

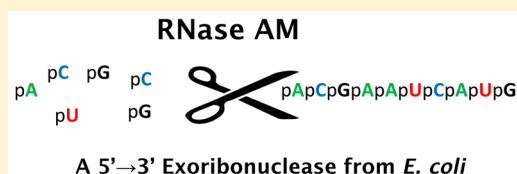
# Discovery of a Previously Unrecognized Ribonuclease from *Escherichia coli* That Hydrolyzes 5'-Phosphorylated Fragments of RNA

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## Supporting Information

**ABSTRACT:** TrpH or YciV (locus tag b1266) from *Escherichia coli* is annotated as a protein of unknown function that belongs to the polymerase and histidinol phosphatase (PHP) family of proteins in the UniProt and NCBI databases. Enzymes from the PHP family have been shown to hydrolyze organophosphoesters using divalent metal ion cofactors at the active site. We found that TrpH is capable of hydrolyzing the 3'-phosphate from 3',5'-bis-phosphonucleotides. The enzyme will also sequentially hydrolyze 5'-phosphomononucleotides from 5'-phosphorylated RNA and DNA oligonucleotides, with no specificity toward the identity of the nucleotide base. The enzyme will not hydrolyze RNA or DNA oligonucleotides that are unphosphorylated at the 5'-end of the substrate, but it makes no difference whether the 3'-end of the oligonucleotide is phosphorylated. These results are consistent with the sequential hydrolysis of 5'-phosphorylated mononucleotides from oligonucleotides in the 5' → 3' direction. The catalytic efficiencies for hydrolysis of 3',5'-pAp, p(Ap)<sub>4</sub>A, and p(dAp)<sub>4</sub>dA were determined to be  $1.8 \times 10^5$ ,  $9.0 \times 10^4$ ,  $4.6 \times 10^4$ , and  $2.9 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ , respectively. TrpH was found to be more efficient at hydrolyzing RNA oligonucleotides than DNA oligonucleotides. This enzyme can also hydrolyze annealed DNA duplexes, albeit at a catalytic efficiency approximately 10-fold lower than that of the corresponding single-stranded oligonucleotides. TrpH is the first enzyme from *E. coli* that has been found to possess 5' → 3' exoribonuclease activity. We propose to name this enzyme RNase AM.



Systematic advances in high-throughput DNA sequencing have resulted in an exponential rise in the number of known and predicted protein sequences in public databases. As of January 2015, the UniProtKB/TrEMBL protein database contained more than 89 million gene sequences.<sup>1</sup> However, functional annotations for the corresponding protein sequences have not kept pace with these efforts, and consequently, the physiological substrates for a significant number of these newly identified enzymes are uncertain, unknown, or incorrectly annotated. One of the many approaches to systematically identifying enzymes of unknown function orchestrates a combination of bioinformatics, structural biology, and focused library screening.<sup>2</sup> Here we describe the utilization of this approach to discover the function of the hypothetical protein known as TrpH or YciV (locus tag b1266) from *Escherichia coli* K12. The protein sequence for this enzyme is encoded in the *E. coli* genome adjacent to the operon for the biosynthesis of tryptophan comprising genes *trpA*–*G*.<sup>3</sup> However, no report was found in the literature assigning any functional role for TrpH in the biosynthesis of L-tryptophan.

TrpH belongs to the polymerase and histidinol phosphatase (PHP) family of proteins, which is a subset of those enzymes from the amidohydrolase superfamily (AHS). Enzymes from the AHS are known to hydrolyze ester and amide functional groups at carbon and phosphorus centers.<sup>4</sup> These enzymes possess a distorted ( $\beta/\alpha$ )<sub>8</sub>-barrel structural fold and up to three divalent metal ions in the active site. The three metal ions have been implicated in activating water or hydroxide as the

nucleophile in these reactions. Enzymes from the PHP family possess a distorted ( $\beta/\alpha$ )<sub>7</sub>-barrel structural fold and a trinuclear metal center within the enzyme active site.<sup>5</sup> The PHP family is further subdivided into three Clusters of Orthologous Groups: cog0613, cog1387, and cog4464.<sup>6</sup> These three COGs have been generically annotated as metal-dependent phosphoesterases, L-histidinol phosphate phosphatase, and protein-tyrosine phosphatases, respectively.<sup>7</sup> We have previously determined the three-dimensional structure and catalytic reaction mechanism of L-histidinol phosphate phosphatase (HPP) from *Lactococcus lactis*.<sup>8</sup> The structure of HPP complexed with L-histidinol and arsenate provided a clear picture for the binding of substrate and the proposed mechanism of hydrolysis. The  $\alpha$ - and  $\beta$ -metal ions are responsible for the nucleophilic activation of hydroxide and enhancement of the electrophilicity of the phosphorus center. The  $\gamma$ -metal ion coordinates the bridging oxygen atom of the phosphomonoester substrate and acts as a Lewis acid to the leaving group alcohol. The phosphorylated substrate is further coordinated to the active site by an ion-pair interaction with the side chains of arginine or lysine.<sup>8</sup>

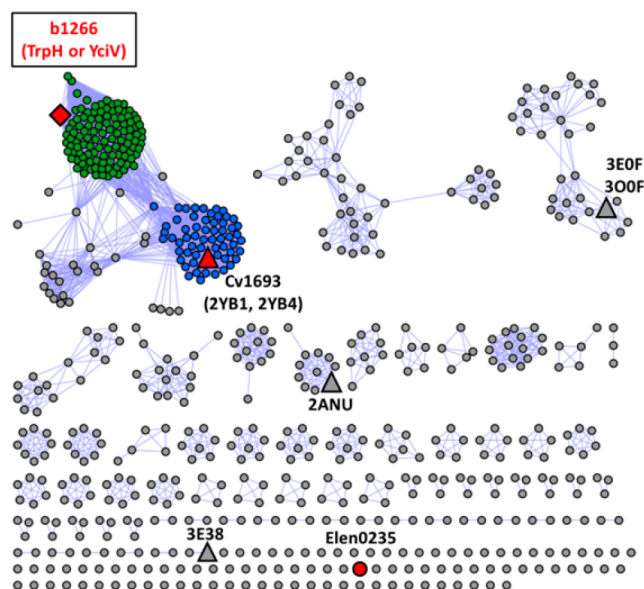
TrpH belongs to cog0613 within the PHP family. The sequence similarity network diagram of cog0613 at an *E* value cutoff of  $1 \times 10^{-60}$  is depicted in Figure 1.<sup>9–11</sup> Currently, there

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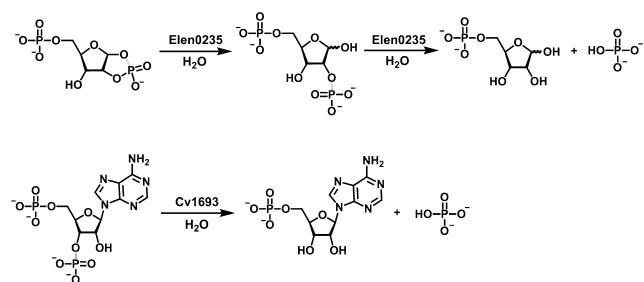




**Figure 1.** Sequence similarity network of cog0613 at an  $E$  value cutoff of  $10^{-60}$ .<sup>9,10</sup> This network was made using Cytoscape (<http://www.cytoscape.org>).<sup>11</sup> Each node represents a nonredundant protein sequence, while each edge (line) represents pairs of sequences that are more closely related than the arbitrary  $E$  value cutoff ( $10^{-60}$ ). The available crystal structures are shown as triangles, and their respective PDB codes are indicated. The enzymes that have been biochemically characterized are colored red. Cv1693 is a 3',5'-nucleotide bisphosphate-3'-phosphatase,<sup>13</sup> while Elen0235 is a cyclic phosphate dihydrolase (cPDH).<sup>12</sup> TrpH (b1266) is the enzyme characterized in this study. Protein sequences that are sufficiently homologous to Cv1693 in sequence ( $E$  value of  $<10^{-70}$ , which is approximately equivalent to a sequence identity of  $>43\%$ ) are colored blue, while those that are similarly homologous to TrpH are colored green. The list of genes shown in green can be found in Table S1 of the Supporting Information.

are four proteins from cog0613 whose crystal structures are available in the Protein Data Bank (PDB): Cv1693 from *Chromobacterium violaceum* (PDB entries 2YB1 and 2YB4), Bad1165 from *Bifidobacterium adolescentis* (PDB entry 3O0F), Tm0559 from *Thermotoga maritima* (PDB entry 2ANU), and Bvu3505 from *Bacteroides vulgatus* (PDB entry 3E38). The physiological functions of two enzymes from cog0613 have been elucidated: Elen0235 from *Eggerthella lenta* and Cv1693 from *C. violaceum*. Elen0235 is a cyclic phosphodiester dihydrolase that catalyzes the hydrolysis of 5-phosphoribose-1,2-cyclic phosphate to ribose 5-phosphate and phosphate via a ribose 2,5-bis-phosphate intermediate (Scheme 1).<sup>12</sup> Cv1693 catalyzes the hydrolysis of 3',5'-bis-phosphonucleotides to 5'-nucleotide monophosphate and inorganic phosphate.<sup>13</sup> Here

**Scheme 1**



we demonstrate that TrpH catalyzes the hydrolysis of 3',5'-bis-phosphonucleotides and the sequential hydrolysis of short oligonucleotides bearing a phosphoryl group at the 5'-terminus.

## MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich, unless indicated otherwise. Genomic DNA for *E. coli* K-12 (ATCC25559) was obtained from the American Type Culture Collection (ATCC). *Pfu* Turbo DNA polymerase, T4 DNA ligase, and restriction enzymes were procured from New England Biolabs. DNA primers and Big Dye were obtained from Integrated DNA Technologies (IDT). Vector pET30a(+) was purchased from EMD Biosciences. *E. coli* BL21(DE3) and XL1 Blue competent cells were obtained from Stratagene. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was conducted using Bio-Rad Mini-protein TGX any-kD gels. The  $P_i$  Colorlock Gold kit for the determination of inorganic phosphate was obtained from Innova Biosciences. Oligonucleotide substrates were purchased from Integrated DNA Technologies, except for p(dAp)dA, p(dAp)<sub>2</sub>dA, dA(dAp), and dA(dAp)<sub>2</sub>, which were obtained from Genelink. The synthesis of the 3',5'-bis-phosphonucleotides was conducted as previously described.<sup>13</sup> High-performance liquid chromatography experiments were conducted using a GE Akta Purifier or Bio-Rad NGC system. UV–visible absorption spectra were recorded using a Spectromax 384 Plus 96-well plate reader from Molecular Devices. Purified guanylate kinase from *Saccharomyces cerevisiae* was a generous gift from the laboratory of T. Begley (Texas A&M University).

**Cloning, Expression, and Purification of TrpH from *E. coli*.** Polymerase chain reaction (PCR) amplification was conducted from the genomic DNA of *E. coli* K-12 using 5'-GCAGGAGCCATATGAGCGACACGAATTATGCAGTGA-TTTACGACCTGC-3' as the forward primer and 5'-CGCG-CTCGAGTAATCCCTCTCTGTGGTGTCTGCGGCTG-TTCC-3' as the reverse primer. The primer pair contained restriction sites for *Nde*I and *Xho*I, respectively. The PCR product was purified with a PCR cleanup kit (Qiagen), doubly digested using *Nde*I and *Xho*I, and ligated into a pET30a(+) vector previously doubly digested with the same set of restriction enzymes.

The recombinant protein was expressed and purified using an iron-free expression procedure described elsewhere.<sup>11</sup> In brief, the ligated plasmid was transformed into *E. coli* BL21(DE3) cells by electroporation. LB cultures (5 mL) containing 50  $\mu$ g/mL kanamycin were inoculated with single colonies and grown overnight. These cultures were used to inoculate 1 L of the same medium at pH 7.2 and allowed to grow at 37 °C until the OD<sub>600</sub> reached 0.15–0.2. The temperature was reduced to 30 °C, and the iron-specific chelator 2,2'-bipyridyl was added to a final concentration of 1.0 mM when the OD<sub>600</sub> reached  $\sim$ 0.4, and 0.25 mM isopropyl D-thiogalactopyranoside (IPTG) was added when the OD<sub>600</sub> reached  $\sim$ 0.6. The temperature was then lowered to  $\sim$ 15 °C, and the cells were shaken for  $\sim$ 16 h, after which they were harvested by centrifugation and stored at  $-80$  °C.

For protein purification,  $\sim$ 3 g of cells was thawed and resuspended in  $\sim$ 60 mL of buffer [25 mM HEPES (pH 7.5), 250 mM KCl, 10% glycerol, and 100  $\mu$ M MnCl<sub>2</sub>] containing 0.4 mg/mL protease inhibitor phenylmethanesulfonyl fluoride (PMSF). Cells were lysed by sonication, and the insoluble cell debris was separated by centrifugation. The supernatant

solution was treated with 10 mL of a 6 mg/mL solution of protamine sulfate in purification buffer. The precipitated DNA was separated by centrifugation after incubation for 30 min. Ammonium sulfate was added to a concentration of 35% saturation, followed by centrifugation, and then to 70% saturation. The precipitated protein was separated by centrifugation, resuspended in 10 mL of purification buffer, and further purified using gel filtration chromatography (GE Superdex 26/600 column). The fractions were analyzed using SDS-PAGE. Fractions containing TrpH were pooled, and the protein concentration was determined by the absorbance at 280 nm using a calculated molar extinction coefficient of  $51450 \text{ M}^{-1} \text{ cm}^{-1}$  and a molecular mass of 32.5 kDa. The purified protein was flash-frozen using liquid nitrogen and stored at  $-80^\circ \text{C}$ . The purity of the protein was estimated to be >95% as judged by SDS-PAGE.

**Metal Analysis.** The metal content of the purified proteins was determined using a PerkinElmer DRC-II inductively coupled plasma mass spectrometer (ICP-MS). Samples were run in a matrix of 1% (v/v) nitric acid. In general, concentrated samples of purified proteins were passed through a desalting column (PD-10 from GE healthcare) pre-equilibrated with metal-free buffer. The buffer was rendered free of metal ions by treatment with Chelex-100 resin (Bio-Rad). The desalted protein was digested with nitric acid ( $\geq 69\%$ , Fluka Analytical) by being heated to  $95^\circ \text{C}$  for 15–20 min. The digested protein was diluted using deionized water such that the final sample contained  $\sim 1 \mu\text{M}$  protein and 1% (v/v) nitric acid.

**Phosphate Detection Assays.** The formation of phosphate was measured using the  $\text{P}_i$  ColorLock Gold kit from Innova Biosciences, according to the manufacturer's directions. The assay conditions included 50 mM HEPES (pH 7.5), 250 mM KCl, 2.0 mM  $\text{MgCl}_2$ , 0.15 mM  $\text{MnCl}_2$ , 0.05 mg/mL BSA, and  $30^\circ \text{C}$ . A set of assays consisted of 6–12 substrate concentrations with 3 or 4 time points taken over a period of 20–40 min. Assays for any given substrate were conducted at an enzyme concentration in the range of 1–50 nM to obtain an inorganic phosphate concentration within the detection limits of the assay kit (1–40  $\mu\text{M}$ ). The color was allowed to develop for 30 min after quenching, and the absorbance was determined at 650 nm. The phosphate concentration of each well was determined using a previously generated standard curve under the same assay conditions. The initial rates at each substrate concentration were determined by linear regression in a plot of phosphate versus time.

**Detection of 5'-Nucleoside Monophosphate.** The release of 5'-AMP, 5'-dAMP, 5'-GMP, or 5'-dGMP by enzymatic hydrolysis of oligonucleotide substrates was measured by monitoring the oxidation of NADH to  $\text{NAD}^+$  by lactate dehydrogenase. This was achieved using a coupling system consisting of the enzymes adenylate kinase or guanylate kinase, pyruvate kinase, and lactate dehydrogenase. Each 250  $\mu\text{L}$  assay contained 250 mM KCl, 2.0 mM  $\text{MgCl}_2$ , 0.15 mM  $\text{MnCl}_2$ , 0.7 mM phospho(enol)pyruvate (PEP), 0.5 mM ATP, 0.3 mM NADH, and 20 units/mL adenylate kinase (or guanylate kinase), 20 units/mL pyruvate kinase, and 20 units/mL lactate dehydrogenase. The concentration of TrpH ranged from 2 to 1000 nM. The assays were conducted at  $30^\circ \text{C}$  and monitored at 340 nm.

**Anion Exchange Chromatography.** Polynucleotide substrates were tested for hydrolysis by TrpH using a Resource Q (1 mL) anion exchange column (GE Lifesciences) and an Akta Purifier system (GE) at a wavelength of 260 nm. The

oligonucleotide (10  $\mu\text{M}$ ) was incubated overnight with 1.0  $\mu\text{M}$  enzyme in a 250  $\mu\text{L}$  reaction mixture containing 25 mM HEPES (pH 7.5), 0.5 mM  $\text{MgCl}_2$ , 200 mM KCl, and 50  $\mu\text{M}$   $\text{MnCl}_2$ . The time-dependent hydrolysis of oligonucleotide substrates by TrpH was monitored using a Resource Q (1 mL) anion exchange column and NGC system (Bio-Rad). In general, the reaction was started by adding the enzyme to a solution containing substrate, and aliquots were removed at specific times. These aliquots were quenched either by being injected directly onto the anion exchange column or by being mixed with hot water (50  $\mu\text{L}$  aliquot added to 200  $\mu\text{L}$  of hot water at  $>90^\circ \text{C}$ ), depending on the interval between two time points.

**Annealing of DNA.** Two single strands of DNA consisting of complementary sequences were mixed in a solution containing 10 mM HEPES (pH 7.5), 50 mM KCl, and 1.0 mM  $\text{MgCl}_2$ , to a final concentration of 100  $\mu\text{M}$  for each strand. The solution was heated at  $95^\circ \text{C}$  for 10 min, cooled slowly to room temperature, and stored overnight at  $4^\circ \text{C}$ .

**Data Analysis.** Kinetic parameters  $k_{\text{cat}}$  and  $k_{\text{cat}}/K_m$  were obtained by fitting the initial velocity data to eq 1 using the nonlinear least-squares fitting program in SigmaPlot 10.0

$$\nu/[E_t] = (k_{\text{cat}}[A])/(K_m + [A]) \quad (1)$$

where  $\nu$  is the initial velocity at substrate concentration  $[A]$ ,  $[E_t]$  is the enzyme concentration,  $k_{\text{cat}}$  is the turnover number, and  $K_m$  is the Michaelis constant.

## RESULTS

**Purification and Optimization of TrpH.** TrpH, expressed under iron-free conditions, and in the presence of exogenously added  $\text{Mn}^{2+}$  ions before protein induction, was found to contain  $\sim 1$  equiv of  $\text{Mn}^{2+}$  per monomer by ICP-MS. When 3',5'-pAp was incubated with TrpH, inorganic phosphate was released as a function of time. The rate of substrate hydrolysis catalyzed by TrpH was found to depend on the concentration of  $\text{Mn}^{2+}$  added to the assay, and the enzyme showed optimal activity at a  $\text{Mn}^{2+}$  concentration of  $\geq 100 \mu\text{M}$ . The presence of KCl in the buffers used for enzyme purification and assays was required for the activity of the enzyme, while the addition of bovine serum albumin (BSA) prevented loss of activity at enzyme concentrations of less than  $\sim 0.5 \mu\text{M}$ .

**Oligonucleotides as Substrates for TrpH.** Short pieces of RNA and DNA, ranging in length from two to five nucleotides, were incubated with TrpH. The RNA and DNA oligonucleotides were phosphorylated at the 5'- or 3'-terminus. TrpH completely hydrolyzed those oligonucleotides that were phosphorylated at the 5'-terminus but did not hydrolyze those oligonucleotides that were only phosphorylated at the 3'-terminus.

**Substrate Specificity of TrpH.** The kinetic parameters for the hydrolysis of RNA and DNA oligonucleotides by TrpH are listed in Table 1. The enzyme exhibited the best catalytic activity toward the hydrolysis of 3',5'-pAp, 3',5'-pGp, and p(Ap)A. Removal of the 5'-phosphoryl group from 3',5'-pAp resulted in a 360-fold reduction in  $k_{\text{cat}}/K_m$  obtained from the kinetic parameters for the hydrolysis of 3'-AMP. The enzyme is able to hydrolyze the 2'-phosphate from 2',5'-pAp with a  $k_{\text{cat}}/K_m$  of  $100 \text{ M}^{-1} \text{ s}^{-1}$ , which is 1000-fold lower than that of 3',5'-pAp. The bis-phosphate mixtures for cytidine, guanosine, and uridine contained 2',5'- and 3',5'-bis-phosphates in a ratio of  $\sim 1:1$ . Therefore, the initial rates of hydrolysis determined for

**Table 1. Kinetic Parameters for TrpH with Various Substrates**

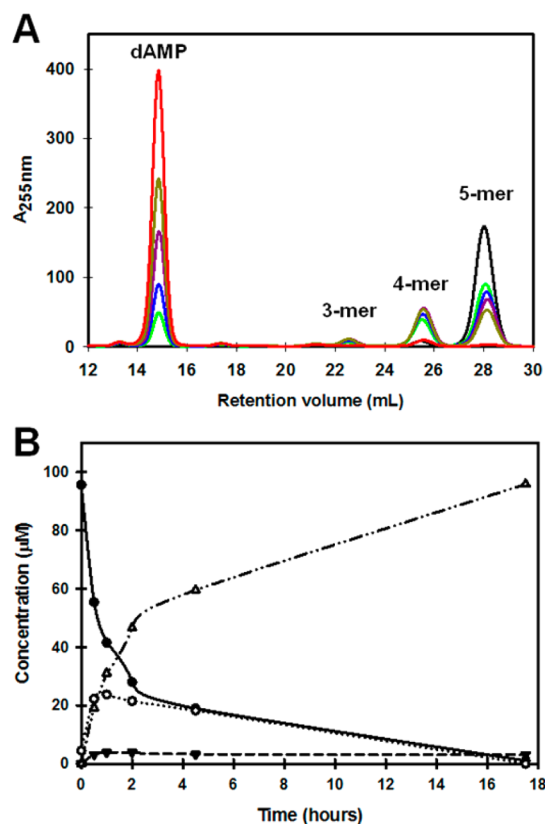
compound	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$K_m$ ( $\mu\text{M}$ )	$k_{\text{cat}}/K_m$ ( $\text{M}^{-1} \text{s}^{-1}$ )
pAp	$9.9 \pm 0.5$	$56 \pm 6$	$(1.8 \pm 0.1) \times 10^5$
2'-deoxy-pAp	$5.6 \pm 0.2$	$53 \pm 6.5$	$(1.1 \pm 0.1) \times 10^5$
pCp (2'/5'/3'/5' mixture) <sup>a</sup>	$6.0 \pm 0.3$	$86 \pm 10$	$(7.0 \pm 0.5) \times 10^4$
2'-deoxy-pCp <sup>a</sup>	$1.9 \pm 0.1$	$114 \pm 10$	$(1.7 \pm 0.1) \times 10^4$
pGp (2'/5'/3'/5' mixture) <sup>a</sup>	$5.5 \pm 0.3$	$45 \pm 9$	$(1.2 \pm 0.2) \times 10^5$
2'-deoxy-pGp <sup>a</sup>	$1.7 \pm 0.1$	$30 \pm 6$	$(5.8 \pm 0.6) \times 10^4$
pUp (2'/5'/3'/5' mixture) <sup>a</sup>	$4.7 \pm 0.2$	$59 \pm 8$	$(8.0 \pm 0.8) \times 10^4$
2'-deoxy-pUp <sup>a</sup>	$2.1 \pm 0.1$	$110 \pm 20$	$(1.9 \pm 0.2) \times 10^4$
2'-deoxy-pTp <sup>a</sup>	$1.6 \pm 0.1$	$32 \pm 5$	$(4.9 \pm 0.6) \times 10^4$
2'-deoxy-pIp <sup>a</sup>	$3.3 \pm 0.3$	$48 \pm 13$	$(6.9 \pm 1.3) \times 10^4$
3'-AMP	$0.22 \pm 0.01$	$420 \pm 80$	$(5.0 \pm 0.6) \times 10^2$
2',5'-pAp	ND <sup>b</sup>	ND <sup>b</sup>	$(1.0 \pm 0.1) \times 10^2$
p(Gp)G	$0.60 \pm 0.07$	$14.0 \pm 7$	$(4.1 \pm 1.8) \times 10^4$
p(Ap)A	$14 \pm 1$	$160 \pm 10$	$(9.0 \pm 0.4) \times 10^4$
p(Ap) <sub>2</sub> A	$3.8 \pm 0.1$	$55 \pm 5$	$(7.0 \pm 0.4) \times 10^4$
p(Ap) <sub>3</sub> A	$3.6 \pm 0.1$	$45 \pm 2$	$(7.9 \pm 0.2) \times 10^4$
p(Ap) <sub>4</sub> A	$2.7 \pm 0.3$	$59 \pm 16$	$(4.6 \pm 0.8) \times 10^4$
p(dAp)dA	$2.5 \pm 0.2$	$149 \pm 20$	$(1.7 \pm 0.1) \times 10^4$
p(dAp) <sub>2</sub> dA	$0.72 \pm 0.04$	$67 \pm 10$	$(1.1 \pm 0.1) \times 10^4$
p(dAp) <sub>3</sub> dA	$1.20 \pm 0.06$	$180 \pm 20$	$(6.8 \pm 0.4) \times 10^3$
p(dAp) <sub>4</sub> dA	$0.79 \pm 0.02$	$280 \pm 20$	$(2.9 \pm 0.1) \times 10^3$

<sup>a</sup>Compounds assayed using a P<sub>i</sub> detection assay. Other substrates were assayed using an adenylate kinase/guanylate kinase–pyruvate kinase–lactate dehydrogenase coupled assay. <sup>b</sup>Not determined.

these substrates were assumed to be for the 3',5'-bis-phosphate isomers. The identity of the purine/pyrimidine base in the 3',5'-bis-phosphonucleotide substrates did not have a significant effect on the kinetic parameters of hydrolysis by TrpH. In general, RNA substrates had catalytic parameters better than those of DNA substrates of the same length. TrpH was able to completely hydrolyze p(dAp)<sub>9</sub>dA when 50  $\mu\text{M}$  substrate was incubated with 1.0  $\mu\text{M}$  enzyme overnight (data not shown). The enzyme was able to hydrolyze p(Ap)<sub>5</sub> at a rate comparable to that of p(Ap)<sub>4</sub>A but unable to hydrolyze (Ap)<sub>4</sub>A at a detectable rate. Substrates must therefore be phosphorylated at the 5'-end of the oligonucleotide, but it makes no difference whether the 3'-end is phosphorylated. These results are consistent with the conclusion that this enzyme hydrolyzes 5'-phosphorylated oligonucleotides from the 5'-end of the substrate.

#### Hydrolysis of Single-Stranded DNA Oligonucleotides.

The DNA fragment p(dAp)<sub>4</sub>dA (100  $\mu\text{M}$ ) was incubated with TrpH (50 nM), and aliquots were removed for analysis by anion exchange chromatography. The time course showed a steady accumulation of 5'-dAMP and consumption of p(dAp)<sub>4</sub>dA (Figure 2A,B). At intermediate time points, p(dAp)<sub>3</sub>dA and p(dAp)<sub>2</sub>dA were detected and confirmed by determining the retention volumes of the corresponding standards under the same conditions. The formation of p(dAp)dA could not be detected. After overnight incubation, p(dAp)<sub>4</sub>dA was completely hydrolyzed to 5'-dAMP. In a similar experiment, 20  $\mu\text{M}$  5'-phosphorylated DNA hexamer (pApdApdGpdCpdApdA) was incubated with 200 nM TrpH, and the hydrolysis was followed using anion exchange chromatography (Figure 3C,D). The enzymatic hydrolysis resulted in the steady accumulation of the deoxynucleoside



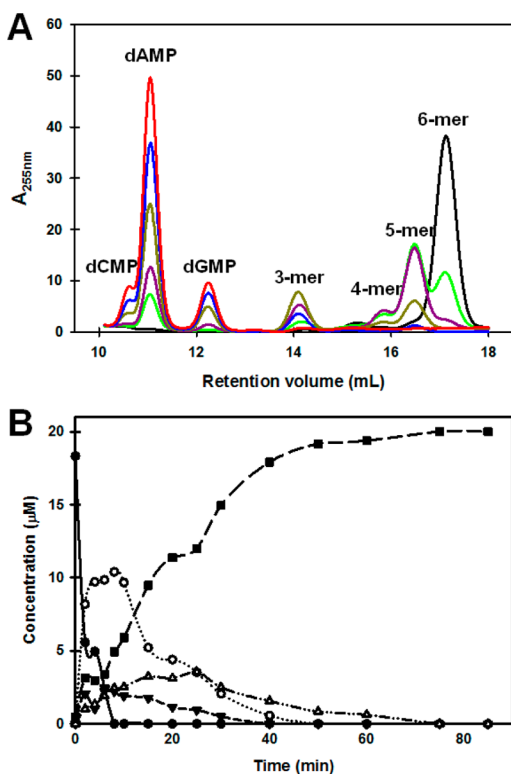
**Figure 2.** Time course for the hydrolysis of DNA oligomer p(dAp)<sub>4</sub>dA by TrpH using anion exchange chromatography. (A) Chromatogram of reaction products during the hydrolysis of 100  $\mu\text{M}$  p(dAp)<sub>4</sub>dA by 50 nM TrpH. The intermediate species, p(dAp)<sub>3</sub>dA and p(dAp)<sub>2</sub>dA, were verified by the addition of standards. Time intervals shown are 0 h (black), 0.5 h (green), 1 h (blue), 2 h (purple), 4.5 h (gray), and 17.5 h (red). (B) Plot of concentration vs time for all the species involved in the hydrolysis of p(dA)<sub>5</sub> by TrpH, shown in panel A. The species shown are 5-mer (●), 4-mer (○), 3-mer (▼), and 5-dAMP (△).

monophosphates with the 5-mer, 4-mer, and 3-mer intermediates detected during the assay.

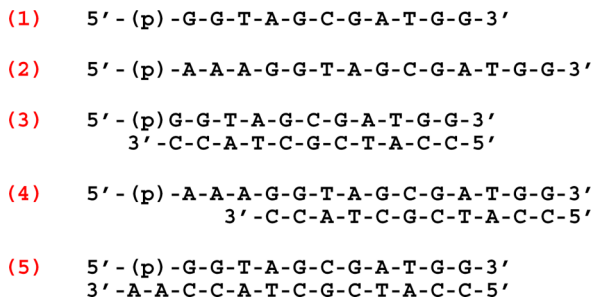
**Hydrolysis of Duplex DNA Oligonucleotides.** DNA substrates 1–5 (Figure 4) were designed such that the 11-mer complementary base sequence had a melting point significantly higher than room temperature ( $T_m \sim 68$  °C).<sup>14</sup> The forward strand (1) was phosphorylated at the 5'-end to ensure that the enzyme will hydrolyze it preferentially as compared to the reverse strand, which was not phosphorylated. For each assay, 20  $\mu\text{M}$  substrate was incubated with 0.4–1.4  $\mu\text{M}$  TrpH, and the initial rate of hydrolysis was determined using the coupling assay for formation of the 5'-phosphomononucleotide. The specific rate constants for the hydrolysis of 20  $\mu\text{M}$  each of 1–5 were 6.0, 4.6, 0.60, 5.4, and 0.31  $\text{min}^{-1}$  respectively. The relative rates of hydrolysis of oligonucleotides 1–5 were calculated to be 1.0, 0.8, 0.1, 0.9, and 0.5, respectively.

## DISCUSSION

**Substrate Specificity of TrpH.** TrpH efficiently catalyzes the hydrolysis of 3',5'-bis-phosphonucleotides as well as the successive hydrolysis of 5'-phosphomononucleotides from the 5'-end of short pieces of RNA and DNA (Scheme 2). The presence of a 2'-hydroxyl group on the oligonucleotide substrates results in a 2–10-fold increase in the catalytic

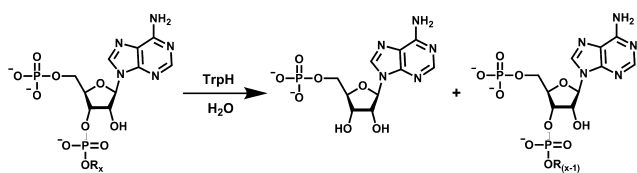


**Figure 3.** Intermediates of hydrolysis of the DNA oligomer p(dAAGCAA) by TrpH analyzed using anion exchange chromatography. (A) UV trace of hydrolysis of 20  $\mu\text{M}$  p(d-AAGCAA) by 200 nM TrpH over time. The final products of hydrolysis, 2'-deoxy-5'-adenosine monophosphate (dAMP), 2'-deoxy-5'-guanosine monophosphate (dGMP), and 2'-deoxy-5'-cytosine monophosphate (dCMP), were verified by running standards separately. Time intervals shown are 0 min (black), 2 min (green), 10 min (purple), 25 min (light green), 40 min (blue), and 85 min (red). (B) Plot of concentration vs time for all the species involved in the hydrolysis of p(d-AAGCAA) by TrpH, shown in panel A. The 5-mer is p(d-AAGCAA). The 4-mer is p(d-GCAA), and the 3-mer is p(d-CAA). The species shown are 6-mer (●), 5-mer (○), 4-mer (▼), 3-mer (△), and 5-nucleoside monophosphates (■).



**Figure 4.** DNA substrates tested as substrates with TrpH.

**Scheme 2**



efficiency of hydrolysis by TrpH compared to that of the corresponding 2'-deoxy substrates. The identity of the purine/pyrimidine base within the 3',5'-bis-phosphonucleotide substrates did not have a significant effect on the kinetic parameters for enzymatic hydrolysis by TrpH. The transient appearance of oligonucleotides of intermediate length formed during the hydrolysis of oligonucleotides (Figure 2) indicates that the enzyme hydrolyzes oligonucleotides bearing a 5'-phosphate in a sequential manner, resulting in the accumulation of 5'-phosphomononucleotide products. The kinetic constants for the hydrolysis of oligonucleotide substrates tend to decrease with the length of the substrates (Table 1). However, the enzyme is capable of hydrolyzing substrates that are at least 14 nucleotides long, as observed from the complete hydrolysis of DNA (DNA substrates 1 and 2) using anion exchange chromatography. The enzyme is capable of hydrolyzing double-stranded oligonucleotides. However, the rate of hydrolysis of double-stranded DNA (3) is approximately 10-fold lower than that of corresponding single-stranded DNA (1). The enzyme is able to hydrolyze an overhang at the 5'-terminus of a double-stranded DNA with a terminal 5'-phosphate (4) at a rate comparable to that of the corresponding single-stranded DNA (2). Double-stranded DNA with a 3'-extension on the reverse strand (5) was hydrolyzed at a rate 2-fold lower than that of 3, and a rate 20-fold lower than that of the single-stranded DNA 1. These results suggest that TrpH is capable of hydrolyzing double-stranded DNA and RNA with frayed ends, but the best substrates identified to date are single-stranded pieces of short RNA oligonucleotides. In summary, the catalytic activity of TrpH can be best described as a 5'-to-3' exonuclease for 5'-phosphorylated oligonucleotides.

**Overview of Known Ribonucleases.** RNA is found primarily as messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). All organisms utilize ribonucleases (RNases) to process RNA during biogenesis and degradation of RNA.<sup>15-17</sup> This processing, in turn, regulates the expression of genetic information in the organism according to changes in environmental conditions. In *E. coli* and other Gram-negative organisms, RNase E, RNase G, and RNase III are the major endonucleases. RNase E cleaves RNA within single-stranded AU-rich regions and shows a preference for RNAs with a monophosphorylated 5'-terminus, and unpaired termini.<sup>18</sup> RNase G is a paralog of RNase E and has a similar substrate specificity. However, RNase G is nonessential and RNase E essential, because *E. coli* cells remain viable when RNase G is knocked out but lose their viability when RNase E is knocked out.<sup>19,20</sup> RNase III, unlike RNase E and RNase G, cleaves RNA within double-stranded regions to yield products that have a characteristic 2 bp overhang at the 3'-end and a monophosphate at the 5'-terminus (similar to substrate 5 shown in Figure 3).<sup>21</sup> The activity of these endonucleases is complemented by the exoribonucleases that degrade the products of the endoribonucleases to the corresponding mononucleotides. Polynucleotide phosphorylase (PNPase), RNase R, RNase II, and oligoribonuclease (Orn) are exonucleases that have been reported to be present in *E. coli*, and all of them possess 3'-5' exonuclease activity. PNPase preferentially degrades RNA with a single-stranded 3'-end by phosphorolysis.<sup>22,23</sup> RNase R and RNase II degrade RNA by hydrolysis, and RNase R is capable of unwinding double-stranded RNA provided the 3'-end is single-stranded for the binding of the enzyme to the RNA.<sup>24,25</sup> The end products of RNA hydrolysis catalyzed by RNase R and RNase II were

found to be 3–6-mers and 1–3-mers, respectively.<sup>25–28</sup> These nanoRNAs are degraded by Orn in *E. coli*, which has been found to be an essential gene.<sup>29</sup> Recently, nanoRNAs have been shown to be involved in priming transcription initiation in bacteria as they can serve as templates for RNA synthesis by DNA-dependent RNA polymerase, which explains the critical role played by Orn in maintaining cell homeostasis.<sup>30</sup> To the best of our knowledge, there have been no reports of a 5′–3′ exonuclease from *E. coli*.

In *Bacillus subtilis* and related Gram-positive organisms, RNase Y and RNase J1 are the major endonucleases.<sup>31</sup> Activity of RNase Y is similar to that of RNase E and is present in organisms in which there is no homologue of RNase E.<sup>32</sup> RNase J was initially identified as an endonuclease<sup>33</sup> but was later shown to possess 5′–3′ exonuclease activity with a strong preference for 5′-monophosphorylated forms of RNA.<sup>34,35</sup> This was the first reported instance of a 5′–3′ exonuclease among bacteria, and the exonuclease activity is similar to that reported here for TrpH. PNPase, RNase R, and RNase PH are other exonucleases that degrade RNA in the 3′–5′ direction.<sup>24,28,36</sup> The degradation of nanoRNA in *B. subtilis* and related organisms is conducted by NrnA/B enzymes, which have been shown to possess nanoRNase activity similar to that of Orn from *E. coli*.<sup>37–39</sup> In bacteria, the enzymes involved in the degradation of RNA form a multimeric complex called an RNA degradosome. These complexes normally contain one or more ribonuclease(s), and an RNA helicase, and have been shown to be important for the efficient degradation of RNA.<sup>40–44</sup>

In eukaryotes, the degradation of RNA is triggered by the removal of the poly(A) tail at the 3′-end by a deadenylase in the deadenylation-dependent pathway or an endonuclease in the deadenylation-independent pathway.<sup>45</sup> Several enzymes have been demonstrated to be involved in the two RNA degradation pathways.<sup>46–48</sup> The exosome, which is a 10–12-subunit protein complex consisting of RNase PH domain enzymes, RNA binding proteins, RNase D-like enzymes, and RNA helicases, is responsible for the 3′–5′ exonucleolytic degradation of RNA.<sup>49</sup> The degradation of RNA in the 5′–3′ direction requires the prior removal of the m7G cap at the 5′-terminus, which exposes the 5′-monophosphorylated terminus to hydrolysis by Xrn1 or Xrn2. Xrn1 is localized in the cytoplasm, while Xrn2 is found in the nucleus. Both enzymes catalyze the exonucleolytic hydrolysis of 5′-monophosphorylated RNA in a processive manner to 5′-nucleoside monophosphates as the final products.<sup>50</sup> In Archaea, RNA is degraded in the 5′–3′ direction by an ortholog of RNase J and in the 3′–5′ direction by the exosome.<sup>17</sup>

**Potential Role of TrpH in *E. coli*.** It is apparent that 5′–3′ exonuclease activity has been found to exist in several phylogenetically distinct organisms. However, this activity has remained elusive in *E. coli* and related organisms. The exploration of the substrate specificity of TrpH has revealed that it is capable of hydrolyzing 5′-monophosphorylated RNA in the 5′–3′ direction in a sequential manner. It is unclear why this activity has not previously been identified in *E. coli*. One possible source of 5′-phosphorylated dinucleotides is the hydrolysis of the bacterial secondary messenger (3′ → 5′)-cyclic-diguanosine monophosphate (c-di-GMP).<sup>51</sup> This cyclic dinucleotide has been shown to be involved in an extensive signal transduction system responsible for regulating biofilm formation, motility, virulence, the cell cycle, differentiation, and other processes.<sup>52</sup> It has been demonstrated that diguanylate cyclases possessing the GGDEF domain are responsible for the

biosynthesis of c-di-GMP from two molecules of guanosine triphosphate (GTP), and enzymes containing the EAL domain catalyze the hydrolysis of c-di-GMP to pGpG. The specific enzyme that catalyzes the hydrolysis of pGpG to 5′-GMP remains unidentified.<sup>52</sup> The substrate specificity of TrpH indicates that it is quite capable of catalyzing the hydrolysis of pGpG to 5′-GMP. TrpH is also capable of hydrolyzing 3′,5′-bis-phosphonucleotides efficiently. However, *E. coli* possesses another enzyme, CysQ, which has been demonstrated to catalyze the hydrolysis of 3′,5′-pAp to 5′-AMP and phosphate during sulfur assimilation.<sup>53,54</sup> Therefore, we deem it unlikely that the hydrolysis of 3′,5′-bis-phosphonucleotides will be the primary physiological function of TrpH.

An examination of the genomic neighborhood of TrpH revealed that one gene, YciO, is strictly conserved immediately downstream of the TrpH gene sequence in the genome of all bacteria that possess a gene copy of TrpH. This gene, with the locus tag b1267 in *E. coli* (gene product YciO), has been annotated as a putative translational factor of unknown function from the SUA5 family. YrdC from *E. coli* belongs to the same family of proteins as YciO and has been found to be involved in the biosynthesis of threonylcarbamoyladenine (t<sup>6</sup>A), a universal modification found at position 37 of tRNAs decoding codons starting with adenosine (ANN).<sup>55</sup> Using complementation studies, it was shown that YciO was not involved in t<sup>6</sup>A biosynthesis, and its molecular function is unknown. Exploring the substrate specificity and physiological role of this gene product (YciO) may help to more completely determine the physiological context of the RNase activity of TrpH within the *E. coli* cell metabolism.

## ■ CONCLUSIONS

We have discovered that the gene product with the locus tag b1266 (TrpH or YciV), which has previously been annotated as a hypothetical protein, is capable of efficiently hydrolyzing single-stranded fragments of RNA that are capped with a 5′-monophosphate in the 5′–3′ direction. This is the first instance of a 5′–3′ exoribonuclease activity reported for an enzyme from *E. coli*. We propose that this RNase be known as RNase AM.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

A list of nonredundant enzyme sequences identified as TrpH orthologs based on a sequence similarity network diagram and the corresponding identifiers and organisms are provided in Table S1. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00192.

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## ABBREVIATIONS

AHS, amidohydrolase superfamily; COG, Cluster of Orthologous Groups; PDB, Protein Data Bank; PHP, polymerase and histidinol phosphatase; ICP-MS, inductively coupled plasma mass spectrometry.

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