of Pip in regulating multidrug resistance, it was purified from a genetically characterized species, Streptomyces coelicolor, utilizing an affinity matrix of the ptr promoter conjugated to magnetic beads. Reverse genetics identified the corresponding locus and confirmed that it encoded Pip, a protein belonging to the TetR family of procaryotic transcriptional repressors. Pip binding motifs were located upstream of the adjacent gene pep, encoding a major facilitator antiporter homologous to ptr. In vivo analysis of antibiotic susceptibility profiles demonstrated that pep conferred elevated levels of resistance only to pristinamycin I (PI), a streptogramin B antibiotic having clinical importance. Purified recombinant Pip was a dimer (in the presence or absence of PI) and displayed a high affinity for its palindromic binding motifs within the ptr promoter and the upstream region of pep. The Pip/ptr promoter complex was dissociated by PI but not by any of the other nonstreptogramin antibiotics that were described previously as transcriptional inducers. These procaryotic regulatory elements served as the basis for the development of systems allowing repression or induction of cloned genes in mammalian and plant cells in response to streptogramin antibiotics (including pristinamycin, virginiamycin, and Synercid®).

Streptomyces are Gram-positive mycelial soil bacteria that undergo a complex developmental program involving morphological differentiation coordinated with biosynthesis of a vast array of structurally diverse secondary metabolites. Although most are of unknown function, many have antimicrobial activity, making this genus the most abundant known source of antibiotics. Streptomyces require extensive collections of resistance genes and corresponding regulatory genes to protect themselves from these endogenous metabolites as well as those produced by competing species. These genes are believed to be the progenitors of resistance determinants acquired by pathogenic bacteria (1, 2) that are progressively eroding the efficacy of antibiotics.

The genetic control and mechanism of tetracycline resistance has been well characterized. The expression of tetA, encoding an integral membrane protein of the major facilitator superfamily (MFS)1 that exports tetracycline, is under the control of the TetR repressor. In the absence of tetracycline, transcription of tetA and the divergent tetR (3) is repressed by TetR. tetA is efficiently expressed only when TetR is released from its operator sites by its association with tetracycline or its analogs. Based on these characteristics, the laboratories of Hillen and Bujard have engineered systems for tetracycline-regulated gene expression in eucaryotic cells (4). An advantage of such systems is that the regulatory protein, along with its DNA recognition motif and ligand, are procaryotic and thus minimize pleiotropic effects within host regulatory circuits.

Pairs of genes homologous to tetR/tetA found within Streptomyces biosynthetic gene clusters serve to respond to and export the cognate antibiotics. For example, ActII-orf1 and TcmR repress promoters controlling divergent structural genes encoding proteins that export actioninhodin (actII-orf2-3) (5) or tetracenomycin (tcmA) (6). However, QacR, a TetR-like repressor of a multidrug resistance gene in Staphylococcus aureus, can bind heterogeneous compounds (7).

Resistance and corresponding regulatory genes present in organisms producing several antibiotics have potentially interesting multidrug recognition capabilities. Streptomyces pristinaespiralis produces the human oral streptogramin antibiotic Pyostacin® (8). Like other streptogramins, it is a mixture of two structurally dissimilar molecules, the streptogramin A component pristinamycin II (PII), a polyunsaturated macrolactone, and the streptogramin B component pristinamycin I (PI), a cyclic hexadepsipeptide (8). The water-soluble form of pristinamycin, Synercid®, recently approved in the United States and Europe for use against most multiple drug-resistant Gram-positive bacteria, is composed of dalfopristin, a 26-sulfonyl derivative of PII, and quinupristin, which is derived from PI by synthetic addition of a (5βR)-[(3S)-quinuclidinyl] thiomethyl group (9).

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1 The abbreviations used are: MFS, major facilitator superfamily; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PI, pristinamycin I; PII, pristinamycin II; bp, base pairs; PCR, polymerase chain reaction; pep, pristinamycin resistance gene from S. coelicolor; ptr, pristinamycin resistance gene from S. pristinaespiralis; tetA, tetracycline resistance gene; tetR, repressor of the tetracycline resistance gene.
A S. pristinaspiralis pristinamycin resistance gene, pptr, encoding a MFS antiporter was cloned in Streptomyces lividans where it provided an increase in resistance to not only PI and PII but also rifampicin (10). Interestingly, the pptr gene was not situated within the pristinamycin biosynthetic cluster (11, 12). It was surprising to find that many Streptomyces hosts, most not known to produce streptogramin antibiotics, nevertheless had systems to control the pptr promoter (13). Studies carried out in S. lividans and Streptomyces coelicolor showed that the pptr promoter (Pptr) was activated not only by PI and PII but also by a wide range of heterogeneous compounds (14). Gel retardation using a DNA fragment encoding the Pptr indicated the activity of a pristinamycin-induced DNA binding protein (Pip).

Preparation of Magnetic Beads Containing the Pptr Promoter Fragment—The promoter fragments were purified after digestion with HindIII and PspI and PpB promoters in E. coli by alkaline lysis and PEG 8000 precipitation (19). Fragments were precipitated in isopropanol and purified on a 1% TBE agarose gel. Fragments were precipitated in isopropanol and purified on a 1% TBE agarose gel.

Preparation of Pip—S. coelicolor J1501 (hisA1 uraA1 strA1 gcp1 SC18 [SCP2]) were used in these studies (17). Systems for cloning in Escherichia coli included pBluescript (Stratagen)XL1 Blue (Invitrogen). pGemT (Promega)XL Blue and M15pRep4(pDS56/RBSII) (18) (provided by Dr. D. Stuber).

Pptr fragment was then mixed with streptavidin-coated magnetic beads (Magnalife, Promega) and incubated with radiolabeled DNA fragments (2–5 ng) for 30 min at 50 °C. After migration, gels were incubated with 2 ml TGED (100 mM NaCl containing 100 μg of poly(dI-dC)poly(dI-dC)) of the Pptr fragment coupled to magnetic particles. The reaction mixtures were then resolved on a 219-bp subcloning it on a 228-bp M15(pRep4)/pDS56/RBSII (18) (provided by Dr. D. Stuber). The promoter fragments were labeled using [γ-32P]dATP and then dialyzed against TGED (100 mM NaCl), en- derived streptogramin antibiotics as controlling agents (15).2

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—S. lividans 1326 and S. coelicolor J1501 (hisA1 uraA1 strA1 gcp1 SC18 [SCP2]) were used in these studies (17). Systems for cloning in Escherichia coli included pBluescript (Stratagen)XL1 Blue (Invitrogen), pGemT (Promega)XL Blue and M15pRep4(pDS56/RBSII) (18) (provided by Dr. D. Stuber). The promoter fragments were purified after digestion with EcoRI and HindIII.

Gel Retardation Assays—The promoter fragments were labeled using [γ-32P]dATP and then incubated on a Quick spin column (Roche Molecular Biochemicals). DNA was precipitated in isopropanol and purified on a 1% TBE agarose gel. Purification of Pip—S. coelicolor J1501 was cultured in 350 ml of YEME. These cultures were then incubated for 20 h at 30 °C. Pip activity was monitored throughout using the gel retardation assay with radiolabeled Pptr fragment.

Cells were harvested from YEME (20 liters) by centrifugation (30 g of wet weight), washed in lysis buffer (200 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% (v/v) glycerol, 50 mM Tris, pH 8.0), resuspended with 150 100 mM NaCl, equilibrated with TGED and monitored by adsorption at 280 nm. The protein was eluted from the column with 10 volumes of buffer providing a linear gradient of NaCl (100–500 mM). Pip activity was observed in fractions containing ~200 mM NaCl. These fractions were dialyzed against TGED + 75 mM NaCl to bring the conductivity equal to that of 100 mM NaCl. The dialyze was then loaded at a rate of 1.5 ml/min onto a 25-ml Sepharose column (XK16/20; Amersham Pharmacia Biotech) equilibrated with TGED + 100 mM NaCl. The column was washed until a stable adsorption base line was attained, and the protein was eluted in a linear gradient of TGED + 500 mM NaCl (seven column volumes). Pip activity was observed in the eluant having conductivity corresponding to 230–300 mM NaCl. The active fractions were pooled and incubated in a suspension (3 ml of TGED + 100 mM NaCl, 300 μg of poly(dI-dC)poly(dI-dC) of the Pptr fragment coupled to magnetic particles. The mix was incubated at room temperature for 1 h while keeping the particles suspended. The particles were collected using a magnet, washed with 2 ml of TGED + 100 mM NaCl containing 100 μg of poly(dI-dC)poly(dI-dC) and 2 ml TGED + 250 mM NaCl. Pip was eluted in a step (1 ml) gradient ranging from 300 mM to 2.5 mM NaCl (in TGED). Column Chromatography—Apparent molecular weight was determined by column size chromatography (Superdex 75 SMART System) in comparison with protein molecular weight standards. Protein band was detected with putative ligand was separated on Superdex75 and further analyzed by HPLC reverse phase chromatography using a C18 column in a gradient of H2O/acetonitrile/trifluoroacetic acid 0.1%.

Peptide and N-terminal Analysis—After SDS-PAGE, proteins were stained with Coomassie blue in 40% methanol/1% acetic acid. The gel containing the protein bands were cut out, rinsed in water, and digested with porcine trypsin (Sigma). Peptides were separated by HPLC (HPLC, C18) in a gradient of acetonitrile/acetonitrile/trifluoroacetic acid 0.1% and then analyzed by Edmann degradation.

Isolation of the pip Gene—PCR reactions were performed in a thermal cycler (Biometra, Göttingen, Germany); reaction products were purified on 5% polyacrylamide TBE gels for analysis and cloning in pCR2 (Stratagene). Amplification of the Pip gene was achieved using degenerate primers corresponding to the two peptides (pep1 (DDEAGCGAAGCG-3) and direct repeats. This fragment was cloned into the PCR cloning vector pGEMT (Promega), and its sequence was confirmed. The cloned fragment was excised with XbaI/HindIII and cloned into pJ4856 (pRM156).

Expression and Purification of Recombinant Pip—The coding region of pip was amplified by PCR using the oligonucleotides P1 and P2 (P1: 5’-TCTGAGACATTTCTACGGTTGGTGGG-3’; P2: 5’-AAGCTTCGCCGCTTGCCTGGG-CCGGC-3’), tagged with a XbaI and HindIII recognition sequences (bold type), using the high fidelity Expand PCR system (Roche Molecular Biochemicals) under the conditions recommended by the manufacturer (but with the addition of 5% MeSO). The fragment was cloned into pGemT (Promega), and its sequence was confirmed. The cloned fragment was excised with XbaI/HindIII and cloned into pJ4856 (pRM156).

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mg/liter kanamycin. Expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 mM. Purification of the protein was done according to the procedure above omitting the DNA affinity step. The purified protein retained full activity for more than a year when stored at −80 °C in 10% glycerol.

Drug Resistance Measurements—Antibiotic resistance was measured on NE medium (11) using either a disc (discs provided by Pasteur Diagnostics) diffusion assay (zones of inhibition were measured after 48 h at 30 °C) or as the concentration of antibiotic lethal for colonial growth (scored after 1 week at 30 °C).

Ultracentrifugation—Sedimentation velocity and sedimentation equilibrium centrifugations were carried out at 20 °C using a solution of 1 mg/ml protein in a TA buffer (10 mM Tris, pH 7.5, 10 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Triton, 10% glycerol) in a XLA Beckman analytical ultracentrifuge equipped with adsorption optics. The sedimentation velocity run was made in a 12-mm double sector cell at 54,000 rpm and scanned at 277 nm. The sedimentation equilibrium run was carried out in the same cell at a rotor speed of 18,000 rpm. Both sectors, one with the protein solution and one with the reference, were filled to a height of 2.5 mm on top of the Fc-43 bottom fluid. The molecular masses were evaluated using a floating base-line computer program that adjusts the base-line absorbance to obtain the best linear fit of ln A versus r² (A = absorbance; r = radial distance). A partial specific volume (v) of 0.73 was assumed. The solution density (1.034 g/ml) and viscosity (1.30 centipoise) were taken from the CRC Handbook.

RESULTS

Purification of Pip from S. coelicolor—Purification of Pip from PI-induced S. coelicolor cultures was performed using various combinations of standard column chromatography. Initial studies established a convenient protocol using DEAE/SP-Sepharose chromatography to enrich Pip. Pip activity was detected by gel mobility shifts resulting from Pip binding to the three operator sites in the ptr promoter (details found under “Experimental Procedures”).

The final purification step, which allowed Pip to be convincingly identified and sequenced, was based on a Pptr DNA affinity matrix (Fig. 1A). The Pptr affinity matrix was prepared from biotin end-labeled Pptr fragment conjugated to streptavidin-coated magnetic beads (“Experimental Procedures”). The Pip-enriched fractions from SP-Sepharose were incubated with the affinity beads. The beads were then sedimented with a magnet and washed repeatedly in low salt buffer. Proteins were stepwise eluted in buffers containing increasing salt concentration (Fig. 1B). Only minor amounts of Pip activity eluted in a buffer containing poly(dI-dC)-poly(dI-dC) (Fig. 1B, lane 3); many proteins were observed by SDS-PAGE in this fraction (Fig. 1A, lane 3). Most Pip activity eluted in fractions of higher salt concentration (Fig. 1B, lanes 4 (300–400 mM), and 5 (600 mM–2.5 M NaCl)). A 28-kDa protein, the major band detected in these high salt washes, coeluted with Pip activity. The Pptr affinity matrix allowed final purification of 10 μg of Pip from ~100 mg of crude protein extract.

Cloning of the S. coelicolor pip gene—The 28-kDa protein was eluted from an SDS-PAGE gel and digested with trypsin to generate peptide fragments that were isolated by HPLC and subjected to N-terminal analysis by Edmann degradation. Four peptide sequences were obtained: pep1, DaVWLOEGR; pep2, HPDPDAGLD; pep3, PWS5SR; and pep4, VAEMLDR. The longest peptides, pep1 and pep2, were also identified in tryptic digests of Pip similarly purified from S. lividans (data not shown). A 500-bp PCR fragment, amplified using the degenerate oligonucleotide primers P1 and P2, was used to probe Southern blots of BamHI-digested S. coelicolor genomic DNA. A hybridizing fragment having a convenient size (~5.5 kilobase pairs) was isolated from the gel and cloned into the BamHI site of pUC18 (pIPs15). Crude extracts prepared from pRM515 transformants had Pip activity in the gel retardation assay (data not shown).

Nucleotide Sequence Analysis of the pip Locus—Nucleotide sequence (accession number AF193856) within the 5.5-kilobase pair BamHI fragment provided further proof that it encoded Pip, the ptr transcriptional regulator. The pip gene sequence predicted a protein with a pI (5.8), mass (28 kDa), and 259 amino acids, and peptide sequences (N-terminal as well as pep1–4) indistinguishable from Pip purified from crude extracts.

The Pip protein sequence was most closely related to RifQ, a putative transcriptional repressor in the rifamycin biosynthetic cluster of Amycolatopsis mediterranei (106/243, 43% identical amino acids) (20), and ActII-orf1, a repressor in the actinorhodin biosynthetic pathway (38 of 122, 31% identical amino acids) (5). The N-terminal region of these proteins contained a helix-turn-helix motif that fitted the consensus of the tetracycline resistance repressor family (TetR) (3).

The nucleotide sequence upstream of the pip gene predicted a transcriptionally coupled open reading frame (Fig. 3; pep) whose amino acid sequence had significant similarity to the MFS 14-spanner drug antiporters (21). The best matches were to streptogramin resistance genes: the S. pristinae spiralis pristinmycin resistance gene (encoding Prr, 73% amino acid identity) (10) and the Streptomyces virginiaeensis virginiamycin resistance gene (encoding VarS, 67% amino acid identity) (22). Nucleotide homology between the pep and ptr loci extended for at least 200 bp upstream of their coding sequences (Fig. 2). Pep had strong similarity (65% identical amino acids) to RifP, encoded by an open reading frame immediately upstream of rifQ (20). Many other proteins having 25–40% identity with Pep were involved either with antibiotic export in Streptomyces.
that produce various antibiotics or with multidrug resistance in diverse bacteria (21).

Regulatory Pip binding motifs identified within the Pptr (13) were also found in the corresponding region upstream of pep (Fig. 2). Only two of the three pip operator sites in the Pptr promoter were conserved in the putative region of S. coelicolor pep promoter (Ppep). The regions of the Ppep which aligned with the Pptr promoter hexamers (Fig. 2) were well conserved (6 of 6 in the –35 hexamer and 5 of 6 in the –10 hexamer). No obvious promoter sequences or ribosome binding sites were detected within the nucleotides separating the end of the pep open reading frame and the pip start codon (37 bp). These data suggested that Pip was located in a position that would allow it to both control pep and to autoregulate its own expression.

In Vivo Functional Analysis of pep—The pep gene along with 205 bp of upstream sequence was cloned into pIJ486 (pRM516) and introduced into S. lividans. Transformants were assayed by disc diffusion tests for antibiotic susceptibility against a spectrum of 60 structurally and functionally dissimilar antibiotics including bacitracin, chloramphenicol, clindamycin, erythromycin, fusidic acid, lincomycin, PI, PII, rifampicin, spiramycin, streptomycin, and tetracycline. The pep gene conferred a detectable elevated resistance only to PI. Subsequent determination of the minimal inhibitory concentration (10 μg/ml) on solid medium showed a 4-fold increase in resistance to PI for strains harboring pRM516 (40 μg/ml).

Pip Sequence-specific Interactions—Recombinant Pip purified from E. coli extract had chromatographic characteristics (on DEAE and heparin) similar to the native protein purified from S. coelicolor. Moreover, both bound specifically to repeated sequence motifs present within Pptr and Ppep having the consensus RTACRSYGTAY. The gel retardation pattern generated by Pip corresponded to the degree of site occupancy, a total of three discrete retarded bands for the Pptr fragment (Fig. 3A, points A–H) as previously documented (13), and two for the Ppep fragment (Fig. 3B, points A–H). These titration profiles on each promoter can be interpreted as a noncooperative binding to each of the operator sites. The binding curve determines an approximate $K_d$ of $10^{-9}$ M.

Ligand Recognition Specificity of Pip—The ligand specificity of Pip was established using gel retardation to test the ability of purified recombinant Pip to bind either Ppep fragment (Fig. 4) or Pptr fragment (data not shown) in the presence of various antibiotics. Pip was released from the Ppep by equimolar amounts of PI. Quinupristin, a chemically modified PI derivative, apparently had 3 orders of magnitude less affinity for Pip. In contrast, a series of other antibiotics that induce the Pptr in vivo, including bacitracin, chloramphenicol, clindamycin, erythromycin, fusidic acid, lincomycin, PI, PII, rifampicin, spiramycin, streptomycin, and tetracycline, did not significantly affect the Pip operator binding (representative results are shown in Fig. 4). These data showed that the PI component of the streptogramin antibiotic complex was the preferred Pip ligand. A binding curve (Fig. 5) representing the inhibition of shift as a function of increasing PI approximates a $K_d$ of $10^{-6}$ M.

Pip (PI-treated or untreated) eluted from a sizing column with an apparent molecular mass of ~50 kDa, indicating a dimeric nonaggregated form under native conditions (Fig. 6). Fractions from the sizing column containing PI-treated or untreated Pep were then analyzed by reverse phase chromatography (Fig. 6) under conditions expected to denature Pep and release the ligand. Indeed, a compound that comigrated with PI...
was detected only in the PI-treated Superdex fraction. Approximate quantification of the HPLC peaks suggested an equimolar binding stoichiometry between the native protein monomer and PI. This proved that the interaction was of a noncovalent nature allowing a reversible association of the antibiotic ligand with this regulatory protein.

**Ultracentrifugation**—Sedimentation equilibrium data predicted Pip to have a molecular mass of 64 kDa (Fig. 7A). This was not significantly different (the significant difference of these measurements is ~5%) from the predicted mass of a Pip dimer (57,032 Da). The addition of PI (Fig. 7B) did not change its measured mass (60 kDa).

Sedimentation velocity analyses (Fig. 7) showed that recombinant Pip purified from *E. coli* was >90% soluble, presumably in its native conformation. Its sedimentation coefficient in the absence of PI ($s_{20,w} = 3.4$) was not significantly altered by the addition of PI ($s_{20,w} = 3.5$).

Thus, Pip was dimeric, and PI did not generate detectable changes in multimerization (dimer) or shape (Pip $f/f_0 = 1.70$; Pip + PI $f/f_0 = 1.58$). Although PI was presumably complexed to Pip, in the absence of dramatic shape changes, the additional mass (853 Da) would not be expected to have a detectable effect on its sedimentation velocity or sedimentation equilibrium-measured molecular weight.

**DISCUSSION**

Pip proved to be a protein with homology and function similar to the TetR transcriptional repressor that recognizes a streptogramin B antibiotic rather than tetracycline. The affinity of Pip for PI measured in vitro ($K_d = 10^{-6}$ M) was similar to that of TetR for tetracycline ($K_d = 10^{-4}$ M). However, in vivo, tetracycline is likely to be complexed with divalent cations such as Mg$^{2+}$ that increase the affinity of tetracycline for TetR more than 1000-fold (23). This lower affinity of Pip for PI in vitro may reflect the absence of a cofactor or imply the existence of an alternative natural ligand in vivo. However, Pip had the same drug recognition specificity for PI as its associated antibiotic resistance gene, Pep. These observations proved that Pip did not mediate multidrug activation of *Pptr* transcription through direct interactions with structurally heterogeneous ligands.
Multidrug resistance can be dependent on global regulators of general stress responses such as sigma factors or transcriptional activators. Mutations in sigma factors that mediate stress responses can decrease antibiotic resistance in \textit{S. aureus} \cite{24} and \textit{E. coli} \cite{25}. In \textit{E. coli}, batteries of efflux systems having overlapping specificity provide resistance to hydrophobic compounds \cite{26}. Several of these loci encode an efflux protein and divergently transcribed TetR homolog \cite{26}. In at least one well characterized system, \textit{acr}, transcription of the transporter gene involves the AraC-like transcriptional activator proteins \textit{marA}, \textit{soxS}, and \textit{rob} \cite{27, 28}. In \textit{Neisseria gonorrhoeae}, a comparable multidrug resistance locus including a TetR-like protein (AcrR), is also activated by an AraC-like protein \cite{29}.

Pip may play a subordinate role as a secondary modulator in a similar multidrug responsive regulatory system. Two Pip homologs, AcrR and MtrR, are not required for initial transcriptional activation during the multidrug resistance response. Instead, they act primarily as repressors, apparently limiting growth inhibitory effects resulting from overexpression of their corresponding MFS pumps \cite{30}. Although compounds that release AcrR and MtrR repressors from their binding sites are unknown, we have identified a specific antibiotic (PI) as a Pip ligand.

Many antibiotic resistance genes are linked to antibiotic biosynthetic clusters in \textit{Streptomyces} \cite{31}. This does not appear to be the case for \textit{pep}. Streptogramins (PI-like) have not been detected in \textit{S. coelicolor}. In addition, its adjacent sequence (4 kilobase pairs downstream of \textit{pep}; \textit{S. coelicolor} cosmid D13)$^3$ did not indicate linkage to known antibiotic biosynthetic genes. Although Pep may be an independent drug resistance element that responds to and provides resistance specifically to PI, its genetic organization was unlike homologous antibiotic resistance loci of Gram-negative organisms.

Hillen and Berens \cite{3} have shown that the characteristic divergent polarity of TetR regulators and MFP genes is important for inducibility to toxic compounds. The arrangement of operator sites having different TetR affinities between the promoters provides for differential expression of the two genes. This apparently allows transcription of \textit{tetR} that is sufficient to effectively repress \textit{tetA} under noninducing conditions. The second characteristic feature of this arrangement is that the \textit{tetA} promoter is fully activated in response to a narrow range of tetracycline concentration \cite{3}. In contrast, the coupled transcriptional organization of \textit{pep} and \textit{pip} does not provide for such an amplified response of the resistance gene relative to its repressor.

Similar linkage of antibiotic export genes to those encoding Pip/TetR-like regulators has been observed in antibiotic biosynthetic gene clusters including: tetracenomycin (\textit{tcmR/tcmA}) \cite{32}, actinorhodin (\textit{actII-orf1/actII-orf2} and \textit{orf3}) \cite{33}, rifamycin (\textit{rijP/rijQ}) \cite{20}, and landomycin (\textit{lanJ/lanK}) \cite{16}. Thus, antibiotic-producing \textit{Streptomyces} species are a rich source of similar regulatory elements responding to a wide variety of structurally diverse secondary metabolic compounds. These elements may have important future applications.

Studies reported here have lead to the design of a family of novel systems for regulated expression of cloned genes in mammalian (15) and plant cells.\textsuperscript{2} These systems are based on bind-

\begin{figure}[h]
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\includegraphics[width=\linewidth]{fig7}
\caption{Analysis of Pip multimerization by ultracentrifugation. Sedimentation equilibrium of Pip (A) or a Pip/PI complex (B) was done for 92 min at 18,000 rpm at 20 °C and scanned at 277 nm. Sedimentation velocity of Pip (C) or a Pip/PI complex (D) was carried out at 54,000 rpm at 20 °C and scanned at 277 nm.}
\end{figure}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Operator} & \textbf{Regulator} & \textbf{Effect} \\
\hline
\textit{pep} & AcrR & Repressed \\
\textit{pip} & MtrR & Repressed \\
\hline
\end{tabular}
\caption{Summary of antibiotic resistance genes and regulators.}
\end{table}

\begin{itemize}
\item R. P. Morris and C. J. Thompson, unpublished data.
\end{itemize}
ing of Pip, or Pip fused to eucaryotic transcriptional activators or repressors, to its operators engineered into eucaryotic promoter sequences. Such constructions have been used to achieve streptogramin-regulated induction or repression of various therapeutic proteins in diverse cell lines. The streptogramin-based expression technology was functionally compatible with a tetracycline-regulated system, thus enabling the selective use of different antibiotics to independently control two different gene activities in the same cell. These may serve as important tools in controlling the timing and levels of gene expression in plant cells, mammalian cells, transgenic animals, and perhaps future human gene therapies.

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