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Cloning, Purification, and Properties of a Phosphotyrosine Protein Phosphatase from *Streptomyces coelicolor* A3(2)

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We describe the isolation and characterization of a gene (*ptpA*) from *Streptomyces coelicolor* A3(2) that codes for a protein with a deduced M_r of 17,690 containing significant amino acid sequence identity with mammalian and prokaryotic small, acidic phosphotyrosine protein phosphatases (PTPases). After expression of *S. coelicolor ptpA* in *Escherichia coli* with a pT7-7-based vector system, PtpA was purified to homogeneity as a fusion protein containing five extra amino acids. The purified fusion enzyme catalyzed the removal of phosphate from *p*-nitrophenylphosphate (PNPP), phosphotyrosine (PY), and a commercial phosphopeptide containing a single phosphotyrosine residue but did not cleave phosphoserine or phosphothreonine. The pH optima for PNPP and PY hydrolysis by PtpA were 6.0 and 6.5, respectively. The K_m values for hydrolysis of PNPP and PY by PtpA were 0.75 mM (pH 6.0, 37°C) and 2.7 mM (pH 6.5, 37°C), respectively. Hydrolysis of PNPP by *S. coelicolor* PtpA was competitively inhibited by dephostatin with a K_i of 1.64 μM; the known PTPase inhibitors phenylarsine oxide, sodium vanadate, and iodoacetate also inhibited enzyme activity. Apparent homologs of *ptpA* were detected in other streptomycetes by Southern hybridization; the biological functions of PtpA and its putative homologs in streptomycetes are not yet known.

Streptomycetes are gram-positive, aerobic, mycelium-forming bacteria that undergo a complex morphological differentiation that includes the sequential production of substrate mycelium, aerial mycelium, and spores (9). Concomitant with morphological differentiation, these organisms undergo a biochemical differentiation during which they produce economically important secondary metabolites. Several studies have been carried out indicating that protein phosphorylation takes place in *Streptomyces coelicolor* and other streptomycetes (14, 33, 42, 47), and a protein kinase that phosphorylates serine and threonine residues in vitro was discovered recently (33). Using monoclonal anti-phosphotyrosine (PY) antibodies 4G10 and IG2, Waters et al. (47) recently showed evidence for protein tyrosine phosphorylation in *Streptomyces hygroscopicus*, *Streptomyces griseus*, *Streptomyces lividans*, and *S. coelicolor*. Furthermore, some unidentified protein tyrosine kinase activities were also observed in extracts of these strains (47), suggesting the presence of proteins in streptomycetes that contain PY residues. We report here the isolation of a gene (*ptpA*) from *S. coelicolor* A3(2) encoding a small, acidic PY protein phosphatase (SA-PTPase) and characterization of its product, PtpA, which has several characteristics similar to those of homologs characterized from mammalian systems. Although PtpA was shown to possess PTPase activity in vitro, its biological function in *S. coelicolor* is still unknown.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Streptomyces azureus*, *Streptomyces peucetius*, and *Streptomyces insignis* were obtained from the American Type Culture Collection as ATCC strains 14921, 29050, and 31913, respectively. *Streptomyces* sp. strain C5 was obtained from the Frederick Cancer Research Center (2). *S. lividans* TK24 and *S. coelicolor* A3(2) strain 1147 were obtained from David A. Hopwood (24). *Escherichia coli* JM83 was used to propagate plasmids for sequencing and restriction analysis. *E. coli* K38/pGP1-2 (39) was used for pT7-7 (45) expression experiments.

Media and growth conditions. *E. coli* was grown in Luria broth (LB; 32) unless otherwise stated. M9 medium (32) was used for pulse-labeling. The enriched medium used for overexpression of PtpA in *E. coli* contained 20 g of Bacto-Tryptone, 10 g of yeast extract, 5 g of NaCl, and 2 g of glycerol per liter and 50 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.2). Plasmids were introduced into *E. coli* by transformation by standard procedures (32). For *E. coli* strains harboring plasmids, the following appropriate antibiotics were added to the media: kanamycin, 75 μg/ml; and ampicillin, 50 μg/ml. *Streptomyces* strains normally were grown in YEME medium (24) supplemented with 34% (wt/vol) sucrose. Methods for handling the *Streptomyces* cultures and routing genetic manipulations were as described previously (24).

Cloning and sequencing of *ptpA*. Chromosomal DNA was isolated from *S. coelicolor* A3(2) 1147 by standard methods (24). A 6.1-kbp *Bam*HI DNA fragment from *S. coelicolor* A3(2) was shot-gun cloned in pUC19 to form pANT305 and was isolated by its ability to hybridize with a 1.0-kbp *Sac*I DNA fragment from *S. insignis* ATCC 31913 containing the *asaA* locus that was previously shown to confer actinorhodin overproduction in *S. lividans* (44). A ca. 2.2-kbp *Sac*I DNA fragment located upstream of the *S. coelicolor asaA* homolog was shot-gun cloned from *S. coelicolor* A3(2) into pUC19 to form pANT333 by using the ca. 0.5-kbp *Bam*HI-*Sac*I fragment from the far left end of the 6.1-kbp *Bam*HI fragment as a probe for in situ hybridizations (Fig. 1).

A 1.7-kbp *Sac*I-*Bam*HI fragment was purified from pANT333 and subcloned into pUC19 to form pANT335 (Fig. 1). A series of nested deletions was generated within the inserts of plasmids pANT333 and pANT335 as described by Henikoff (23), and both strands of the insert DNA were sequenced by the dideoxy chain termination method (40) with Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio), double-stranded templates, and α-thio-^{35}S-dCTP (Dupont-New England Nuclear, Boston, Mass.). 7-Deaza-dGTP was substituted for dGTP to reduce compressions. Homology searches were carried out with BLAST (1), and sequence alignments were optimized with the PILE-UP and LINE-UP algorithms within the University of Wisconsin Genetics Computer Group programs (12).

Construction of the *ptpA* expression plasmid. The 686-bp *Mlu*I-*Bam*HI fragment containing the *S. coelicolor ptpA* gene was purified and used as the template in PCRs for the generation of *ptpA* containing appropriate restriction sites at both ends. The primers synthesized were 5′-GTGAATTCCGATGACCTACC GCGTCTG-3′ for the 5′ end (containing an *Eco*RI site) and 5′-GTGGATCCG CAGCCCGGCCCGCACCGCG-3′ for the 3′ end (carrying a *Bam*HI site). The amplified DNA fragment was digested with *Eco*RI and *Bam*HI and ligated into pUC19 to make pANT383. The nucleotide (nt) sequences of four individual synthesized *ptpA* constructs were confirmed by sequencing. A clone containing the proper sequence was subcloned into pT7-7 to make the expression construct pANT384.

Pulse-labeling of PtpA. Cultures of *E. coli* K38(pGP1-2/pANT384) were grown overnight in 1 ml of LB containing kanamycin and ampicillin at 30°C. A 50-μl aliquot of this culture was added to 2 ml of fresh LB containing kanamycin and

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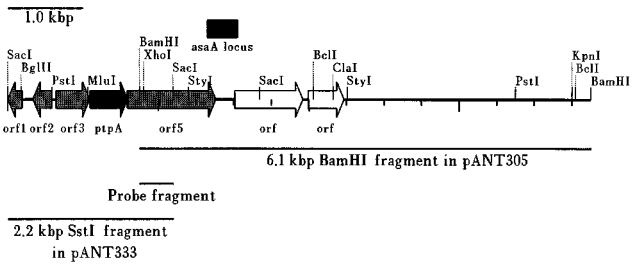

FIG. 1. Restriction map of *S. coelicolor* DNA described in this study. The 6.1-kbp *Bam*HI DNA fragment of *S. coelicolor* was originally isolated by Southern hybridization to *S. insignis asaA*. The 2.2-kbp *Sac*I DNA fragment containing *ptpA* was obtained using the marked 0.5-kbp *Bam*HI-*Sac*I fragment as a probe. Three complete ORFs (*orf-2*, *orf-3*, and *ptp-A*) and two partial ORFs (*orf-1* and *orf-5*) are contained in the sequenced 2.2-kbp *Sac*I fragment described in the text. The region responsible for the activation of sporulation and antibiotic biosynthesis (30) is labeled the *asaA* locus.

ampicillin, and the mixture was shaken for ca. 3 h at 30°C to an optical density (OD) at 590 nm of 0.4. Cells from 1 ml of this culture were collected by centrifugation and washed five times with 1 ml of M9 medium before resuspension in 2 ml of M9 medium containing kanamycin and ampicillin plus 0.02% (wt/vol) of 18 amino acids (all natural amino acids except cysteine and methionine). The cultures were incubated for another 60 to 180 min at 30°C and then incubated at 42°C for 20 min with shaking for heat induction. Rifampin was added at a final concentration of 200 μg/ml, and the culture was kept at 42°C for another 15 min. The culture was then incubated again at 30°C for 20 min. L-[^{35}S]methionine (40 μCi in 4 μl) was added to the culture, and at time points between 5 min and 1 h, aliquots of 0.5 ml were removed, pelleted by centrifugation, and resuspended in 50 μl of cracking buffer (60 mM Tris-HCl [pH 6.8], 1% [vol/vol] 2-mercaptoethanol, 10% [vol/vol] glycerol, 0.01% [wt/vol] bromophenol blue, 1% [wt/vol] sodium dodecyl sulfate [SDS]). Before loading, the sample was boiled for 3 min; 10- to 20-μl samples were loaded on the gels. After electrophoresis, the polyacrylamide gel was sprayed with intensifier (Dupont-New England Nuclear) and then dried. X-ray film (X-Omat; Kodak, Rochester, N.Y.) was exposed to the dried gel overnight at −70°C with an intensifying screen.

Enzyme assays. Acid phosphatase activity was monitored by end point assays in a buffer composed of 100 mM sodium citrate (pH 6.0), 1.0 mM EDTA, and 20 mM *p*-nitrophenylphosphate (PNPP) as previously described (49). The assay was optimized with respect to protein concentration and time so that the end points were all run within linear regions.

PTPase activity was determined by an end point assays in a reaction mixture containing 10 mM L-tyrosine phosphate, 100 mM sodium citrate (pH 6.5), and 1 mM EDTA at 37°C as previously described (35). The molar extinction coefficient for phosphate at 750 nm is 19,000 $M^{-1} \cdot cm^{-1}$ (30); the assay was optimized with respect to protein concentration and time so that the end points were all run within linear regions.

PTPase activity of PtpA on a commercially synthesized phosphopeptide (NH_2-T-S-T-E-P-Q-Y[PO_4]-Q-P-G-E-N-L-COOH; Upstate Biotechnology Inc., Lake Placid, N.Y.; tyrosine phosphatase assay kit 2 [21]) was determined in a solution containing 1 mM bis-Tris (pH 6.5) buffer and phosphopeptide in the concentration range of 0 to 750 μM. The reactions were started by adding the purified PtpA (range, 0 to 1.8 μg) at 37°C for 40 min in a final volume of 25 μl. The reactions were stopped by the addition of 100 μl of malachite green solution, which was followed by incubation at room temperature for 15 min. The free phosphate released from the peptide was determined by measuring the A_{630} according to the manufacturer's instructions. This assay was not optimized with respect to time, since shorter time periods resulted in activities approaching the lower limit of detection.

Purification of PtpA. For purification of PtpA, a single colony of *E. coli* K38(pGP1-2/pANT384) was used to inoculate 50 ml of LB containing kanamycin and ampicillin, which was incubated overnight at 30°C with shaking at 250 rpm. Ten milliliters of this overnight culture was used to inoculate 500 ml of enriched medium containing both kanamycin and ampicillin, which was shaken at 30°C for 4.5 h until an OD at 590 nm of 1.5 was reached. Expression of *ptpA* was induced by shifting the temperature to 42°C for 40 min and then by further incubation at 28°C for another 3 h. Cells were collected and washed once with 40 ml of lysing buffer (25 mM Tris-HCl [pH 7.5], 25 mM sucrose, 1 mM $MgCl_2$, 0.1 mM ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid [EGTA], 10% [vol/vol] glycerol). The cells were collected and resuspended in 20 ml of lysing buffer plus 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mg of leupeptin and 10 μg of aprotinin per ml (complete lysing buffer).

All subsequent protein purification procedures were carried out at 4°C. The mycelia were broken with a French pressure cell at 15,000 lb/in^2. The supernatant was collected after centrifugation at 10,000 × *g* for 30 min. Proteins in the supernatant were fractionated with ammonium sulfate precipitation. The proteins precipitated in the 40-to-60% (wt/vol) fraction were collected by centrifugation and dissolved in a minimum volume of complete lysing buffer containing dithiothreitol and the protease inhibitors. This protein solution was desalted with Sephadex G-25 NAP-5 (Pharmacia Biotech, Inc., Piscataway, N.Y.), and the final protein concentration in the desalted solution was adjusted to 30 mg/ml with lysing buffer. The desalted protein solution was then loaded onto a Superose-12 fast protein liquid chromatography gel filtration column (Pharmacia), and the proteins were eluted in a 2× volume of elution buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1 mM EGTA) at a flow rate of 0.5 ml/min. The active fractions were collected, pooled, and concentrated by ultracentrifugation through a PM10 membrane (Amicon, Inc., Beverly, Mass.). The concentrated protein (ca. 0.1 mg/ml) was loaded onto a Mono Q HR 10/10 column (Pharmacia) equilibrated with 20 mM Tris-HCl (pH 8.0). The protein bound on the column was washed with 40 ml of 20 mM Tris-HCl (pH 8.0) buffer and eluted by increasing the NaCl concentration gradually in a combination linear-step gradient of 0% NaCl (25 min), 0 to 15% NaCl (10 min), 15% NaCl (25 min), 15 to 30% NaCl (20 min), 30% NaCl (16 min), 30 to 100% NaCl (20 min), and 100% NaCl (8 min) at a flow rate of 1 ml/min. PtpA activity was monitored with PNPP (pH 6.0) as described previously, and the fractions with the highest activity of acid phosphatase were collected. The homogeneity of the purified PtpA was confirmed by SDS-12% (wt/vol) polyacrylamide gel electrophoresis (PAGE [28]) and then by Coomassie blue R-250 staining. PtpA concentrations were determined by the dye-binding method described by Bradford (6).

Protein sequencing. Purified PtpA was run on an 15% SDS-PAGE gel, transferred to a polyvinylidene difluoride membrane, and sequenced by automatic Edman degradation as previously described (29).

pH optimum. Acid phosphatase (with PNPP as substrate) and PTPase (with PY as substrate) activities at different pH values were compared with the following buffers: for pH 5.0 to 5.7, 100 mM sodium acetate; for pH 6.0 to 6.5, 100 mM sodium citrate; and for pH 7.0 to 7.5, 100 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES). All reactions were carried out at 37°C.

Kinetic and inhibition constants. The kinetics of the acid phosphatase and PTPase activities of PtpA were determined at 37°C at the optimum pH for each reaction, i.e., pH 6.0 for acid phosphatase and pH 6.5 for PTPase. On the basis of preliminary experiments, nine substrate concentrations were used that spanned the concentration range of approximately 0.5× to 10× the K_m.

The inhibition profiles of the acid phosphatase and PTPase activities by different chemicals were determined by first exposing the enzymes to the required concentrations of the chemicals in the reaction buffers for 2 min on ice. For inhibition assays, the reactions were initiated by the addition of 10 mM PNPP or 10 mM L-tyrosine phosphate at 37°C. Four inhibitor concentrations and four substrate concentrations were used for the determination of K_i values. Duplicate sets of data were obtained at each concentration.

Materials. All primers were obtained from the Ohio State Biochemistry Instrumentation Center. Unless otherwise noted, all biochemicals and enzymes were purchased from Sigma Chemical Co. (St. Louis, Mo.). L-[^{35}S]methionine and α-thio-^{35}S-dCTP were purchased from Dupont-New England Nuclear (Boston, Mass.).

Nucleotide sequence accession number. The nucleotide sequence described in this paper has been deposited with GenBank under accession number U37580.

RESULTS

Gene cloning. The 2.2-kbp *Sac*I DNA fragment containing *ptpA* was isolated by DNA walking upstream from the *S. coelicolor asaA* locus (Fig. 1) (30), the *S. insignis* homolog of which was previously shown to confer actinorhodin antibiotic overproduction on *S. lividans* (44). This DNA was located to *Ase*I fragment H (17 fragments in total) of the *S. coelicolor* chromosomal physical map (26a, 27). Figure 2 shows the nucleotide sequence of the 2.2-kbp *Sac*I fragment containing *ptpA*.

Analysis of ORFs. FRAME (4) and CODON PREFERENCE (52) computer algorithms indicated the presence of a partial open reading frame (ORF) and a complete ORF reading right to left and two complete ORFs and a partial ORF reading divergently left to right within this 2.2-kbp sequence. A sequence of 74 nt separates the divergently reading genes.

The partial *orf*, designated *orf-1*, and *orf-2* and *orf-3* appear to encode membrane-spanning proteins as determined by hydrophobicity plots of their amino acid sequences (data not shown). However, the deduced products of *orf-1*, *orf-2*, and *orf-3* are not closely related to any sequence in the databases as determined by BLAST (1) analysis.

The fourth gene, which is here named *ptpA*, encodes a 164-amino-acid protein with a deduced M_r of 17,690 that has 46, 38,

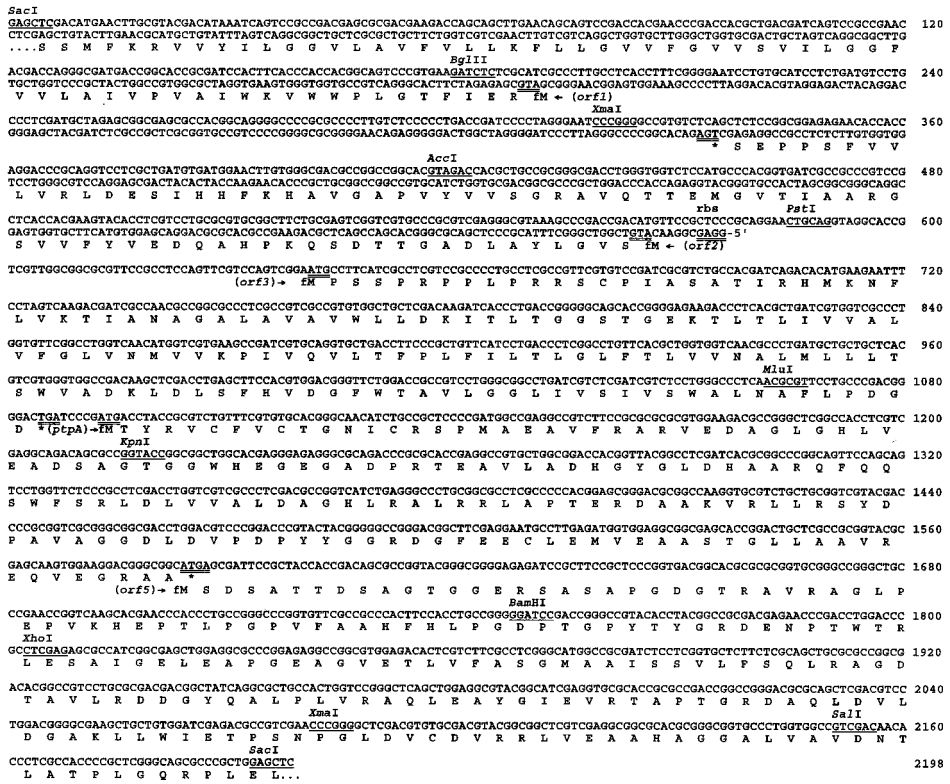

FIG. 2. Nucleotide sequence of the 2,198-bp *Sac*I DNA fragment containing *ptpA* identified in Fig. 1. For the genes reading right to left, the second strand reading in the opposite direction has also been included. When double stranded sequence is given, the top strand reads in the direction 5′ to 3′. The deduced amino acid sequences of the proposed translation products are also given below the nucleotide sequence. The numbers at the right indicate nucleotide positions with respect to the beginning *Sac*I site. Potential ribosome-binding sites and initiation and stop codons are doubly underlined, and major restriction sites are singly underlined. Stop codons are indicated by asterisks.

and 37% amino acid sequence identity with SA-PTPases from *Schizosaccharomyces pombe* (34), human erythrocytes (49), and bovine heart tissue (50), respectively (Fig. 3), as well as a high degree of similarity with several other small, acidic phosphatases from mammalian and prokaryotic sources (Fig. 3). In the N-terminal region, in which the active site cysteine is located, the sequence identity between PtpA and bovine heart SA-PTPase is ca. 81% (17 of 21 identical residues). *S. coelicolor* PtpA contains 4 cysteine residues, only 2 of which are conserved with the mammalian SA-PTPases. Both of the

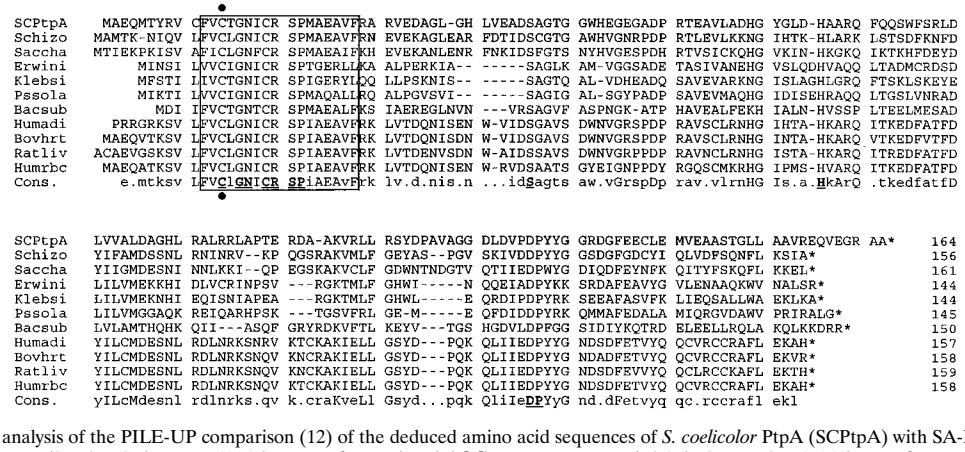

FIG. 3. LINE-UP analysis of the PILE-UP comparison (12) of the deduced amino acid sequences of *S. coelicolor* PtpA (SCPtpA) with SA-PTPases from *S. pombe* (Schizo [34]), *S. cerevisiae* (Saccha; SwissProt P40347), *E. amylovora* (Erwini [7]), *K. pneumoniae* (Klebsi; GenBank D21242), *P. solanacearum* (Pssola; GenBank U17898), *B. subtilis* (Bacsub; SwissProt P39155), human adipocytes (Humadi [41]), bovine heart (Bovhrt [50]), rat liver (Ratliv [31]), and human erythrocytes (Humrbc [13]). The consensus sequence (Cons.) generated by LINE-UP is given at the bottom. Underlined residues are identical in all proteins, capitalized residues are found in at least 8 of 11 proteins, and residues in lowercase type were found in a majority of the proteins. The highly conserved active-site Cys residues (FVCxGNICRSPx AEAxF) for these types of PTPases are N-terminally positioned (see box; the active-site cysteine [references 11 and 53] is indicated with a bullet). Dashes indicate gaps generated by PILE-UP. The parameters used were 3.0 for gap weight and 0.1 for gap length weight.

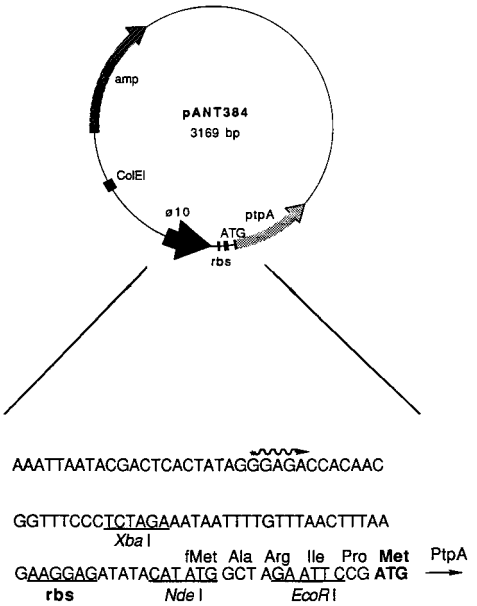

FIG. 4. Structure of pANT384. This plasmid was constructed by inserting the PCR-generated *ptpA* DNA sequence into vector pT7-7 for the expression of *S. coelicolor* PtpA in *E. coli* through a translational fusion. The transcriptional initiation site from vector pT7-7 is marked by an asterisk and wavy arrow. The translational start codon from the vector corresponds to formylmethionyl (fMet), the first amino acid in the recombinant product. The ATG of the sixth codon is the original translational start site of *ptpA*. rbs, ribosome-binding site.

conserved Cys residues are located in the active-site region (11).

The apparent translation initiation codon of *S. coelicolor ptpA* is separated from the stop codon of upstream *orf-2* only by 5 nt. A presumed weak ribosome-binding site (GGA) is located 8 nt upstream from the ATG initiation codon of *ptpA*, within the 3′ end of *orf2*. Both the relative positioning and the strength of this Shine-Dalgarno sequence for ribosome binding are consistent with those of other streptomycete genes (43). At the 3′ end, *ptpA* apparently overlaps the downstream gene, *orf-5*, by 4 nt. The partial *orf* immediately downstream of *ptpA* encodes the N terminus of a deduced protein with 34% amino acid identity with *Saccharomyces cerevisiae* Cys3 (36) and *E. coli* MetB (15).

Protein expression and purification. For the expression of *S. coelicolor ptpA* in *E. coli*, a fusion protein was generated by using the *E. coli* expression vector pT7-7 (45). A modified *S. coelicolor ptpA* gene was synthesized by PCR, was sequenced for verification, and was subcloned into the expression vector to make pANT384 (Fig. 4). The fusion PtpA overexpressed by pANT384 was designed to add five extra amino acids (fM-A-R-I-P-) to the N-terminal end of the protein by the sequence between the ATG start codon in the vector sequence and the newly introduced *Eco*RI site used for subcloning (Fig. 4). Consideration was also given to *E. coli* codon usage, since some difficulties have been previously observed in expressing some streptomycete proteins in *E. coli* (18). Overexpression of a polypeptide with an M_r of about 21,000 was observed after pulse-labeling (Fig. 5A). The molecular weight of the purified fusion polypeptide as measured by SDS-PAGE was bigger than that predicted from the sequence of the fusion protein (predicted M_r, 18,258) (Fig. 5B).

Most of the recombinant fusion PtpA was present in inclusion bodies at the original temperature used for the experiment (32°C); however, when the cultures were grown at 28°C, the amount of soluble, fusion PtpA increased severalfold. The enzymatic activity of the PtpA fusion product was initially assayed by its ability to cleave PNPP at pH 5.0. However, since *E. coli* also contains several acid phosphatases (38), there was significant host background activity. Nevertheless, recombinant *S. coelicolor* PtpA activity in cultures grown at 28°C conferred a threefold-greater level of activity than total host acid phosphatase activity, and the clone-specific activity was easily monitored during purification.

The PtpA fusion protein was purified to homogeneity from heat-induced cultures of *E. coli*(pANT384) by precipitation in the 40 to 60% fraction with ammonium sulfate, filtration through Superose-12, and elution with 30% (wt/vol) NaCl from a Mono Q anion-exchange column. Approximately 200 μg of pure protein (Fig. 5B) was obtained from 100 ml of recombinant culture. The N-terminal sequence of the purified fusion protein was Ala-Arg-Ile-Pro-Met, exactly as predicted, minus the N-terminal formylmethionine, from the deduced amino acid sequence of the recombinant PtpA-fusion protein (Fig. 4), indicating that it was the recombinant product rather than a host acid phosphatase.

Identification of enzymatic activity. Purified recombinant fusion PtpA cleaved PY with a specific activity of ca. 1.0 μmol/min · mg (pH 6.0) but did not cleave either phosphoserine or phosphothreonine to the detectable limit of the assay. The pH optima for cleavage of PNPP and PY by the purified enzyme were found to be 6.0 and 6.5, respectively (30); all subsequent enzyme assays were run at optimal pH values.

To determine if PtpA could cleave a known mammalian PTPase substrate, a commercial phosphopeptide was used as substrate for the recombinant fusion PtpA. Recombinant PtpA weakly dephosphorylated the phosphopeptide NH_2-T-S-T-E-P-Q-Y[PO_4]-Q-P-G-E-N-L-COOH (21) at pH 6.5 in a protein-dependent (Fig. 6) and substrate-dependent (30) manner. However, no hydrolysis activity above background was detected at pH 7.4 (30), the pH recommended by the manufacturer for use with mammalian PTPases. This pH requirement

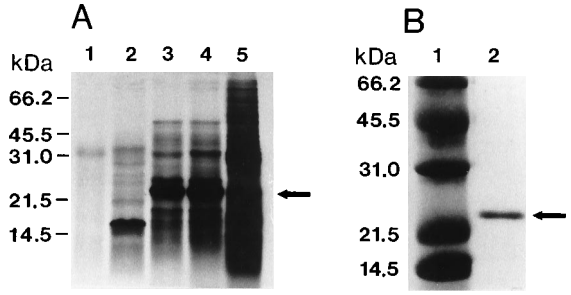

FIG. 5. PtpA expression in *E. coli*. (A) Autoradiogram of pulse-labeled *S. coelicolor* PtpA expressed in *E. coli*(pGP1-2/pANT384) in the presence or absence of rifampin. The positions of molecular mass standards (bovine serum albumin [66.2 kDa], hen egg white ovalbumin [45.5 kDa], bovine carbonic anhydrase [31.0 kDa], soybean trypsin inhibitor [21.5 kDa], and hen egg white lysozyme [14.5 kDa]) are indicated at the left. Lanes: 1, *E. coli*(pGP1-2/pT7-7) control induced in the presence of rifampin (no protein was significantly labeled, indicating that the gene expression was abolished; 2, a mutant allele of *ptpA* generated by PCR inserted into pT7-7 and introduced into *E. coli*(pGP1-2) to serve as a negative control (upon induction in the presence of rifampin, this mutant plasmid was shown to give a translational product smaller than that expected for PtpA); 3 and 4, *E. coli*(pGP1-2/pANT384) induced at 42°C (labeled PtpA is indicated by the arrow); 5, proteins labeled in the absence of rifampin treatment. (B) SDS-PAGE of purified PtpA, stained with Coomassie brilliant blue R-250. The size standards (lane 1) used were the same as those in panel A. Lane 2, purified recombinant fusion PtpA after the Mono Q step. The single band corresponding to *S. coelicolor* PtpA is indicated by an arrow.

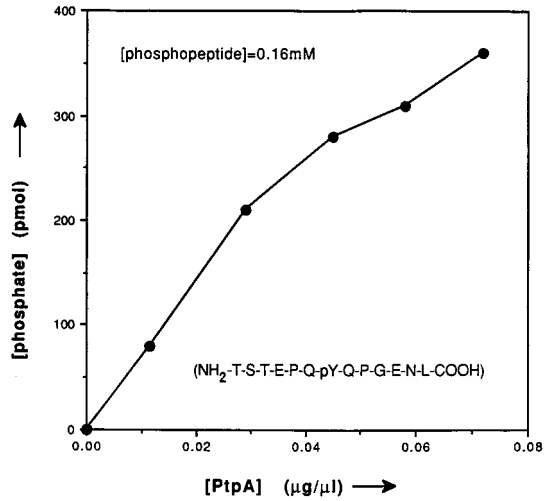

FIG. 6. Ability of *S. coelicolor* PtpA to dephosphorylate the commercial phosphopeptide NH_2-T-S-T-E-P-Q-Y[PO_4]-Q-P-G-E-N-L-COOH. Orthophosphate released from the phosphopeptide in 60 min at 37°C (pH 6.5) was analyzed as a function of PtpA concentration in this assay. Each datum point is the result of duplicate assays.

is consistent with the pH optimum with PY and PNPP as substrates.

Kinetic features. The K_m for recombinant fusion *S. coelicolor* PtpA with PNPP as substrate was determined to be 0.75 mM (pH 6.0; 37°C), which is similar to that for the bovine heart PTPase with the same substrate (50). The maximum rate of velocity (V_{max}) for PTPase with PNPP as substrate was 4.85 μmol/min · mg. With PY as substrate, the K_m and V_{max} were determined to be 2.7 mM (pH 6.5; 37°C) and 2.16 μmol/min · mg, respectively.

Inhibition assays. Specific inhibitors of PTPases, orthovanadate (17), dephostatin (25), phenylarsine oxide (16, 54) and iodoacetic acid (54), were tested for their abilities to inhibit the cleavage of PY by purified recombinant PtpA. All inhibitors were effective, although at widely different concentrations. With the same concentrations of purified PtpA (0.027 μg/μl) and substrate (10 mM PY), the concentrations of inhibitors required to inhibit enzyme activity by 50% were approximately 0.3 μM for dephostatin, 0.02 mM for phenylarsine oxide, 0.8 mM for orthovanadate, and 5 mM for iodoacetic acid. Dephostatin was found to be a competitive inhibitor of recombinant, fusion PtpA with a K_i of 1.64 μM for cleavage of PNPP.

Homologs of *ptpA* in other streptomycetes. A 183-bp DNA fragment (from nt 1039 to 1221; Fig. 2) containing the 5′ end of *S. coelicolor ptpA* (which includes the most highly conserved region and active site) was used in Southern hybridization experiments to probe the DNA of other streptomycetes to determine whether they contain apparent homologs of this gene. *Streptomyces* sp. strain C5 and *S. insignis* each contained a single *Sac*I DNA fragment of 5.1 kbp to which the probe bound at high stringency (0.4× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]; 0.1% SDS), *Streptomyces azureus* contained a 3.1-kbp *Sac*I fragment to which the probe hybridized, and *S. coelicolor* contained only the 2.2-kbp *Sac*I fragment shown in Fig. 1 to which the probe hybridized. *S. lividans* TK24 contained three *Sac*I fragments (2.2, 3.6, and 5.0 kbp) which hybridized to the probe at high stringency, suggesting that multiple homologs of this gene may exist in that strain. No *ptpA* homolog was observed in *Streptomyces peucetius* DNA by Southern analysis under the conditions tested (30).

DISCUSSION

A gene was found in the chromosomal DNA of *S. coelicolor* that encodes an SA-PTPase (PtpA). PtpA represents the first example of a streptomycete PTPase and is one of the few examples of prokaryotic proteins proven to exhibit PTPase activity. A gene (*iphP*) encoding a PTPase with a molecular mass of ca. 30 kDa has been cloned from the cyanobacterium *Nostoc commune* UTEX 584 (37). The enzyme, IphP, which displayed both protein phosphoserine and PTPase activities (37), has significant sequence similarity with vaccinia virus VH1 (19), another phosphatase with dual substrate specificity (37). The *N. commune* PTPase may be located extracellularly (37), suggesting that it may not be involved in a signal transduction pathway. A second bacterial PTPase previously described is *Yersinia pseudotuberculosis* YopH (20), an extracellular protein encoded by a plasmid-borne gene that functions to dephosphorylate host PY proteins (5). It has been postulated (37) that *Y. pseudotuberculosis* YopH, a proven virulence determinant (5, 20), might have arisen from a eukaryotic source through lateral gene transfer. *S. coelicolor* PtpA does not have significant sequence identity with either *N. commune* IphP or *Y. pseudotuberculosis* YopH. Not only is PtpA much smaller than the other two prokaryotic PTPases, but the conserved active site of PtpA is N-terminally located, whereas the conserved active sites of IphP and YopH are located toward the C-terminal portions of these proteins (20, 37).

Very recently, a gene (*amsI*) encoding a small phosphatase was found to be clustered with exopolysaccharide biosynthesis genes in *Erwinia amylovora* (7). The gene product, AmsI, was postulated to function in regeneration of undecaprenol monophosphate, the inner membrane lipid carrier involved in exopolysaccharide biosynthesis (7). *S. coelicolor* PtpA is closely related to *E. amylovora* AmsI (Fig. 3 and 7).

S. coelicolor PtpA and *E. amylovora* AmsI belong to a family of highly conserved, small M_r, <20,000), acidic PTPases (10, 22, 56) (Fig. 3 and 7). SA-PTPases contain an N-terminally located, highly conserved active site (FVCxGNICRSPxAE AxF) (Fig. 3). Analog-probing experiments indicate that SA-PTPases contain an active-site Cys residue; a thioester is formed as an enzyme bound intermediate in the reaction (50). X-ray crystallography of the bovine heart PTPase (53) recently confirmed that Cys-12 is the nucleophilic active-site Cys residue and that Arg-18 is in the active site, as is Asp-129, which is hypothesized to donate a proton to the leaving group (55). SA-PTPases have been found in a wide variety of mammalian tissues, including human erythrocytes (13, 49), bovine heart tissue (50), human placenta tissue (46), bovine liver tissue (8), human adipose tissue (41), chicken muscle tissue (3), and rat liver tissue (31). Additionally, a gene encoding an SA-PTPase was recently cloned from the fission yeast *S. pombe* by screening for clones that rescued temperature-sensitive *cdc25-22*, a protein phosphatase involved in the regulation of mitosis (34). A partial gene for an SA-PTPase homolog also was found clustered with genes encoding phycobilisome chromoproteins in the cyanobacterium *Synechococcus* sp. strain WH8020 (48). The sequences for genes encoding SA-PTPase homologs in *Bacillus subtilis*, *Pseudomonas solanacearum*, and *Klebsiella pneumoniae* (Fig. 3 and 7) have been submitted to GenBank. Analysis of this family of proteins reveals that the *E. amylovora* (7), *P. solanacearum*, and *K. pneumoniae* SA-PTPases, all of which are found associated with exopolysaccharide biosynthesis genes (7 [GenBank accession numbers D21242 and U17898]), are similar, that the mammalian SA-PTPases form a tight cluster, and that the two yeast SA-PTPases are more similar to each other than to other SA-PTPases (Fig. 7). *S.*

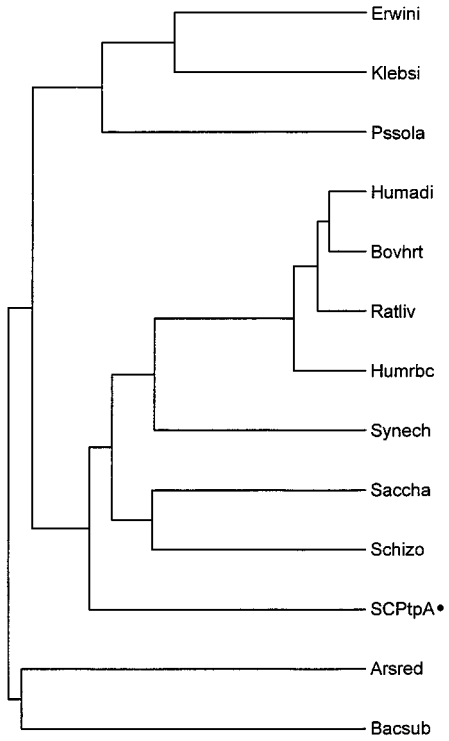

FIG. 7. Dendrogram of PILEUP analysis (12) of SA-PTPases from different sources showing similarity within the group. Abbreviations for SA-PTPases from each source are as follows: *S. coelicolor* PtpA, SCPtpA (see bullet); *S. pombe*, Schizo (34); *S. cerevisiae*, Saccha (SwissProt P40347); *E. amylovora*, Erwini (7); *K. pneumoniae*, Klebsi (GenBank D21242); *P. solanacearum*, Pssola (GenBank U17898); *B. subtilis*, Bacsub (SwissProt P39155); human adipocytes, Humadi (41); bovine heart, Bovhrt (50), rat liver, Ratliv (31); human erythrocytes, Humrbc (13); *Synechococcus* species, Synech (48); and arsenate reductase, Arsred (26). The parameters used were 3.0 for gap weight and 0.1 for gap length weight.

coelicolor PtpA falls within the extended group containing the mammalian and yeast enzymes. The *B. subtilis* SA-PTPase homolog, clustered with genes of the *spoIIR* locus (SwissProt P39155), on the other hand, is more similar to arsenate reductase (26) than to the other SA-PTPases (Fig. 7). The presence of SA-PTPase homologs in such a wide variety of prokaryotes raises interesting questions about their evolution and origin, especially since they seem to be clustered with such different types of genes in the different groups of prokaryotes. However, of this group of prokaryotic genes, only the product of *S. coelicolor ptpA* has thus far been shown to contain in vitro PTPase activity.

PtpA dephosphorylated a commercial peptide containing a single PY residue, a strong indication that it is capable of dephosphorylating PY residues in proteins. *S. coelicolor* PtpA also hydrolyzed PY but did not show any activity against phosphoserine or phosphothreonine, indicating that, at least among the substrates tested, it exhibits specificity for phosphotyrosyl residues. Waters et al. (47) recently demonstrated the putative presence of PY-containing proteins in several streptomycetes, including *S. coelicolor*. Moreover, we have found that dephostatin and phenylarsine oxide, both of which are specific inhibitors of PTPases which inhibited PtpA in vitro, inhibit growth of *S. coelicolor* in minimal medium (30, 51), suggesting that PtpA activity may be vital to the growth of *S. coelicolor*.

The biological function of PtpA is currently unknown. *S. coelicolor ptpA* is potentially translationally coupled with the upstream gene *orf-3* because there is only a 5-nt gap between these two genes (Fig. 2), and the 3′ end of *ptpA* overlaps the downstream gene, *orf-5*, which appears to encode a protein involved in sulfur amino acid metabolism. Considering that in bacteria, genes with related functions often are grouped together in operons, we speculate that *S. coelicolor* PtpA may be involved in the regulation of sulfur amino acid metabolism.

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