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NOTES

The *Streptomyces coelicolor* Polynucleotide Phosphorylase Homologue, and Not the Putative Poly(A) Polymerase, Can Polyadenylate RNA

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A protein containing a nucleotidyltransferase motif characteristic of poly(A) polymerases has been proposed to polyadenylate RNA in *Streptomyces coelicolor* (P. Bralley and G. H. Jones, Mol. Microbiol. 40:1155-1164, 2001). We show that this protein lacks poly(A) polymerase activity and is instead a tRNA nucleotidyltransferase that repairs CCA ends of tRNAs. In contrast, a *Streptomyces coelicolor* polynucleotide phosphorylase homologue that exhibits polyadenylation activity may account for the poly(A) tails found in this organism.

The sequencing of genomes of microorganisms has provided important information about microbial gene function and expression, not only for the subject organism but also for other microorganisms through comparison of DNA and protein sequence relationships. Classification of gene products into functional groups based on sequence similarity is commonly employed in the annotation of genomic DNA sequencing data and often enables the prediction of a locus' function (see, for example, reference 27). However, assignment of biological role on the basis of DNA or protein sequence alone may be problematical; whereas sequence comparisons may identify orthologous loci and discover evolutionary relationships among genes, related proteins may carry out dissimilar functions in different species, and conversely, dissimilar proteins may perform the same function.

Polyadenylation at the 3′ ends of RNA molecules is a post-transcriptional process that occurs in all three phylogenetic domains and has been shown to prominently affect a number of cellular processes, including RNA transport, translation, splicing, and RNA stability (for reviews, see references 8 and 39). Polyadenylation affects RNA stability in vivo (12, 14, 31, 46), in part by accelerating transcript digestion by 3′-5′ exoribonucleases (40, 45). In *Escherichia coli*, polyadenylation is carried out principally by poly(A) polymerase I (PAP I; EC 2.7.7.19), which can add poly(A) in vivo to both primary transcripts (4, 12, 14, 31, 45, 46) and their decay intermediates (4, 28, 45). PAP I is a member of the nucleotidyltransferase (NTF) superfamily, which also includes tRNA NTF (EC 2.7.7.25), also known as CCA-adding enzyme (15, 48). Different members of the NTF superfamily have distinctly different biochemical actions despite very considerable sequence homology; thus, it has not been practical to infer the enzyme action of NTF superfamily members from their structural features (48). NTF superfamily members classified as tRNA NTFs specifically add or repair CCA triplets at the 3′ ends of tRNAs (7), whereas family members designated as PAPs can add continuous stretches of principally adenosines to the 3′ ends of almost any RNA (4, 28). Determination of the specific function of the individual members of the NTF superfamily must therefore be dependent on analysis of the enzymatic activities. It is noteworthy that *E. coli* PAP I can use all four nucleoside triphosphates for making long homoribopolymers in vitro and also shows high binding activity to poly(U), poly(A), and poly(C) [but not poly(G)] (47). This lack of preference for adenosines is also seen in vivo when the enzyme is overexpressed beyond its usually tightly regulated steady-state level (28). Neither the gene for PAP I nor tRNA NTF is an essential gene in *E. coli* (26, 49).

While the genomes of certain microorganisms, including *Bacillus subtilis* and *Synechocystis* strain PCC6803, do not encode a PAP of the NTF family (34, 37), they nevertheless contain polyadenylated RNAs (10, 37, 38). Polyadenylation also occurs in PAP I mutant strains of *E. coli* (20, 30), and in this case it is mediated, at least in part, by polynucleotide phosphorylase (PNPase; EC 2.7.7.8) (29). This protein, which was originally discovered by its ability to catalyze the formation of polyribonucleotides and later shown to function in vivo largely as a 3′-5′ phosphorylytic exoribonuclease (11), more recently was shown to have functional overlap in *E. coli* with tRNA NTF and PAP I (29, 36). Interestingly, PNPase-like proteins can not only remove poly(A) tails both in vivo and in vitro (37, 44, 45) but also add poly(A) tails in vivo to mRNA in *E. coli* (29), *Synechocystis* strain PCC6803 (37), and plants (23).

Streptomyces species are GC rich (~72%) gram-positive bacteria that exhibit a morphologically and physiologically complex development (5). These soil-living bacteria produce a variety of antibiotics and other medically and agriculturally important secondary metabolites. The genome of *Streptomyces*

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coelicolor recently has been sequenced and annotated (1), facilitating use of this species as a model organism for global analysis of altered gene expression during *Streptomyces* development (17). *S. coelicolor* transcripts have been shown to undergo polyadenylation in vivo (2, 3; B. Sohlberg J. Huang, and S. Cohen, unpublished observations), and global analysis of *S. coelicolor* transcripts at single-gene resolution indicates that mRNA polyadenylation in *S. coelicolor* is widespread among individual mRNAs (J. Huang, J. Shi, B. Sohlberg, and S. N. Cohen, unpublished data). During our investigations of RNA polyadenylation in *S. coelicolor*, we discovered that a PAP I-like locus identified in the *S. coelicolor* genome by sequence analysis and hypothesized to be a mediator of polyadenylation (3) instead functions as an NTF that adds CCA termini to the 3′ ends of tRNA but lacks detectable ability to polyadenylate RNA. Here we report these findings and additionally show that the *S. coelicolor* PNPase homologue can act as a PAP and is likely to be a principal producer of poly(A) tails in this organism, as has been speculated from the heterogeneous nature of these tails (2).

The genes for the putative NTF and *Streptomyces pnp* were amplified by PCR of genomic DNA from *S. coelicolor* A3(2) M145 and the following primer pairs: 5′SCNTF (5′-GAA ATT AAT ACG ACT CAC TAT AGG GTT AAC TTT AAC TTT AAG AAG GAG ATA TAC ATA TGC CGA ACG CCA ACG AAG TC-3′) and 3′SCNTF (5′-CCG AAT TCA GTG GTG GTGGTG GTG GTG GTG GTG TGC GTT GTC CGG GTG CTG AGC-3′); and 5′SA/SCPNP (5′-CCG GAA TTC CAT ATG GAG AAC GAG AAC CAC-3′) and 3′SCPNP (5′-TCA GGA TCC TCA GTG GTG GTG GTG GTG GTG GTG GTG CTT GTC GGC GTC GTC-3′). The products were digested with restriction enzymes and ligated into expression vectors digested with the same enzymes. The NTF gene was inserted into pET21a (Novagen) and pIJ6021, kindly provided by Mervyn Bibb (John Innes Centre, Norwich, United Kingdom) (42), whereas the *pnp* gene was inserted into pET11a (Novagen). *E. coli pnp* was amplified from *E. coli* genomic DNA using the primers ECPNPFWD (5′-NNA GAT CTN NCA TAT GCG CAG AAG ATC GGG T-3′) and ECPNPREV (5′-AGG ATC CTC AGT GGT GGT GGT GGT GGT GGT GCT CGC CCT GTT CAG CAG CCG G-3′). The gene was then inserted into pET11a. The strain and construct expressing *E. coli* PAP I (9) was kindly provided by Yanan Feng (Stanford University, Stanford, Calif.). The *E. coli* vectors were introduced by transformation into BL21(DE3)/pLysS, and the protein expression was induced by 1 mM isopropyl-β-D-thiogalactopyranoside. *Streptomyces lividans* TK21 (16) was used as host for the pIJ6021-SCNTF construct, and protein production was induced by thiostrepton as described previously (42). All His-tagged proteins were purified according to the HisBind purification manual (Novagen), except that the matrix used was Ni-NTA agarose from Qiagen. The plasmid pJSE371, containing the gene for the PNPase-like protein (GPSI) from *Streptomyces antibioticus* (19), was a kind gift from George H. Jones (Emory University, Atlanta, Ga.). The *S. antibioticus pnp*/*gpsI* gene from pJSE371 was cloned into pGEX-6P-1 (Amersham Pharmacia), and the resulting vector was used for production of glutathione *S*-transferase (GST)-tagged *S. antibioticus* PNPase-like protein in the PNPase-deficient *E. coli* strain CAN244 (PNP$^-$) (35) according to the GST manual from Amersham Pharmacia.

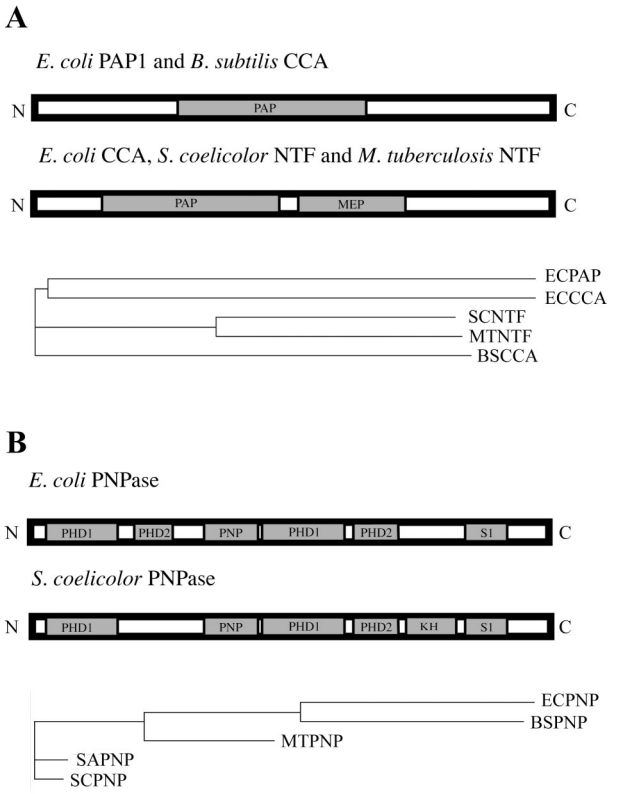

FIG. 1. Schematic structure and sequence comparison of members of the NTF family and the PNPase family. (A) The sequences from *E. coli* PAP I (ECPAP), *E. coli* CCA-adding enzyme (ECCCA), *S. coelicolor* A3(2) NTF (SCNTF), *B. subtilis* CCA-adding enzyme (BSCCA), and *M. tuberculosis* NTF (MTNTF) were selected for comparison and analyzed using the Conserved Domain Architecture Retrieval Tool (CDART) on the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov). The shaded segments indicate the conserved regions in each protein. PAP is a domain common among PAPs (pfam01743), whereas MEP is a common feature of metal dependent phosphohydrolases (smart00471). Sequence relationships are also presented as a dendrogram. (B) The sequences from *C. coli* PNPase (ECPNP), *S. antibioticus* PNPase-like protein (SAPNP), *S. coelicolor* A3(2) PNPase-like protein (SCPNP), *B. subtilis* PNPase (BSPNP), and *M. tuberculosis* PNPase-like protein (MTPNP) were selected and analyzed as described for panel A.

GGG-RNA I transcripts (9) were internally labeled with [α-^{32}P]CTP (NEN) using the MEGA T7short script kit from Ambion. The transcripts were purified by denaturing polyacrylamide gels. Native tRNA was purchased from Boehringer and used without further purification. The multiple bands seen in Fig. 2B reflect the heterogeneity of the tRNA preparation ("X" indicates an unknown species in the tRNA preparation that can be labeled with ATP by the NTF). The template for *supF* tRNA^{tyr} was amplified from pJA11 and transcribed as previously described (9). For polyadenylation assays, labeled RNA I was incubated with the purified proteins in a reaction mixture containing 250 mM NaCl, 10 mM $MgCl_2$, 2 mM K_2HPO_4, 1 mM dithiothreitol, 1 mM phosphoenolpyruvate, 0.6 U of pyruvate kinase (Sigma), and 10 U of RNase inhibitor (Promega) at 37°C for the indicated times. The reaction was

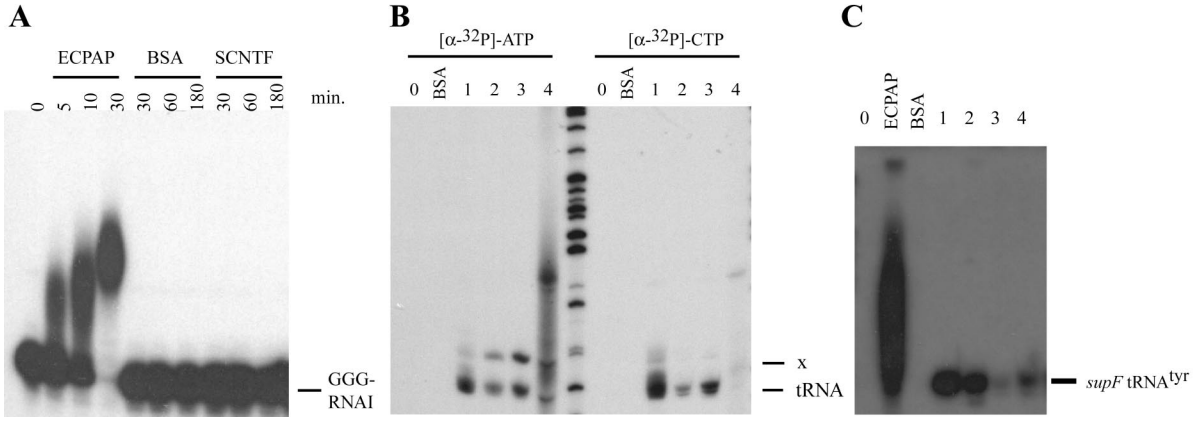

FIG. 2. Polyadenylation and CCA repair assays with *S. coelicolor* NTF. (A) In vitro-transcribed and labeled GGG-RNA I was incubated with His-tagged PAP from *E. coli* (ECPAP), bovine serum albumin (BSA), or His-tagged NTF from *S. coelicolor* (SCNTF) for the indicated times. (B) CCA repair assay with native tRNA. Native, purified tRNA was incubated with His-tagged *S. coelicolor* NTF. The left part of the gel indicates that the reaction mixture contained ATP with trace amounts of [α-^{32}P]ATP, whereas the right part of the gel shows the result when the reaction mixture contained CTP with trace amounts of [α-^{32}P]CTP. Lanes: 0, no protein added; BSA, BSA added; 1, SCNTF from *E. coli*; 2 and 3, SCNTF overexpressed and purified from *S. lividans*; 4, PAP from *E. coli*. X, unknown species. (C) CCA repair assay with in vitro-transcribed tRNA. Lanes: 0, no protein added; ECPAP, PAP from *E. coli* added; BSA, BSA added; 1 and 2, SCNTF from *E. coli*; 3 and 4, SCNTF from *S. lividans*.

stopped by adding proteinase K buffer (50 mM Tris-HCl [pH 7.9], 10 mM EDTA, 10 mM NaCl, 0.2% sodium dodecyl sulfate) and 50 μg of proteinase K (Invitrogen) per sample. The samples were incubated at 30°C for 10 min and extracted with phenol, and then the RNA was precipitated by ethanol-sodium acetate and left at −20°C for 30 min before centrifugation and drying. The dried samples were redissolved in formamide dye mix, denatured, and separated on 8% polyacrylamide gels containing 8.3 M urea. Polyadenylation assays with PNPase and the PNPase-like proteins were performed in a reaction mixture containing 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 50 mM NaCl, 0.1 mM dithiothreitol, 0.5 mM ADP, and 0.5 μg of yeast tRNA per μl. CCA end repair assays were performed by incubating unlabeled tRNA with purified proteins in a reaction buffer (50 mM glycine–NaOH [pH 9.4], 5 mM $MgCl_2$, 0.5 mM ATP [or 0.5 mM CTP]) with either [α-^{32}P]ATP or [α-^{32}P]CTP (for tracing) at 37°C for 15 min. The samples were extracted with phenol, precipitated with ethanol, dried, and redissolved in formamide dye mix. The samples were then denatured and run on denaturing polyacrylamide gels containing 8.3 M urea.

***S. coelicolor* A3(2) encodes only one protein containing an NTF motif.** When we screened the genome of *S. coelicolor* A3(2) M145 (1) for putative protein-encoded open reading frames whose sequence was suggestive of possible polyadenylation activity, we found only one protein predicted to contain a motif characteristic of NTFs (15, 48). This protein, encoded by gene SCO3896, contains motifs similar to those identified in *E. coli* PAP I and other NTFs; *E. coli* PAP I and *Bacillus subtilis* CCA-adding enzyme contain only the PAP domain (Fig. 1A). Another gene, predicted to be a PNPase (1), encodes a protein that has limited homology to NTFs, as has been noted for the PNPases of other bacteria (48). The PNPase-like proteins in Fig. 1B contain a conserved domain for PNPase (PNP, pfam03726), an S1 RNA-binding domain (S1, smart00316), and repeats of domains from RNase PH (PHD1, pfam01138, and PHD2, pfam03725). In addition to these domains, the PNPase-like proteins from *S. coelicolor*, *S. antibioticus*, *B. subtilis*, and *Mycobacterium tuberculosis* contain the KH RNA-binding domain (KH, pfam00013), located between the second tandem repeat of the PHD 1 and PHD2 domains and the S1 domain. The KH domain is not present in *E. coli* PNPase.

Since both genes were judged to have the potential to polyadenylate RNA, we overexpressed and purified both proteins for use in further experiments. These were designated as SCNTF and SCPNP, respectively (Fig. 1A and B, respectively).

The *Streptomyces* protein encoded by gene SCO3896 does not have polyadenylation activity but instead adds CCA to tRNA. GGG-RNA I, a small antisense RNA that is known to be polyadenylated by purified *E. coli* PAP I (9), showed a time-dependent conversion of substrate to slower migrating bands, characteristic of polyadenylation, when treated with this enzyme in the presence of ATP (Fig. 2A). In contrast, no detectable polyadenylation of GGG-RNA I by SCNTF was observed under the same conditions. In agreement with the previous finding that unpaired terminal nucleotides on RNA enable polyadenylation of this substrate by PAP I (9, 45), incubation of the SCNTF protein with a collection of native tRNAs in the presence of [α-^{32}P]ATP (Fig. 2B) resulted in the incorporation of this labeled nucleotide, while attachment of [α-^{32}P]CTP to the substrate by PAP I was barely detectable. This finding is consistent with earlier evidence that PAP I incorporates C's less efficiently than A's into tails. On the other hand, incubation of SCNTF with the tRNA resulted in the addition of either ATP or CTP to the tRNA, indicating that the *Streptomyces* NTF homologue is a CCA-adding enzyme rather than a PAP. Similar results were observed when synthetic *supF* tRNA^{tyr} was used as the substrate: as shown in Fig. 2C, *E. coli* PAP I generated long poly(A) additions on the tRNA whereas *Streptomyces* SCNTF was able to add only 1 to 3 nucleotides to the 3′ end. Thus, the activities of NTF were the same for the two sources of tRNA and not dependent on whether it had been overexpressed and purified from *E. coli* or *S. lividans*.

***Streptomyces* PNPase-like protein can polyadenylate RNA in**

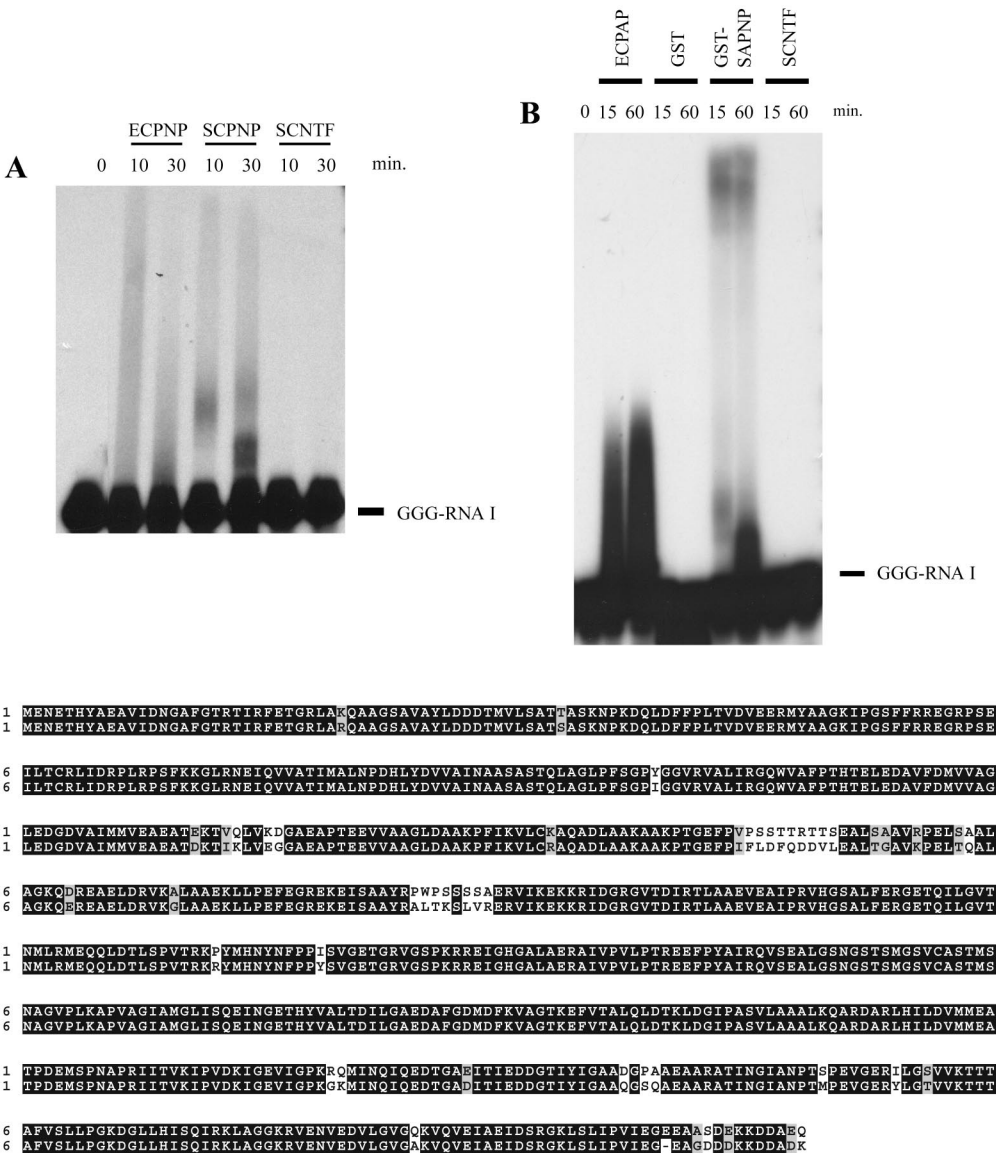

FIG. 3. Polyadenylation assay with PNPase and SCNTF using GGG-RNA I as the substrate. (A) Polyadenylation assays with His-tagged PNPase-like protein from *E. coli* or *S. coelicolor* and with His-tagged *S. coelicolor* NTF for the indicated times. Lanes: 0, no protein added; ECPNP; PNPase from *E. coli*; SCPNP, PNPase-like protein from *S. coelicolor*; SCNTF, NTF from *S. coelicolor*. (B) Polyadenylation assay with GST-tagged PNPase-like protein from *S. antibioticus* and SCNTF. Lanes: 0, no protein added; ECPAP, PAP from *E. coli*; GST, GST alone; GST-SAPNP, GST-tagged *S. antibioticus* PNPase-like protein; SCNTF, *S. coelicolor* NTF. (C) Sequence comparison between the PNPase-like proteins from *S. antibioticus* and *S. coelicolor*. The two amino acid sequences were aligned and visualized using the BoxShade server (http://www.ch.embnet.org/software/BOX_form.html). The sequences have 93% identity.

vitro. We considered the PNPase homologue GPSI [originally named after its ability to synthesize (p)ppGpp (19)] to be the second-most likely mediator of RNA polyadenylation in *Streptomyces* spp.; the *E. coli* PNPase has been known from its initial characterization to be able to generate polyribonucleotides and only later became recognized as one of the major exoribonucleases in *E. coli*. Additionally, Bralley and Jones (2) have observed that RNA tails in *S. coelicolor* are heterogeneous, as has been observed for 3′ additions by *E. coli* PNPase (29). To learn whether the *Streptomyces* PNPase-like protein is able to polyadenylate RNA, we performed modified polyadenylation assays without the addition of phosphate, which triggers the exoribonucleolytic activity of PNPase. Purified PNPase-like protein and NTF from *Streptomyces* spp. and PNPase from *E. coli* were incubated with labeled RNA and ADP. Figure 3A shows that the *Streptomyces* PNPase-like protein is able to utilize ADP for polyadenylation of RNA. *E. coli* PNPase can also utilize ADP, whereas *Streptomyces* NTF was not active in these assays. These results show that the *Streptomyces* PNPase-like protein, in addition to being a phosphorylytic exoribonuclease, can act as a PAP.

As the His-tagged *Streptomyces* PNPase-like protein we used had been purified from *E. coli*, we wanted to confirm that the polyadenylation activity we observed was due to the *Strepto-*

myces PNPase-like protein and not to contamination by *E. coli* PNPase that might possibly be incorporated into PNPase heteromultimers. The PNPase-like gene from *S. antibioticus*, which is the best characterized *Streptomyces* PNPase-like protein (18, 19) and is also the only PNPase-like protein to have been crystallized (41), was employed for this purpose. The *S. antibioticus* and *S. coelicolor pnp*/*gpsI* genes are very closely related (Fig. 3C), with over 93% identity (1, 19). The *S. antibioticus* PNPase/GPSI gene from plasmid pJSE371 was transferred into a pGEX vector so that a GST-PNPase fusion could be expressed and purified from an *E. coli* host lacking endogenous PNPase. Figure 3B shows that, indeed, the GST-PNPase fusion protein was able to polyadenylate RNA I, whereas GST or NTF did not polyadenylate the same RNA substrate.

The finding that the only NTF predicted from *S. coelicolor* genome DNA sequence analysis is a CCA-adding enzyme and does not participate in the polyadenylation process raises the question of where in phylogeny these structurally related proteins diverged evolutionarily to display disparate enzymatic activities. tRNA requires a CCA triplet at the 3′ end in order to function; as *Streptomyces* and many other bacterial species generate tRNAs lacking CCA ends, these bacteria require CCA-adding enzymes to produce functional tRNAs. On the other hand, species such as *E. coli* produce tRNA primary transcripts that already contain functional 3′ ends and thus do not need the CCA-adding and -repairing activities of these enzymes. Two separate gene sequences that contain NTF motifs occur not only in *Proteobacteria* but also in the *Thermus*/*Deinococcus*, *Spirochaetales*, and *Planctomyces*/*Chlamydia*/*Verrucomicrobium* groups, suggesting the presence of both a tRNA NTF (i.e., putative CCA-adding enzyme) and a PAP in these organisms. Potentially, PAP I may have evolved from duplication of a gene encoding the CCA-adding enzyme in one of these species. Interestingly, even among *Firmicutes*, which includes the *Bacillus*/*Clostridium* and *Actinobacteria* groupings, there are species differences, and among *Bacillacea*, *B. subtilis* contains one gene that includes an NTF motif whereas *B. halodurans* has two (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser). We have not been successful in generating an *S. coelicolor pnp* mutant, suggesting that, unlike *E. coli* and *B. subtilis*, but like *Synechocystis* strain PCC6803, this organism requires PNPase for viability.

PNPase is a multifunctional enzyme and can act both as an exoribonuclease and a polyribonucleotide synthetase. It was believed that the intracellular concentrations of phosphate in *E. coli* are too high for PNPase to act as a tail maker and that the enzyme acts solely in vivo in this species as an RNase; however, recently it was found that *E. coli* PNPase has PAP activity in cells grown in Luria broth, which is plentiful in phosphate (29). Other bacterial species living under conditions where phosphate is limiting may have separate means of modulating the synthetic and exonucleolytic activities of PNPase-like proteins, for example, modifications of PNPase or interactions between PNPase and either RNA or other cellular proteins. PNPase can exist in *E. coli* in several different forms (32), and potentially, these forms may interact differentially with substrates or with other cellular proteins. Both PNPase and PAP I are associated in vitro (for a review, see reference 6) and in vivo (24, 25, 32) with other proteins in *E. coli*. In addition to interacting with RNase E in vitro, *E. coli* PAP I interacts in vitro with several DEAD box RNA helicases (33) and both in vitro and in vivo with the Sm-like protein Hfq, which has been shown to affect mRNA stability and improve poly(A) tail production (13, 21, 43). The *Streptomyces* PNPase-like protein interacts physically with the *Streptomyces* RNase E homologue RNase ES (22).

The mechanism that enables preferential incorporation of adenosine residues by PNPase into polyribonucleotide tails that include other nucleotides is also not known. However, the finding that *E. coli*, and possibly other bacteria, have proofreading systems that interact with PAP I and ensure that this enzyme only includes A's in the tails suggests that the composition of tails is biologically important (28, 47). *E. coli* normally lives in a nutrient-rich environment where ATP may serve as a natural source for polyadenylation. *Streptomyces* species inhabit environments with limited supplies of nutrients. Polyadenylation of RNA by ADP may provide a way to preserve ATP and energy for other purposes.

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