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The Gene *ygdP*, Associated with the Invasiveness of *Escherichia coli* K1, Designates a Nudix Hydrolase, Orf176, Active on Adenosine (5′)-Pentaphospho-(5′)-adenosine (Ap_5A)*

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Maurice J. Bessman‡, Joseph D. Walsh, Christopher A. Dunn, Jyothishmathi Swaminathan, John E. Weldon, and Jianying Shen

From the Department of Biology and the McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218

***ygdP*, a gene associated with the invasion of brain microvascular endothelial cells by *Escherichia coli* K1 (Badger, J. L., Wass, C. A., and Kim, K. S. (2000) *Mol. Microbiol.* 36, 174–182), the primary Gram-negative bacterium causing meningitis in newborns, has been cloned and expressed in *E. coli*. The protein, YgdP, was purified to near homogeneity and identified as a member of the Nudix hydrolase subfamily of dinucleoside oligophosphate pyrophosphatases. It catalyzes the hydrolysis of diadenosine *tetra*-, *penta*-, and *hexa*-phosphates with a preference for diadenosine *penta*-phosphate, from which it forms ATP and ADP. The enzyme has a requirement for a divalent metal cation that can be met with Mg^{2+}, Zn^{2+}, or Mn^{2+} and, like most of the Nudix hydrolases, has an alkaline pH optimum between 8.5 and 9. This is the second identification of a gene associated with the invasiveness of a human pathogen as a member of the Nudix hydrolase subfamily of dinucleoside oligophosphate pyrophosphatases, and an examination of homologous proteins in other invasive bacteria suggests that this may be a common feature of cellular invasion.**

Recently, Badger and colleagues (1) identified a gene, *ygdP*, associated with the invasiveness of *Escherichia coli* K1, the primary Gram-negative bacterium causing neonatal meningitis. We are interested in this gene for two reasons. First, it codes for a distinct amino acid signature sequence (Sequence 1) categorizing it as a member of the Nudix hydrolases, a widespread family of enzymes catalyzing the hydrolysis of nucleoside diphosphate derivatives (2).

G*X*$_5$**E***X*$_7$**RE***U**X***EE***X**G***U*** (*U* = Ile, Leu, Val)

SEQUENCE 1

A recent BLAST (3) search has identified more than 600 members of the Nudix hydrolase family in over 200 species ranging from viruses to humans, and we have been systematically cloning, expressing, purifying, and identifying their functions. Furthermore, *ygdP* is of interest because we have previously demonstrated that another gene associated with invasion, *ialA* from *Bartonella bacilliformis*, the only bacterium known to invade human red blood cells, designates a Nudix hydrolase active on Ap_4A[1] (4, 5). In this paper we describe the cloning and expression of *ygdP* and the purification and characterization of the gene product, YgdP (Orf176). It has been identified as a member of the same subfamily of Nudix hydrolases active on Ap_4A, Ap_5A, and Ap_6A. Thus, the ability to invade cells by these two very different human pathogens is associated with Nudix hydrolases specific for members of the diadenosine oligophosphate family, suggesting that this interesting class of compounds is involved in the invasion process.

EXPERIMENTAL PROCEDURES

Materials

Enzymes used in cloning were from Stratagene, Life Technologies Inc., or United States Biochemical Corp. Biochemicals were from Sigma, and oligonucleotide primers were from Integrated DNA Technologies. PET11b and HMS174(DE3) were from Novagen. HiLoad® Superdex® and DEAE-Sepharose were from Amersham Pharmacia Biotech.

Methods

Cloning—Gene *ygdP* (GenBank™ accession number AE000366) was amplified from *E. coli* K12 genomic DNA prepared in this laboratory. Forward primers were constructed to contain an *Nde*I site and reverse primers to contain a *Bam*HI site. After digestion with *Nde*I and *Bam*HI, the gene was inserted into the corresponding sites of pET11b to form pOrf176 under control of a T7 promoter and was engineered into HMS174(DE3) for expression.

Purification of YgdP Protein—Two liters of LB medium containing 100 μg/ml ampicillin were inoculated with 40 ml of an overnight culture of pOrf176:HMS174(DE3), grown to an A_{600} of 0.8, and induced with isopropyl-β-D-thiogalactopyranoside (1 mM final concentration). After 3 h at 37 °C, the cells were harvested by centrifugation, washed, resuspended in 4 volumes of TE buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA) and disrupted in a French press. Cell debris was removed by centrifugation at 5000 × *g*, and the protein concentration was adjusted to 10 mg/ml by the addition of TE buffer. Two liters of cells cultured and treated in this manner yielded ~30 ml of extract (Fraction I).

Fraction I was adjusted to 1 M ammonium sulfate, applied to an anion exchange column (DEAE-Sepharose, 2.5 × 24 cm), and eluted with TE buffer. Active fractions were pooled and concentrated by precipitation in 60% saturated ammonium sulfate, and the precipitate was dissolved in a minimal volume of TE buffer (Fraction II).

Fraction II was applied to a gel filtration column (HiLoad® Superdex®)and eluted with TE buffer containing 200 mM NaCl. Active fractions were combined and concentrated by precipitation in 60% ammonium sulfate, dialyzed against TE buffer containing 100 mM ammonium sulfate, and stored at −80 °C.

Enzyme Assay—This assay measures the conversion of a phospha-

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‡ To whom correspondence should be addressed. Tel.: 410-516-7316; Fax: 410-516-5213; E-mail: zoot@jhu.edu.

[1] The abbreviations used are: Ap_4A, adenosine (5′)-tetraphospho-(5′)-adenosine; Ap_3A, adenosine (5′)-triphospho-(5′)-adenosine; Ap_5A, adenosine (5′)-pentaphospho-(5′)-adenosine; Ap_6A, adenosine (5′)-hexaphospho-(5′)-adenosine.

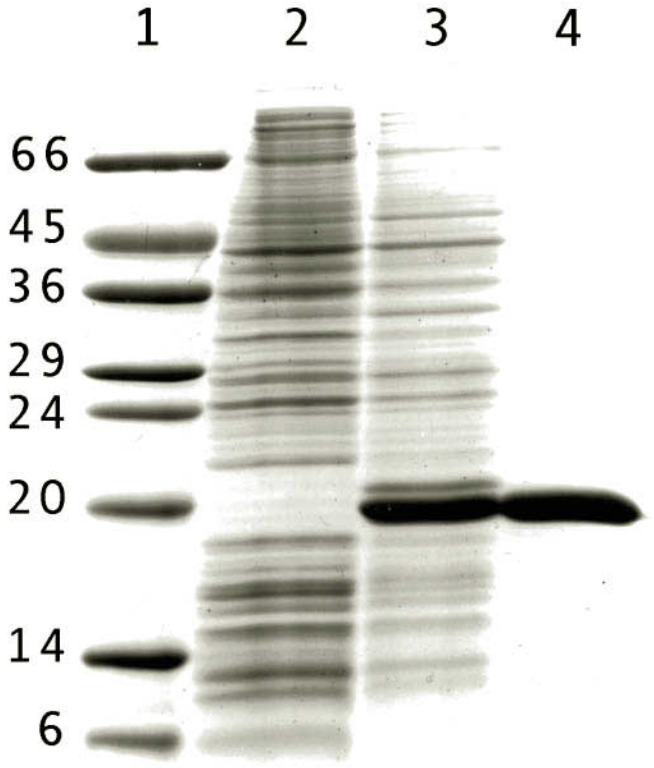

FIG. 1. **Expression and purification of YgdP.** A 15% polyacrylamide gel containing 1% SDS stained with Coomassie Blue included the following: *lane 1*, reference proteins of bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa) trypsinogen (24 kDa), trypsin inhibitor (20 kDa), α-lactalbumin (14.2 kDa), and aprotinin (6.5 kDa); *lanes 2* and *3,* 10 μg of a crude extract of cells containing pET11b (without insert) or pOrf176, respectively; *lane 4*, 1 μg of Fraction III containing approximately the same number of enzyme units as *lane 3*.

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YgdP    SWQFPQGGINPGESAEQAMYRELFEEVGL
IalA    LWQFPQGGIDEGEEPLDAARRELYEETGM
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FIG. 2. **A comparison of the amino acid sequences of YgdP and IalA.** The *bolded* amino acids represent the Nudix signature sequence (2), and the *boxed* amino acids show the amino acid identities in this region.

TABLE I
Substrate specificities of YgdP and IalA

All substrates were present at a concentration of 1 mM in the standard assay. The favored substrate for each enzyme under these conditions was set at 100. The actual value for YgdP was 3.3 nmol of Ap_5A hydrolyzed and for IalA, 12.3 nmol of Ap_4A hydrolyzed.

Substrate	Relative activity	
	YgdP	IalA[a]
Ap_3A	<1	<1
Ap_4A	14	100
Ap_5A	100	77
Ap_6A	92	67

[a] These data are taken from Ref. 4.

tase-insensitive substrate to a phosphatase-sensitive product.

The standard incubation mixture contained in 50 μl: 2 mM substrate, 50 mM Tris-HCl, pH 9.0, 5 mM $MgCl_2$, 4 units of calf intestinal alkaline phosphatase, and 0.1–1 milliunit of enzyme. After 15 min at 37 °C, the reaction was terminated by the addition of 250 μl of 4 mM EDTA and assayed for P_i by the procedure of Ames and Dubin (6). A unit of enzyme hydrolyzes 1 μmol of substrate/min under these conditions. Note that for every μmol of Ap_5A hydrolyzed, 5 μmol of P_i is produced.

RESULTS

Expression and Purification of the YGDP Protein—Cloning of *ygdP* and expression of YgdP protein in host HMS174(DE3)

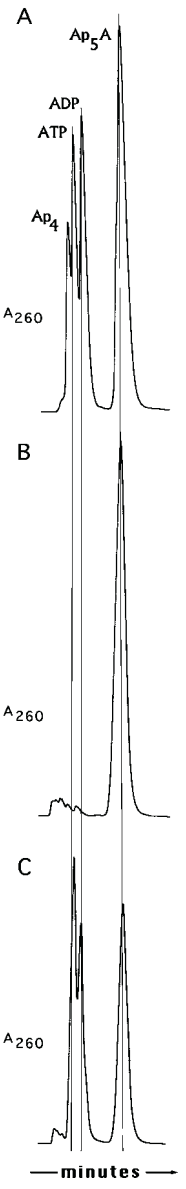

FIG. 3. **Products of the Ap_5A pyrophosphatase reaction.** A scaled-up standard reaction mixture (omitting alkaline phosphatase) was incubated at 37 °C. Aliquots were removed at 0, 5, 10, 15, and 20 min, applied to a calibrated reverse phase C_{18} column, and analyzed by high performance liquid chromatography. The 20-min tracing is shown (*panel C*) along with the zero time profile (*panel B*) and the standards (*panel A*). The earlier time points showed a similar pattern, and Ap_4A was not detected throughout the course of the reaction.

produced a strongly visible band on an SDS-polyacrylamide gel that was not found with the parent vector. Treatment of the cell extract as outlined under "Methods" yielded ~12 mg of highly purified protein as seen in Fig. 1. Usually, our first step in a purification scheme is the removal of nucleic acids with streptomycin. However, in this instance, YgdP precipitated with the nucleic acids fraction and then resisted all efforts at further purification. This was most likely a result of the high concentration of basic amino acids in the protein (net charge, 12; pI, 11.1) interacting with the polyanionic nucleic acids. An alternate procedure involving the adsorption of the nucleic acids on a polycationic resin (DEAE-Sepharose) was successful in separating the protein for further purification by gel filtration.

Identification of Enzymatic Activity—The presence in YgdP of the signature sequence characteristic of the Nudix hydrolases strongly implicated a nucleoside diphosphate derivative

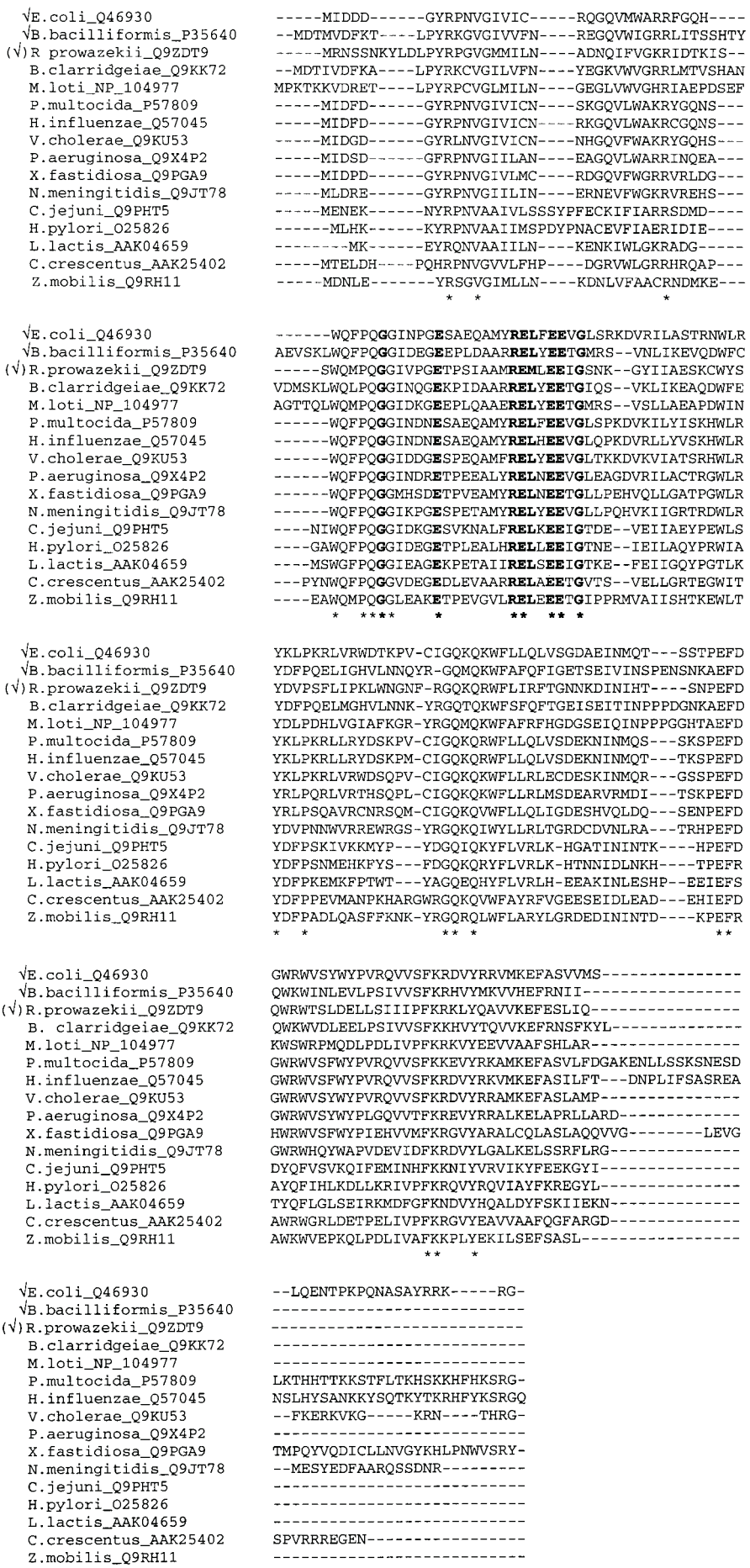

FIG. 4. **Open reading frames homologous to YgdP.** Open reading frames (with GenBank™ accession numbers) uncovered in a recent BLAST (3) search using the amino acid sequence of YgdP as the query are aligned with the CLUSTAL (24) algorithm. The Nudix signature sequence is shown in *bold type*, and *asterisks* designate only those amino acids identical for all 16 proteins, although considerable homologies and similarities are evident throughout the sequences. The *check marks* denote identified diadenosine oligophosphate pyrophosphatases. The identification of the *R. prowazekii* orthologue is from an unpublished observation (see "Discussion" and Footnote 3).

as a likely substrate of the enzyme. Furthermore, the association of *ygdP* with invasion and the strong homology of the protein with IalA, the invasion protein of *B. bacilliformis* (see Fig. 2), suggested that Ap_4A, the substrate of the *Bartonella* enzyme, was also likely to be a substrate for YgdP. Accordingly, several members of the diadenosine oligophosphate family were tested as substrates. As expected, the enzyme hydrolyzed Ap_4A, but Ap_5A was hydrolyzed at a higher rate. This is shown in Table I along with data from the *Bartonella* enzyme IalA for comparison. As with IalA, YgdP had little or no activity toward the lower homologue of the diadenosine oligophosphate family, Ap_3A, and with the higher homologue, Ap_6A, the rate was approximately 70% of the favored substrate. YgdP did not act on other nucleoside diphosphate derivatives such as ADP-ribose, NADH, UDP-glucose, etc., typical substrates of various members of the Nudix hydrolase family (data not shown)

Products of the Reaction—The standard assay was scaled up and modified to omit alkaline phosphatase. After 0, 5, 10, 15, and 20 min of incubation, aliquots were applied to a calibrated reverse phase column and subjected to high performance liquid chromatography. Fig. 3 is a tracing of the zero time and 20 min values. Intermediate time points (not shown) had similar profiles allowing for their relative incubation times. A visual inspection of the chromatogram shows the formation of ATP and ADP at the expense of Ap_5A. Quantification of the nucleotide species by integrating the areas under the respective curves indicated that at 20 min, 24 nmol of Ap_4A were hydrolyzed with the concomitant formation of ~26 nmol of ATP and 27 nmol of ADP. No Ap_4A was detectable throughout the course of the incubation. The stoichiometry of the hydrolytic reaction is described by the following equation: $Ap_5A + H_2O \rightarrow ATP + ADP$. In corresponding experiments, Ap_4A led to the formation of ATP and AMP, and Ap_6A yielded 2 mol of ATP (data not shown). Thus with all three substrates ATP was produced, suggesting that the nucleophilic attack was on the gamma or delta phosphorus.

Other Properties of the Enzyme—YgdP behaves like a monomer in solution, eluting from a gel filtration column at a position characteristic of a 20-kDa protein. It has an absolute requirement for a divalent cation and is maximally active at 10 mM Mg^{2+} and to a lesser extent with Zn^{2+} and Mn^{2+}. Like most of the other Nudix hydrolases YgdP has an alkaline pH optimum between 8.5 and 9.0, and under optimal conditions it has a specific activity of ~3.3 units mg^{-1} protein, equivalent to a k_{cat} of 1 s^{-1} and K_m of 0.36 for Ap_5A and a k_{cat} of 0.1 and K_m of 0.07 for Ap_4A.

DISCUSSION

Here we present a second example in which a gene associated with the invasiveness of a human pathogen has been shown to be a member of the Nudix hydrolase subfamily of dinucleoside oligophosphate pyrophosphatases. In *B. bacilliformis*, the invasion-associated gene *ialA* codes for an Ap_4A pyrophosphatase (4, 5). In this paper it has been demonstrated that *ygdP*, associated with the invasiveness of *E. coli* K1, designates an enzyme preferring diadenosine pentaphosphate. Actually, both enzymes are active on Ap_4A, Ap_5A, and Ap_6A, as shown in Table I, and so the differences in their relative specificities may only reflect small changes in the architecture of the active site brought about by random amino acid substitutions in this region. The area containing the Nudix signature sequence (see Fig. 2) encompasses the catalytic site of members of the Nudix hydrolase family (7, 8), and the enzymatic activity is particularly sensitive to amino acid substitutions (7, 9, 10). Recently, the three-dimensional structure of the Ap_4A Nudix hydrolase from *Lupinus angustifolius* has been solved (11), and the crystal structure of YgdP is currently under investigation.[2] A comparison of these two structures may provide insight into the physical basis for the small differences in specificity.

The dinucleoside oligophosphates themselves were discovered in 1966 as a byproduct of aminoacyl-tRNA synthetase reactions (12), and since that time there have been numerous reports of their presence intracellularly in many different cell types, as well as extracellularly in chromaffin granules, secretory cells, synaptosomes, and synaptic vesicles (for review see Ref. 13). All of these substrates, including Ap_3A, have been implicated in diverse physiological responses such as inhibition of ATP-sensitive K^+ channels (14), activation of purinoceptors (15), regulation of cell differentiation and apoptosis (16), and involvement in pain transduction (17). They have thus been categorized as a novel class of signaling molecules (18). Although there have been a plethora of reports on the distribution and putative function of this family of molecules, there is little hard evidence on their mechanism of action. One attractive hypothesis concerning the role of YgdP and IalA in cellular invasion relates to the observation that the diadenosine oligophosphates are involved in cellular stress responses. As a result of heat shock or oxidative stress, the concentrations of these compounds, termed "alarmones," can increase more than 100-fold over their endogenous level (19, 20). YgdP and IalA might function by reducing the concentrations of these signaling molecules during the invasion process. If this were the case, the location of these enzymes in the cell would be an important determinant of their efficacy. In this regard, it has been shown that members of the diadenosine oligophosphate hydrolases from a variety of animal tissues are *ecto*-enzymes located on the cell surface (21–23). We are presently raising antibodies against pure YgdP in order to address this question.

Although at present YgdP and IalA are the only two members of the diadenosine oligophosphate pyrophosphatases that have been characterized as invasion associated enzymes, it seems more than coincidental that this subfamily of the Nudix hydrolases is somehow involved in the invasion process. Fig. 4 depicts a recent BLAST (3) search against YgdP and an alignment using CLUSTAL (24). Thirteen of the 16 organisms listed are well known invasive animal or plant pathogens, and a 14th, *Mesorhizobium loti*, is a root nodulating symbiont involved in nitrogen fixation. Preliminary characterization of the *Rickettsia prowazekii* homologous protein (third entry in Fig. 4) indicates that it is an Ap_5A pyrophosphatase,[3] and it is likely that many if not all of the other proteins listed in Fig. 4 are dinucleoside oligophosphate pyrophosphatases.

In addition to *ialA* and *ygdP*, other genes of unknown function are associated with the invasion process in *B. bacilliformis* (25, 26) and *E. coli* K1 (1). The identity of *ialB*, a gene supplementary to *ialA* and that is required for the invasion of human red blood cells by *B. bacilliformis*, has remained a mystery, because searches did not reveal significant similarity to other proteins in the data banks. We have recently uncovered an open reading frame (GenBank™ accession number BAB50118) in the newly sequenced *M. loti* genome (27) (Fig. 4, *line 5*) that is 36% identical and 54% similar to IalB. It would be of interest to see whether the two unidentified genes from these widely different species complement each other and perhaps provide some clue as to the role played in the invasion process by this interesting family of dinucleoside oligophosphates and their cognate pyrophosphatases.

[2] S. B. Gabelli, M. J. Bessman, and L. M. Amzell, unpublished observations.

[3] Gaywee, J., Radulovic, S., Bessman, M. J., Kim, K. S., and Azad, A. F. (2001) Poster B-262, presented at the 101st General Meeting, American Society of Microbiology, Orlando, FL (May 20–24, 2001).

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