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Article

The First Poly(A) Polymerase from *Alphaproteobacteria*

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Abstract

Bacterial poly(A) polymerases (PAPs) play an important role in RNA metabolism but remain poorly characterized outside *Gammaproteobacteria*. Here, we cloned and biochemically characterized the first PAP from *Alphaproteobacteria*, specifically from *Marinobacter lipolyticus* (Mli PAP). Using homology-based screening against *E. coli* PAP-1, we identified Mli PAP, sharing 54.8% sequence identity with its *E. coli* counterpart. The enzyme was expressed in *E. coli* but formed insoluble inclusion bodies; the active enzyme was purified as a fusion protein with the DsbA protein and used for functional assays. Mli PAP exhibited optimal activity at 30 °C and similar thermostability to *E. coli* PAP-1. ATP was the preferred substrate, with K_m comparable to *E. coli* PAP-1 (1.61 mM and 1.70 mM, respectively), and Mg^{2+} (10 mM) was identified as the optimal cofactor. Mli PAP displayed salt-dependent activity, with the most effective polyadenylation in KCl and inhibition by NaCl and ammonium salts, contrasting with the halophilic nature of its host. This study provides the first functional insights into PAPs from *Alphaproteobacteria*, broadening the understanding of PAP diversity and biochemical properties, as well as the potential applications of PAPs in biotechnology.

Keywords: *pcnB*; polyA-polymerase; PAP; polyadenylation; *Marinobacter lipolyticus*

1. Introduction

Polyadenylation in bacterial cells plays a major role in RNA degradation, where polyA tails serve as a signal for RNAses-mediated cleavage. In *E. coli*, poly(A) talis are mostly synthesized in vivo by a specific poly(A) polymerase (PAP-1), and at least two other enzymes are also capable of producing them, including PAP-2 and polynucleotide phosphorylase. Together with closely related CCA-transferases, PAPs are included in the nucleotidyltransferase superfamily, where bacterial PAPs and eukaryotic CCA-transferases stand as class II enzymes, while eukaryotic PAPs and bacterial CCA-transferases are class I enzymes.

Despite early discovery in the 1960s, the details of polyadenylation and its role in cells remain not fully understood [1]. Thus, only two bacterial PAPs were cloned and biochemically characterized, namely, PAP-1 from *E. coli* and PAP from *Geobacter sulfurreducens* [2–4]. At least two other enzymes, *E. coli* PAP-2 [5] and PAP from *Pseudomonas putida* [6], have also been discovered, but these enzymes were purified directly from host organisms without cloning and their functions in cells were not directly studied. Bacterial PAPs were only recovered from *Gammaproteobacteria* (*E. coli* and *P. putida*) and *Desulfuromonadia* (*G. sulfurreducens*). This lack of biochemical data is striking comparing to close counterparts of PAPs, bacterial CCA-transferases that were discovered in a wide range of bacterial taxa:

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Gammaproteobacteria, *Bacilli*, *Actinomycetes*, *Aquificae*, *Deinococci* and *Cyanophyceae*. While a few CCA-transferases and a few PAP from bacteria were characterized in vitro [7,8], their prediction in silico using amino acid sequences remains a challenging task. Homology of these nucleotidyltransferases is high and only one signature sequence has been reported for bacterial PAPs, which does not allow us to robustly distinguish PAPs and CCA-transferases [9,10].

The *pcnB* gene encoding bacterial PAPs is thought to originate from a duplicated CCA-transferase gene and was inherited by *Gamma*- and *Betaproteobacteria* [11]. In some other taxa including *Alphaproteobaceria*, *Aquificae*, *Baceroides*, etc., the *pcnB* gene could arise by horizontal transfer, while in other phyla, the gene could be transmitted vertically. Studying putative PAPs outside of *Gammaproteobacteria* can shed light on how these enzymes changed after horizontal transfer. Being a sister class to *Gammaproteobacteria*, *Alphaproteobacteria* can be a good starting point for these studies because their PAPs are relatively close to *E. coli* PAP-1 and can be identified in silico based on homology to this enzyme. Also, replication of *Alphaproteobacteria* plasmids can be controlled by the same mechanism involving polyadenylation by PAP as ColE1-type plasmids from *Gammaproteobacteria* [12].

Recently, PAPs have become an important tool for biotechnology because of booming development of mRNA vaccines for treatment of various pathological conditions from viral infection to cancer [13,14]. Poly(A) tails can be attached to therapeutic mRNA during transcription, being encoded in the respective plasmid. However, this approach is complicated by insertions and deletions in a plasmid sequence encoding poly(A) tail. These altercations lead to length inconsistency of poly(A) tails, affecting the stability of mRNA. Usage of PAPs allows us to overcome this issue but a spectrum of available PAPs is narrow, including yeast PAP and *E. coli* PAP-1. Other bacterial PAP can be superior considering thermal stability and reaction efficacy, yet these enzymes are not biochemically characterized. In vitro transcription coupled with polyadenylation at temperatures higher than 37 °C and in high-salt buffers can reduce dsRNA impurities and omit extra purification steps of the intendent mRNA products [15]. Thus, the manufacturing cost and immunogenicity of mRNAs may be reduced, facilitating scaled-up production of these therapeutics.

Thus, the absence of biochemical data not only hampers studying of novel PAPs, but also restricts usage of bacterial PAPs in practical applications.

In the present work, we have cloned and biochemically characterized a novel poly(A)-polymerase from *Marinobacter lipolyticus*, a halophil discovered in saline soil in Spain. This enzyme, being the first PAP discovered from *Alphaproteobacteria*, was compared with known *E. coli* PAP-1 regarding optimal temperature, thermal stability, salt concentration and cofactors.

2. Results

2.1. Search for the Mli PAP Gene and Purification of Mli PAP

To search for new bacterial PAPs, we aligned the amino acid sequence of *E. coli* PAP-1 in Protein BLAST 2.17.0 against proteins from *Alphaproteobacteria* using the following criteria: the length, 400–600 a.a.; the presence of all PAP structural domains, conservative D69, D71, E108 positions; and the signature of bacterial PAPs [LIV][LIV]G[R/K][R/K]Fx-[LIV]h[HQL][LIV]. Candidate proteins with additional domains were discarded to avoid the presence of other enzymatic activities. The retrieved putative PAPs organized into a range based on their similarity to *E. coli* PAP-1, and among 37 candidates, we selected a putative PAP from *Marinobacter lipolyticus* (GenBank: EON91085.1) based on its high similarity to *E. coli* PAP-1 and the host's habitat. *M. lipolyticus* is an aerobic moderate halophile discovered in saline soil of southern Spain. The optimal growth conditions of 7.5% NaCl, 37 °C and pH 7.5 assume a low probability of horizontal gene transfer directly

from *E. coli* that growth is inhibited by 4–7% NaCl. Thus, the gene of *M. lipolyticus* PAP (Mli PAP) has unlikely been transferred from *E. coli* or its close relatives. Alignment of Mli PAP with yeast PAP1 and other characterized bacterial PAPs, *E. coli* PAP-1 and *Geobacter sulfurreducens*, is presented in Figure 1.

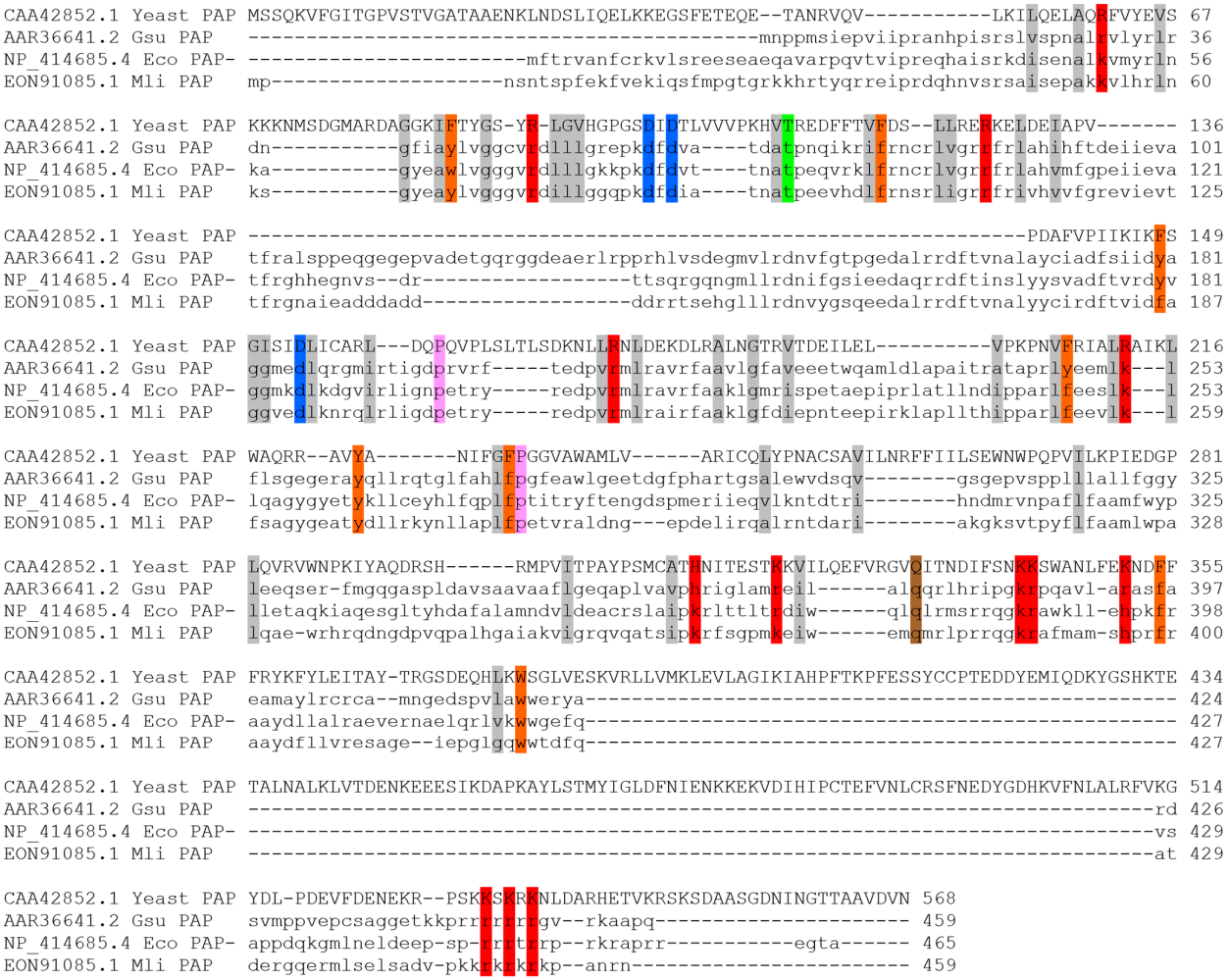

Figure 1. Alignment of Mli PAP, other bacterial PAPs and yeast PAP1. Amino acid sequences were aligned using ClustalW and visualized in Sequence Manipulation Suite. The colored background indicates conservative amino acid residues.

Homology of the three aligned PAPs is relatively limited: 43.7% between *E. coli* PAP-1 and PAP from *Geobacter sulfurreducens*, 54.8% between *E. coli* PAP-1 and Mli PAP, and 43.8% between PAP from *Geobacter sulfurreducens* and Mli PAP. Being one of the search criteria for a new PAP, [LIV][LIV]G[R/K][R/K]Fx-[LIV]h[HQL][LIV] was conservative among all three PAPs. Interestingly, the N-terminal half of these proteins was more conservative than the C-terminal part. In *E. coli* PAP-1, the C-terminal region is responsible for interplay with degradosome proteins, RNase E, DEAD-box RNA helicases, while the N-terminal part hosts catalytically active a.a. residues. Plausibly, the same structure–function relationships can be found in other bacterial PAPs. Also, the N-terminus of *Geobacter sulfurreducens* PAP is 20 a.a. shorter than that of *E. coli* PAP-1, and 20 additional a.a. (positions 115–134) are inserted.

The main properties of the candidate Mli PAP and other bacterial PAPs are listed in Table 1.

Table 1. Properties of known bacterial PAPs.

Host	GenBank ID	Length, a.a.	MW, kDa	pI	Net Charge at pH 7.0
M. lipolyticus	EON91085.1	459	52.8	9.52	13.4
E. coli	NP_414685.4	465	53.9	9.67	17.8
G.sulfurreducens	AAR36641.2	459	51.1	7.24	0.51

While Mli PAP and *E. coli* PAP-1 are highly charged proteins with a pI value close to 9.5, *G. sulfurreducens* PAP is strikingly different without a high net positive charge. Notably, the more conservative N-terminal part of the latter protein also has a significantly lower net charge than the two other PAPs.

After cloning in the pET23a vector, Mli PAP and *E. coli* PAP-1 were expressed in *E. coli* BL21 (DE3) pLysS cells following purification of recombinant PAPs. Unfortunately, while *E. coli* PAP-1 purification using a combination of metal-chelate and ion-exchange chromatography was successful, we did not manage to isolate Mli PAP despite all our efforts (Figure 2).

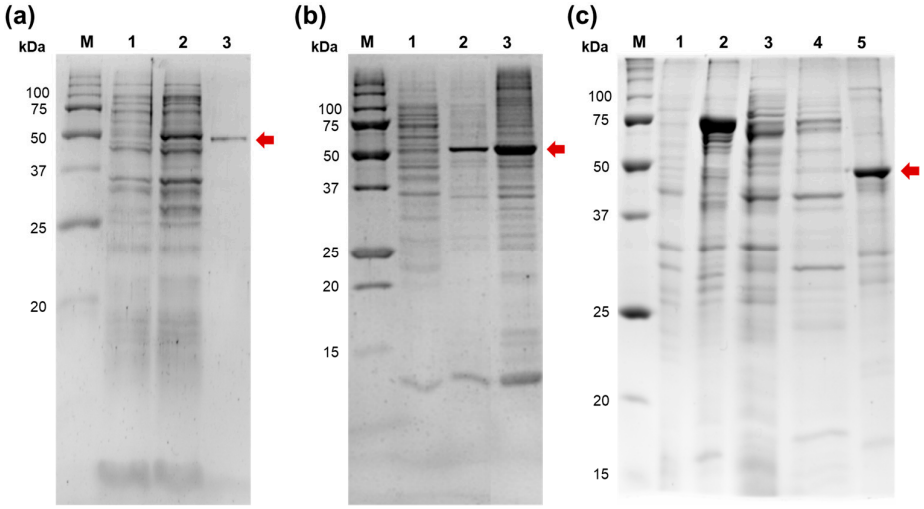

Figure 2. Expression and purification of *E. coli* PAP-1 and Mli PAP. The enzymes were expressed in *E. coli* strain BL21 (DE3) pLysS. (**a**) Purification of *E. coli* PAP-1. Soluble *E. coli* PAP-1 was purified using affinity and ion-exchange chromatography. 1—*E. coli* lysate before expression of *E. coli* PAP-1, 2—crude lysate after expression, 3—purified *E. coli* PAP-1. (**b**) Partial purification of Mli PAP. Mli PAP was extracted from insoluble inclusion bodies by a high-salt buffer, 1—soluble protein fraction after expression of Mli PAP, 2—solubilized Mli PAP, 3—insoluble inclusion bodies. (**c**) Purification of Mli PAP as a fusion protein. Mli PAP was purified as a fusion with the DsbA protein followed by DsbA removal by TEV protease. 1—*E. coli* lysate before expression of DsbA-Mli PAP, 2—crude lysate after expression, 3—soluble protein fraction after expression, 4—DsbA-Mli PAP after the first affinity chromatography, 5—purified Mli PAP after DsbA removal. M—Precision Plus Protein standards (Bio-Rad, Hercules, CA, USA). PAPs are marked by an arrow.

E. coli PAP-1 is notoriously known for its tendency to aggregate and being insoluble after lysis, plausibly because of a high net positive charge. However, a high ionic strength in all purification steps and 2 M urea in lysis allowed us to recover active *E. coli* PAP-1 despite its low percentage in a soluble protein fraction. The same approach was ineffective for isolation of Mli PAP; virtually all Mli PAP was insoluble after lysis without traceable poly(A)-polymerase activity in supernatant. Similarly to our previous experience with *E. coli* PAP-1, insoluble Mli PAP retained its specific activity, allowing for purification of the enzyme from inclusion bodies. Up to 20% of active Mli PAP could be extracted from inclusion bodies by resuspension in a high-salt buffer. Despite a C-terminal His-Tag, Mli

PAP binding with a Ni-charged IMAC resin was ineffective, leaving most of the protein in a flow-throw, while the eluted Mli PAP was contaminated by cellular proteins to a high degree. Supplying the binding buffer by 2 M urea, 1% Triton X-100, 5 mM beta-mercaptoethanol, or charging IMAC by Co^{2+} did not allow for purification of Mli PAP using metal-affinity chromatography. Denaturation of inclusion bodies with Mli PAP by 8 M urea followed by purification using Ni-charged IMAC resin allowed us to isolate the relatively pure enzyme (Supplementary Figure S1). However, Mli PAP was inactive after either flash renaturation by dilution or dialysis at +4 °C. Renaturation of Mli PAP bound with Ni-charged IMAC resin by a slow reverse gradient of urea (8–0 M) was also unsuccessful (Supplementary Figure S1). Surprisingly, Mli PAP also did not bind with any other used resin, including anion-exchanging Macro-Prep HighQ, cation-exchanging Macro-Prep HighS, affine Heparin SepFast Media, or hydrophobic Macro-Prep t-Butyl HIC in pH 6–9 (Supplementary Figure S2). The solubilized enzyme was stable for up to several months in a buffer containing 1 M NaCl without loss of specific activity. It should also be noted that no poly(A)-activity was detected in control probes before induction of Mli PAP, ensuring that poly(A)-activity was associated with the recombinant Mli PAP but not host *E. coli* proteins.

Mli PAP used for further characterization was purified as a fusion with the DsbA, a small periplasmic *E. coli* protein used as a solubilization tag. The fusion DsbA-Mli PAP was also mostly insoluble after lysis; however, the remaining amount of the soluble recombinant protein was enough for further purification by affinity and ion-exchange chromatography. After purification, DsbA was removed by TEV protease, leaving isolated Mli PAP (Figure 2c). Notably, biochemical properties of Mli PAP solubilized from inclusion bodies and purified as a fusion protein were identical, indicating that the obtained results are robust.

2.2. Biochemical Properties of Mli PAP

2.2.1. Optimal Temperature and Thermostability

After purification, we studied the biochemical properties of Mli PAP using fluorescently labeled (r)A_{20} oligonucleotide. Here, we assessed the specific activity of Mli PAP in the reaction buffer for *E. coli* PAP-1 and used this estimation for planning further experiments where working conditions assumed less than 5–30% of elongated substrate. Molar activity of Mli PAP was not assessed due to the presence of contaminating proteins.

Reaction temperature is one of the main parameters defining efficacy of amplification. Suboptimal temperature leads to slower poly(A) synthesis or poly(A) polymerase thermal inactivation. To examine the optimal reaction temperature for Mli PAP, we carried out poly(A) tails synthesis at 25–60 °C with a step of 5 °C and quantified the reaction products. The results of the optimal temperature assay are presented in Figure 3.

Optimal temperature was almost the same for both enzymes, 30 °C for Mli PAP and 30–35 °C for *E. coli* PAP-1, which was in agreement with the optimal growth temperature of both host bacteria, 37 °C [16]. It should be noted that Mli PAP and *E. coli* PAP quickly lose activity at temperatures higher than 35 °C, becoming almost inactive at 45 °C. Next, we tested the thermostability of Mli PAP. Thermal stability defines the ability of an enzyme to remain active after heating. The enzyme was incubated at 30–60 °C for up to 60 min in the reaction buffer for *E. coli* PAP-1, recommended by NEB, without ATP following poly(A)-polymerase activity analysis. The results of the assay are presented in Figure 4.

E. coli PAP-1 and Mli PAP completely lost activity after incubation for 15 min at 50–60 °C and retained only 20–40% of their specific activity after 60 min at 30–40 °C, demonstrating relatively low thermal stability. However, both PAPs retained 20–30% of their specific activity in the presence of the (r)A_{20} oligonucleotide substrate after incuba-

tion for 60 min at 50–60 °C. This stabilizing effect is similar to that observed for reverse transcriptases and DNA polymerases, which are stabilized by RNA and DNA templates, respectively. Thus, prolonged incubation of bacterial PAPs may require supplementation of the incubation buffer with RNA.

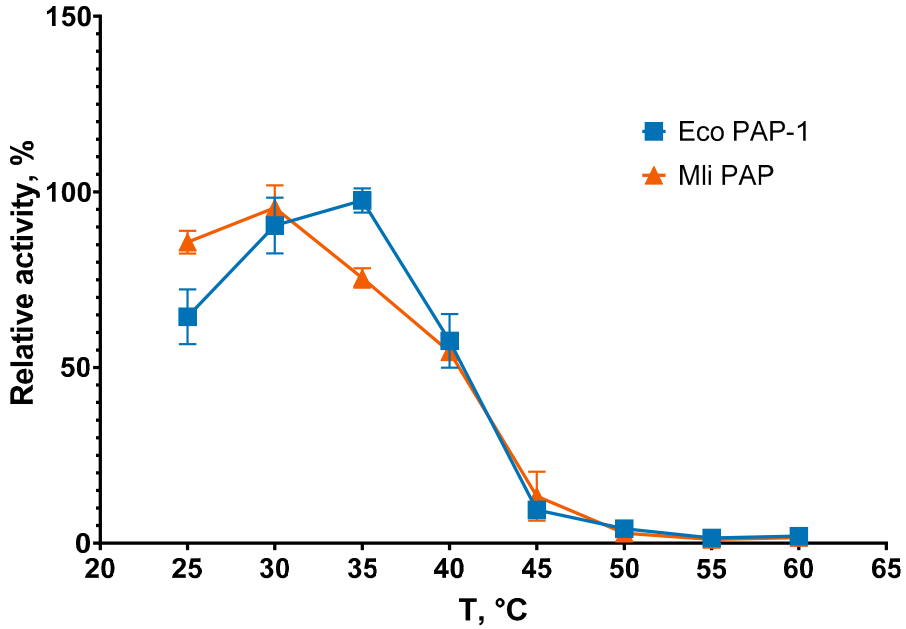

Figure 3. Optimal temperature assay. The optimal temperature for Mli PAP was determined in a poly(A) polymerase activity assay using fluorescently labeled (r)A_{20} oligonucleotide substrate. *X*-axis marks the reaction temperature; *Y*-axis designates the relative specific activity. Each experiment was triplicated; error bars demonstrate SD.

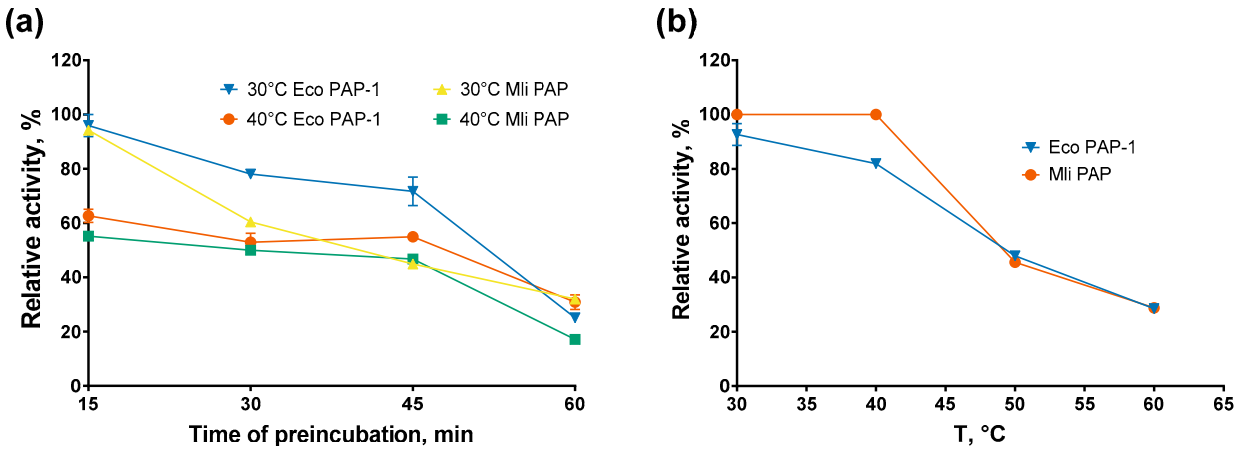

Figure 4. Thermostability assay. (**a**) Preincubation without the RNA substrate, (**b**) preincubation with the RNA substrate. Enzymes were incubated at 30–90 °C for up to 60 min following a poly(A)-polymerase activity assay using fluorescently labeled (r)A_{20} oligonucleotide substrate. The *X*-axis marks the reaction temperature (**a**) or the preincubation temperature (**b**); the *Y*-axis designates the relative specific activity. Each experiment was triplicated; error bars demonstrate SD.

2.2.2. Optimal Reaction Buffer

At the next step of Mli PAP characterization, we tested its activity under various conditions, including pH, salts, and cofactors. Optimal composition of the reaction buffer provides the best possible reaction efficacy, and knowing an enzyme's ability to synthetize poly(A) tails in varying conditions also allows us to perform multiple enzymatic reactions in a single tube. Salt concentration, together with pH, affects the enzyme's conformation and its interaction with the respective substrate and other compounds. Cofactors often change

the specificity of an enzyme, enabling chemical transformation of unusual substrates. In our experimental setup, firstly, we titrated Mg^{2+}, Mn^{2+}, Co^{2+} as cofactors, followed by titration of salts at various pH values. The concentration of the buffer reagent was 50 mM in all the experiments. For divalent cations, the ATP concentration was fixed at 0.5 mM. The results of the cofactor titration experiments are presented in Figure 5.

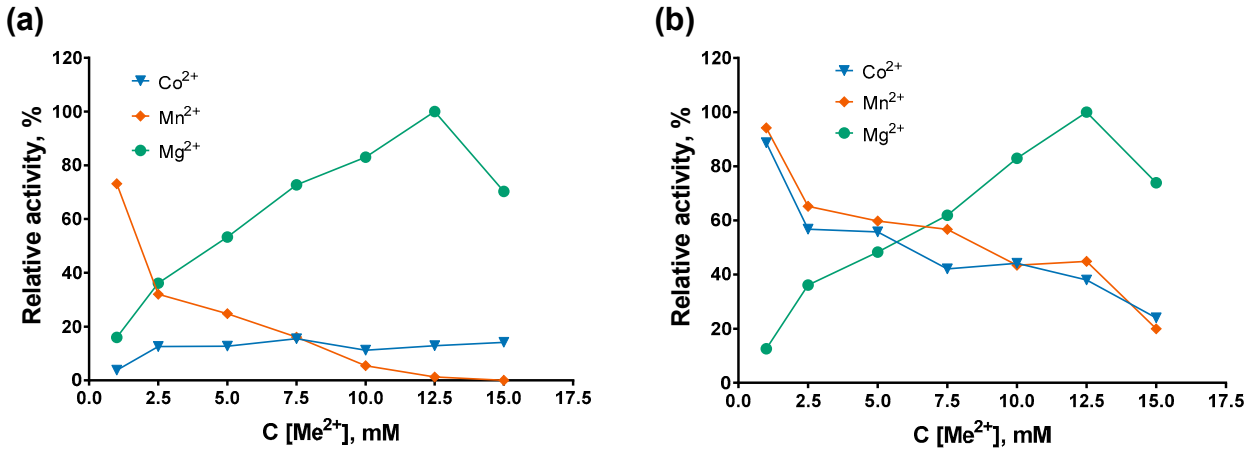

Figure 5. Optimal cofactors. (**a**) Mli PAP, (**b**) *E. coli* PAP-1. Cofactors were titrated in reaction mixes for a poly(A)-polymerase activity assay using fluorescently labeled (r)A_{20} oligonucleotide substrate. Other components of the reaction buffer were similar to the reaction buffer for *E. coli* PAP-1 recommended by NEB. The *X*-axis marks the cofactors' concentrations; the *Y*-axis designates the relative specific activity. Each experiment was triplicated; error bars demonstrate SD.

Among the three tested divalent cations, Mg^{2+} was the preferred cofactor for both enzymes, and the highest activity was observed at a magnesium concentration of 12.5 mM, which was used in all subsequent experiments. *E. coli* PAP-1 was more promiscuous than Mli PAP, efficiently synthesizing poly(A) tails in the presence of 1 mM Co^{2+} or Mn^{2+}, retaining 88–95% of its maximal activity. In contrast, Mli PAP retained 73% activity with 1 mM Mn^{2+} but showed less than 20% activity with all tested Co^{2+} concentrations. Plausibly, exchange of magnesium to other cations in PAP's active site resulted in the decreased activity.

After cofactors, we titrated various salts in the range of 0–200 mM at pH 7.0–9.0 and assessed the specific activity of the studied PAPs. The titration results are presented in Figures 6 and 7.

The specific activity of both PAPs decreased monotonically with increasing concentrations of ammonium salts, either $(NH_4)_2SO_4$ or NH_4Cl, with Mli PAP being slightly more tolerant of NH_4Cl than *E. coli* PAP-1. In contrast, while *E. coli* PAP-1 exhibited a similar activity profile in the presence of NaCl and KCl (with peak activity at 100 mM salt), Mli PAP lost activity at NaCl concentrations $\geq$ 125 mM and showed maximal activity at 150–175 mM KCl. Notably, both enzymes displayed their highest activity in KCl. Regarding pH, both PAPs were the most active at higher pH values in the presence of NaCl and KCl, whereas no clear pH dependence was observed with $(NH_4)_2SO_4$ or NH_4Cl. Overall, these results are difficult to reconcile, as inhibition by ammonium ions coincided with the requirement for relatively high KCl concentrations to achieve maximal activity. The preference of Mli PAP for KCl may be related to its tendency to aggregate in low-salt buffers and weak binding to ion-exchange resins. As the optimal buffer for Mli PAP, we chose 50 mM Tris-HCl, 150 mM KCl, pH 9.0.

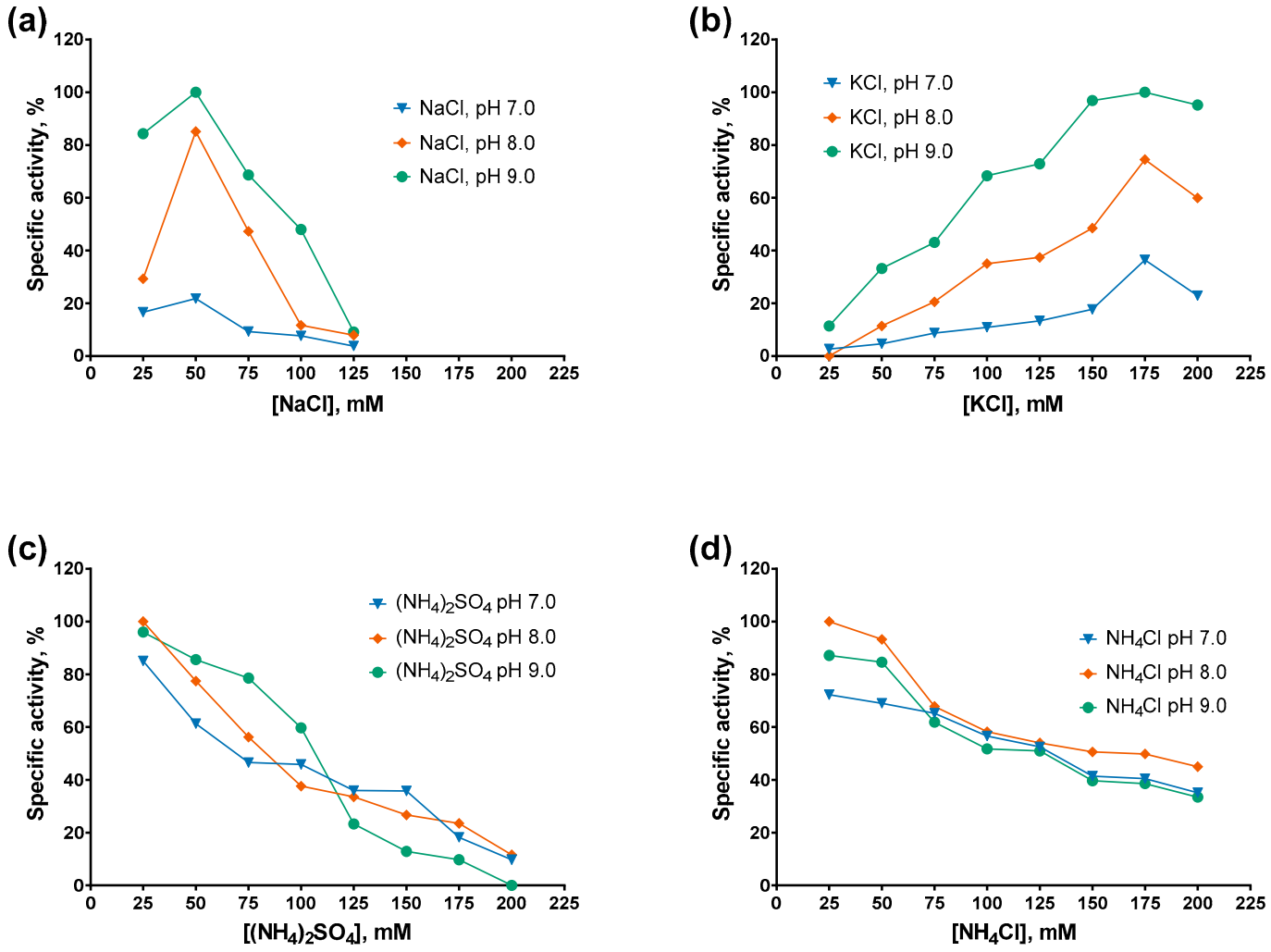

Figure 6. Optimal salts and pH for Mli PAP: (**a**) NaCl, (**b**) KCl, (**c**) $(NH_4)_2SO_4$, (**d**) NH_4Cl. Salts were salts in the range of 0–200 mM at pH 7.0–9.0 in reaction mixes for a poly(A)-polymerase activity assay using fluorescently labeled (r)A_{20} oligonucleotide substrate. The *X*-axis marks the salts concentrations; the *Y*-axis designates the relative specific activity. Each experiment was triplicated; error bars demonstrate SD.

2.2.3. Nucleotide Specificity and K_m for ATP

Poly(A)-polymerases utilize ATP as the monomer for synthetizing of poly(A) tails. However, in vitro, *E. coli* PAP-1 also inserts CMP, UMP and with a much-lower-efficacy GMP in the growing RNA strand. Thus, *E. coli* PAP-1 is not absolutely specific to ATP and the same could be said for Mli PAP. To test this hypothesis, we titrated all four canonical NTPs in the range of 0.25–10 mM with a fixed concentration of Mg^{2+} and calculated the Km values. The results of the titration experiments are presented in Figure 8.

As expected, ATP was the preferential substrate for *E. coli* PAP-1 and Mli PAP. Only short, elongated products were observed when CTP or UTP were used, while the substrate remained intact with GTP. Saturation was not reached even with 10 mM CTP and UTP, demonstrating a discrimination ability of the studied PAPs in our experimental setup, where both enzymes were able to utilize CTP and UTP as a substrate, but with much less efficacy than ATP. Following NTPs' titration, we defined K_m for ATP by titrating ATP and adjusting reaction time of the polyadenylation assay. The resulting K_m(ATP) values for *E. coli* PAP-1 and Mli PAP were almost identical—1.70 mM and 1.61 mM, respectively—indicating a high degree of similarity between the two enzymes.

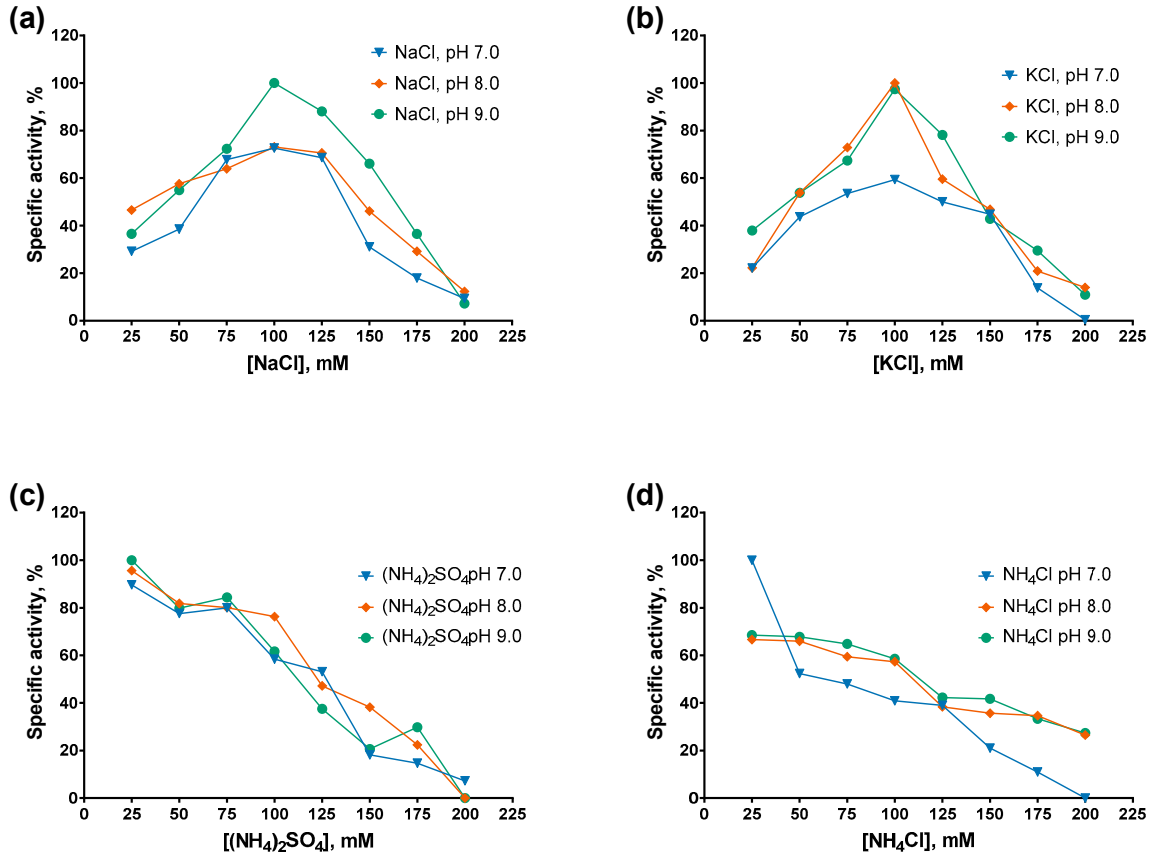

Figure 7. Optimal salts and pH for Mli PAP: (**a**) NaCl, (**b**) KCl, (**c**) $(NH_4)_2SO_4$, (**d**) NH_4Cl. Salts were titrated in the range of 0–200 mM at pH 7.0–9.0 in reaction mixes for a poly(A)-polymerase activity assay using fluorescently labeled (r)A_{20} oligonucleotide substrate. The *X*-axis marks the salts concentrations; the *Y*-axis designates the relative specific activity. Each experiment was triplicated; error bars demonstrate SD.

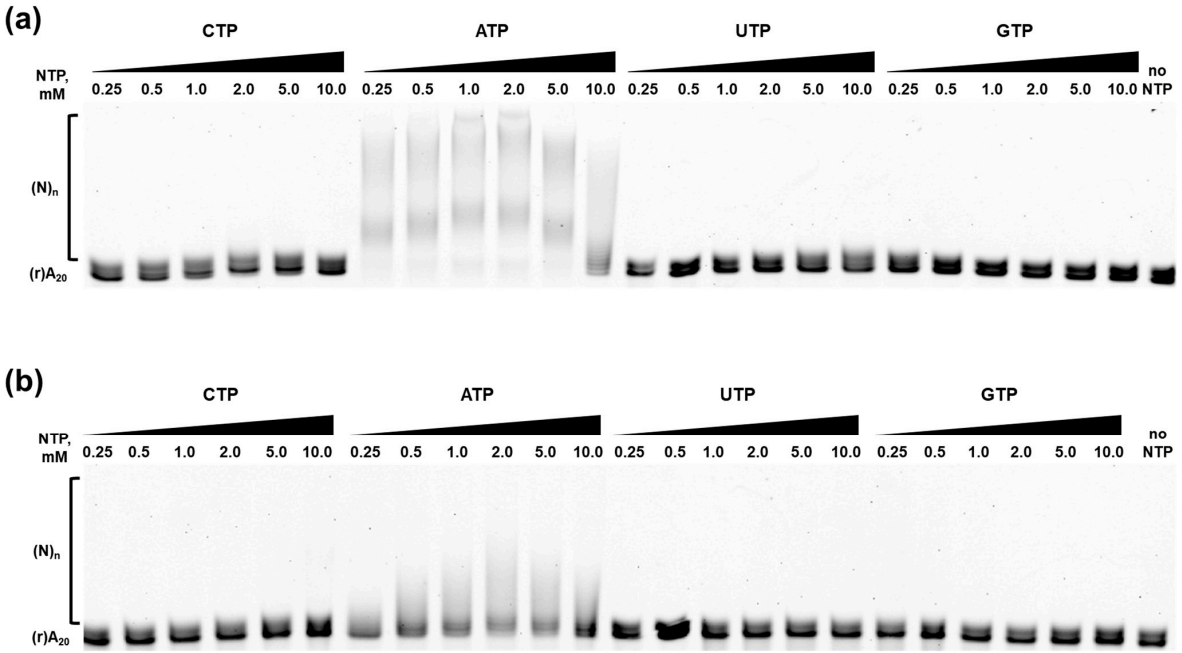

Figure 8. Nucleotide specificity of *E. coli* PAP-1 (**a**) and Mli PAP (**b**). NTPs were titrated in the range of 0.25–10 mM in reaction mixes for a poly(A)-polymerase activity assay using fluorescently labeled (r)A_{20} oligonucleotide substrate. NTPs and their concentration are marked above each line, (r)A_{20} demonstrates the substrate's bands, and $(N)_n$ marks the polyadenylated substrate.

3. Discussion

Polyadenylation in *E. coli* was discovered in the early 1960s; however, unlike many other enzymes involved in nucleic acid metabolism, PAPs remain understudied. To date, only two bacterial PAPs have been cloned and biochemically characterized, and one additional PAP has been isolated directly from *P. putida*. This scarcity of experimental data makes in silico prediction of new PAPs a non-trivial task, given the high sequence similarity between tRNA nucleotidyltransferases and PAPs. As a result, despite the rapid development of mRNA therapeutics, modern biotechnology currently relies on only two PAPs for in vitro polyadenylation: *E. coli* PAP-1 and yeast PAP. In this study, we selected *Alphaproteobacteria* as a source of new PAPs because members of this class are believed to have acquired PAPs through horizontal gene transfer from *Gammaproteobacteria*, reducing the likelihood of inadvertently cloning a tRNA nucleotidyltransferase instead of a PAP.

E. coli PAP is known to be toxic to host cells, and its overexpression can lead to cell death. However, we did not observe any abnormalities in cell growth following induction of either *E. coli* PAP-1 or Mli PAP. It should be noted that we did not specifically assess host–cell viability, and potential toxicity of Mli PAP toward *E. coli* may not manifest as cell lysis. In this context, more detailed studies, including complementation experiments using a *pcnB*-knockout *E. coli* strain, would be informative and could clarify the impact of a heterologous bacterial PAP on *E. coli* growth.

Previously, *E. coli* PAP-1 was reported to be prone to aggregation and to require relatively high ionic strength for stability in solution [3,4]. In our experiments, *E. coli* PAP-1 was also largely insoluble after cell lysis and rapidly lost activity when stored in low-salt buffers. A similar effect was observed for Mli PAP, suggesting that poor solubility may be a common characteristic of bacterial PAPs, as their related enzymes, tRNA nucleotidyltransferases, have also been reported to be insoluble [17]. In the study by Shi et al., *E. coli* tRNA nucleotidyltransferase was successfully purified and retained activity after urea denaturation followed by renaturation on Ni–NTA resin. However, neither *E. coli* PAP-1 nor Mli PAP retained activity after a denaturation–renaturation cycle in our study; so, we attempted to purify both enzymes under non-denaturing conditions. A sufficient amount of soluble *E. coli* PAP-1 was available for successful purification. In contrast, Mli PAP expressed in *E. coli* appeared to be completely insoluble. Both PAPs retained detectable activity in inclusion bodies, and we used this observation as the basis for attempts to purify Mli PAP by metal-chelate, ion-exchange, and affinity chromatography. However, Mli PAP solubilized from inclusion bodies using a high-salt buffer failed to bind to any of the tested resins despite extensive variation in pH and ionic strength. We managed to purify Mli PAP using only a solubilization tag, the DsbA protein, N-terminally fused to Mli PAP. The fused protein, while also being mostly insoluble, remained in a soluble fraction in a sufficient amount for purification and further analysis. It also should be emphasized that no poly(A) polymerase activity was detected in *E. coli* cells prior to Mli PAP induction, indicating that the results obtained were robust and that no detectable background activity from host poly(A) polymerases interfered with our assays.

Regarding biochemical properties, *E. coli* PAP-1 and Mli PAP demonstrated similar thermostability and optimal temperatures, although their cofactor and salt preferences differed slightly. Both host organisms are mesophiles with optimal growth temperatures near 37 °C; therefore, the observed optimal temperature and thermostability of the PAPs are consistent with expectations for enzymes from mesophilic bacteria. However, *E. coli* PAP-1 showed higher activity in the presence of Mn^{2+} and Co^{2+} and retained more activity in NaCl compared with Mli PAP. Unexpectedly, Mli PAP was strongly inhibited by NaCl but not by KCl, despite its native host, *M. lipolyticus*, tolerating much higher NaCl concentrations than *E. coli* (up to 25% vs. 7–11% *w*/*v*, respectively) [16,18]. This discrepancy may hint

toward stabilization of Mli PAP through interactions with other *M. lipolyticus* proteins that are absent in *E. coli*. The nearly linear inhibition by NH_4^+ may be related to the Hofmeister series, in which NH_4^+ is among the strongest kosmotropic cations. Given the aggregation tendency of PAPs, inhibition by NH_4^+ may reflect salt-induced precipitation. However, this explanation is preliminary and requires direct experimental validation. Structural studies would also provide an insight into the difference between the two studied PAPs, demonstrating possibly different binding with an RNA substrate or altered structure of active sites.

The nucleotide specificity of both PAPs was identical: ATP was the preferred substrate, while CMP and UMP were incorporated with much lower efficiency, and GMP incorporation was not detected. This observation partially contradicts previous reports suggesting that *E. coli* PAP-1 can utilize not only ATP but also other NTPs as substrates for RNA elongation [19]. In the study by Yehudai-Resheff and Schuster, the incorporation rates of non-ATP nucleotides were higher than those observed in our experimental system. This discrepancy may be attributed to differences in used reaction buffer compositions and substrates, as we used a short (r)A_{20} oligonucleotide rather than a longer in vitro-transcribed RNA. Relaxed substrate specificity may be an in vitro artifact caused by somehow suboptimal reaction conditions. In the presence of Mn^{2+}, DNA polymerases incorporate NTPs much more efficiently than when Mg^{2+} is used as a cofactor. For *E. coli* DNA polymerase I, increasing pH from 7.6 to 9.1 results in increased misincorporation rate of ribonucleotides [20]. Nevertheless, both *E. coli* PAP-1 and Mli PAP exhibited relaxed, but not absolute, nucleotide specificity, suggesting the existence of mechanisms that enhance PAP NTPs' selectivity in vivo.

Several limitations of the present study should be mentioned. First, we did not use long RNA substrates, although RNA's secondary structure is known to affect the activity of *E. coli* PAP-1 [21]. Whether the RNA structure modulates poly(A) tail synthesis by Mli PAP remains an open question requiring further investigation. Second, NTP specificity was evaluated qualitatively but not quantitatively, leaving room for speculation regarding subtle differences in substrate preference between the two PAPs. Nevertheless, the similar K_m(ATP) values measured for *E. coli* PAP-1 and Mli PAP suggest that both enzymes may incorporate alternative NTPs in a comparable manner. Third, the efficacy of Mli PAP in actual practical applications remains unknown since the respective comparison with *E. coli* PAP-1 and the yeast PAP requires extensive additional experiments. However, this study reveals the true potential of various PAPs for usage in biotechnology, including not only efficacy of polyadenylation but also their stability and tolerance to various inhibitors that can appear in reaction mixes.

In summary, we cloned and characterized the biochemical properties of the first poly(A) polymerase identified in *Alphaproteobacteria*, specifically, *Marinobacter lipolyticus*. The newly characterized PAP exhibited properties broadly similar to those of *E. coli* PAP-1, providing a foundation for further studies on the diversity and mechanistic features of bacterial PAPs.

4. Materials and Methods

4.1. Search and Selection of Mli PAP Coding Sequence

The amino acid sequence of *E. coli* PAP-1 was used as a reference for the search of potential bacterial PAPs using Protein BLAST 2.17.0 against proteins from *Alphaproteobacteria*. Criteria for cloning were length of 400–600 a.a., the presence of all PAP structural domains (head, neck, body and leg), conservative positions corresponding to D69, D71, and E108 involved in catalysis in PAP I *E. coli* [11], and the characteristic signature of bacterial PAPs [LIV][LIV]G[R/K][R/K]Fx-[LIV]h[HQL][LIV] [9]. The retrieved putative PAPs were

organized in a range based on their similarity to *E. coli* PAP-1. The selected coding sequence of a putative PAP from *Marinobacter lipolyticus* (GenBank: EON91085.1) was synthesized and cloned into the pET23a vector by Shanghai RealGene Bio-tech, Inc. (Shanghai, China), resulting in a plasmid pPAP-Mli followed by cloning into pBADM52 vector, resulting in a plasmid pPAP-Mli2.

4.2. Expression and Purification of PAP from Marinobacter Lipolyticus

A starter culture of *E. coli* BL21 (DE3) pLysS (Promega, Madison, WI, USA) strain harboring either the plasmids pPAP-Mli or pPAP-Mli2 was grown to OD600 = 0.8 in LB medium with 100 μg/mL ampicillin at 37 °C. In a LiFlus GX fermenter (Biotron Inc., Bucheon, Republic of Korea), 4 L of LB with 100 μg/mL ampicillin was inoculated with 20 mL of the starter culture, and the cells were grown to OD600 = 0.6 at 37 °C. The expression of Mli PAP was induced by adding IPTG up to 1 mM concentration. After induction for 12 h at 18 °C, the cells were harvested by centrifugation at 4000× *g* and stored at −70 °C.

For partial Mli PAP purification, the cell pellet with pPAP-Mli was resuspended in the lysis buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 1 mM PMSF, 10 mM DTT, 10% glycerol, 1% Triton X-100, 1 mg/mL lysozyme), and incubated for 30 min at 37 °C, followed by sonication. After lysis, the soluble fraction was separated by two consequent centrifugation steps at 20,000× *g* for 30 min. The insoluble fraction was resuspended in the resuspension buffer (50 mM Tris-HCl pH 8.0, 1 M NaCl, 1 mM PMSF), incubated at 4 °C for 14 h and clarified by centrifugation at 25,000× *g* for 30 min. The resulting supernatant was supplemented by 0.01% NaN_3 and stored at +4 °C. The protein concentration of was measured using a standard Bradford assay.

For full Mli PAP purification, after the lysis of the cell pellet with pPAP-Mli2 under the above-described conditions, the soluble proteins were precipitated ON by 60% $(NH_4)_2SO_4$, followed by centrifugation at 20,000× *g* for 30 min. The resulting pellet was suspended in the resuspension buffer (50 mM $NaPO_4$, pH 7.0, 1 M NaCl, 5 mM β-mercaptoethanol) and loaded onto a 2 mL IMAC column (Bio-Rad, Hercules, CA, USA) pre-equilibrated with buffer A1 (50 mM $NaPO_4$, pH 7.0, 0.5 M NaCl, 5 mM β-mercaptoethanol), followed by washing the column with 25 mL of the buffer A with 1 M NaCl. Bound proteins were eluted using 10 column volumes and a 0–100% linear gradient of buffer B1 (buffer A1 with 0.5 M imidazole). After affinity chromatography, the fractions with the DsbA-Mli PAP fusion protein were pooled and loaded onto a 2 mL Macro-Prep DEAE Resin (Bio-Rad, column, Hercules, CA, USA) pre-equilibrated with buffer C1 (50 mM $NaPO_4$, pH 7.0, 0.3 M NaCl, 5 mM β-mercaptoethanol). The column was washed with 10 mL of buffer C, and bound proteins were eluted by 10 column volumes and a 0–100% linear gradient of buffer D (50 mM $NaPO_4$ pH 7.0, 1.5 M NaCl, 5 mM β-mercaptoethanol). The fractions with DsbA-Mli PAP were pooled and the fusion protein was hydrolyzed by TEV protease (Biolabmix, Novosibirsk, Russia), followed by further affinity chromatography under the same conditions for removal of His-tagged TEV protease and DsbA. Purified Mli PAP remained in the flow-throw and was dialyzed against a storage buffer (20 mM Tris-HCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 50% glycerol, pH 7.5), and stored at −20 °C. All the fractions from each step were analyzed by SDS-PAGE. The purity of the isolated Mli PAP was not lower than 95%. The concentration of purified Mli PAP was measured using a standard Bradford assay.

4.3. Expression and Purification of PAP-1 from E. coli

E. coli PAP-1 was cloned into the pET36b vector (Novagen, Madison, WI, USA) using the restriction–ligation method and NheI and XhoI restriction sites, resulting in the plasmid pET-PAP-Eco as described in our previous work [22]. A starter culture of *E. coli* BL21 (DE3)

pLysS harboring the plasmid pET-PAP-Eco was grown to OD600 = 0.6 in LB medium with 100 μg/mL kanamycin at 37 °C. In a LiFlus GX fermenter (Biotron Inc., Bucheon, Republic of Korea), 4 L of LB with 100 μg/mL kanamycin was inoculated with 20 mL of the starter culture, and the cells were grown to OD600 = 0.6 at 37 °C. The expression of *E. coli* PAP-1 was induced by adding IPTG up to 1 mM concentration. After induction for 12 h at 18 °C, the cells were harvested by centrifugation at 4000× *g* and stored at −70 °C.

For protein purification, the cell pellet was resuspended in a lysis buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM PMSF, 2 M urea, 5 mM β-Mercaptoethanol, 1 mg/mL lysozyme), and incubated for 30 min on ice, followed by sonication. After lysis, the soluble fraction was separated by two consequent centrifugation steps at 20,000× *g* for 30 min. Soluble proteins were precipitated ON by 60% $(NH_4)_2SO_4$, followed by centrifugation at 20,000× *g* for 30 min. The resulting pellet was suspended in the lysis buffer and loaded onto a 5 mL IMAC column (Bio-Rad, Hercules, CA, USA) pre-equilibrated with buffer A (50 mM $NaPO_4$, pH 7.0, 0.5 M NaCl, 5 mM β-mercaptoethanol), followed by washing the column with 25 mL of the buffer A with 1 M NaCl. Bound proteins were eluted using 10 column volumes and a 0–100% linear gradient of buffer B (buffer A with 0.5 M imidazole). After affinity chromatography, the fractions with *E. coli* PAP-1 were pooled and loaded onto a 2 mL Macro-Prep HighS Resin (Bio-Rad, column, Hercules, CA, USA) pre-equilibrated with buffer C (50 mM $NaPO_4$, pH 7.0, 0.3 M NaCl, 5 mM β-mercaptoethanol). The column was washed with 10 mL of buffer C, and bound proteins were eluted by 10 column volumes and a 0–100% linear gradient of buffer D (50 mM $NaPO_4$, pH 7.0, 1.5 M NaCl, 5 mM β-mercaptoethanol). The fractions with *E. coli* PAP-1 were pooled, dialyzed against a storage buffer (20 mM Tris-HCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 50% glycerol, pH 7.5), and stored at −20 °C. All the fractions from each step were analyzed by SDS-PAGE. The purity of the isolated *E. coli* PAP-1 was not lower than 95%. The concentration of purified *E. coli* PAP-1 was measured using a standard Bradford assay.

4.4. Polyadenylation Assay

The specific activity of poly(A) polymerase was analyzed using elongation of the fluorescently labeled (r)A_{20} oligonucleotide. The reaction mixes (10 μL) contained a 1× reaction buffer for *E. coli* PAP-1 (50 mM Tris-HCl, 250 mM NaCl, 10 mM $MgCl_2$, pH 8.0), 1 mM ATP, 10 pmol of (r)A_{20}-oligonucleotide with 5′-terminal FAM, and an indicated amount of PAP. The reactions were started by the addition of the enzyme and immediately transferred to a preheated thermocycler, followed by incubation for 25 min at 37 °C. After incubation, the reactions were quenched by the addition of 10 μL of formamide and denatured by heating for 5 min at 95 °C. The reaction products were analyzed using denaturing PAGE in a 18% acrylamide gel with 7 M urea and quantified using the ImageLab software version 6.0.1 (Bio-Rad, Hercules, CA, USA).

4.5. Thermal Stability, Optimal Temperature, Ion Concentration and Nucleotide Specificity

The thermal stability of Mli PAP and *E. coli* PAP-1 was studied by heating the enzyme and then assaying the poly(A)-polymerase activity. The aliquots of the polymerase activity reaction buffer (described above) without ATP containing an identical amount of Mli PAP were incubated at temperatures from 30 °C to 60 °C, increasing by 10 °C per step for 15–60 min. The reactions were chilled on ice, and poly(A) polymerase activity was measured as mentioned above.

The temperature optimum of Mli PAP and *E. coli* PAP-1 was defined by measuring the poly(A)-polymerase activity at temperatures from 25 °C to 60 °C, increasing by 5 °C per step, with other conditions identical to those described above for the poly(A) polymerase activity assay. The reactions were initiated by adding the aliquots of enzymes to mixes

containing all other components, including the primed template. The mixes were preheated for 5 min before the addition of enzymes.

The optimal ion concentrations were examined as in the poly(A) polymerase activity assay using 25–200 mM NH_4Cl, KCl, NaCl, $(NH_4)_2SO_4$ or 1–15 mM $MgCl_2$, $MnCl_2$, $CoCl_2$.

After optimization of reaction conditions, nucleotide specificity of Mli PAP was defined in the optimized reaction buffer (50 mM Tris-HCl, 150 mM KCl, 10 mM $MgCl_2$, pH 8.0) by titration of NTPs in reaction mixes for the poly(A) polymerase activity assay in the range of 0.25–10.0 mM. The polyadenylation assay was conducted for 30 min at 37 °C.

4.6. Data Analysis

The relative amount of the polyadenylated substrate was calculated—Ratio = I(elongated products)/(I(substrate) + I(elongated products))—and used to estimate by a linear regression model an enzyme amount necessary to elongate 50% of the substrate initially added to the reaction. All calculations were performed in the GraphPad Prism 8.0.1 software (Insight Venture Management, New York, NY, USA).

5. Conclusions

In the present study, we successfully cloned and biochemically characterized the first poly(A) polymerase from *Alphaproteobacteria*, specifically, *Marinobacter lipolyticus* (Mli PAP). Due to the absence of the active soluble enzyme after expression in *E. coli* and the absence of binding with various chromatography resins, Mli PAP was partially purified from inclusion bodies and purified as a fusion protein with DsbA, following cleavage of DsbA. Despite challenges in solubility and purification, the enzyme retained robust polyadenylation activity when extracted from inclusion bodies. Mli PAP exhibited optimal activity at 30 °C, comparable thermostability to *E. coli* PAP-1, strict ATP specificity, and a preference for Mg^{2+} as a cofactor. Notably, it demonstrated distinct salt dependence, with maximal activity in KCl and strong inhibition by NaCl and ammonium salts, highlighting functional divergence from its halophilic host and from *E. coli* PAP-1. These findings expand the biochemical understanding of bacterial PAPs beyond *Gammaproteobacteria* and provide a foundation for exploring PAP diversity, evolutionary mechanisms, and potential biotechnological applications, particularly in RNA-based therapeutics.

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Abbreviations

The following abbreviations are used in this manuscript:

PAP	poly(A)-polymerase
Mli	*Marinobacter lipolyticus*

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