A New Subfamily of the Nudix Hydrolase Superfamily Active on 5-methyl-UTP (riboTTP) and UTP

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Running title: The riboTTPase Subfamily of the Nudix Hydrolases
Summary

A new subfamily of the Nudix hydrolases, identified by conserved amino acids upstream and downstream of the Nudix box, has been characterized. The cloned, expressed, and purified orthologous enzymes have major activities on the non-canonical nucleoside triphosphate 5-methyl-UTP (riboTTP) and the canonical nucleotide, UTP. In addition to their homologous signature sequences and their similar substrate specificities, the members of the subfamily are inhabitants of, or related to the bacterial rhizosphere. We propose the acronym and mnemonic, utp, for the gene designating this unique UTPase.
The Nudix hydrolases, so named because they catalyze the hydrolysis of nucleoside diphosphates linked to some other moiety \( x \) (1), constitute a superfamily of enzymes with representatives in all three kingdoms. The members of this superfamily can be identified by a highly conserved amino acid signature sequence called the Nudix box viz:

\[
G\ldots E\ldots \ldots \ldots U\ldots \ldots \ldots E\ldots \ldots \ldots G
\]

Sequence 1

in which "\( x \)" represents any amino acid, and \( U \) is a bulky hydrophobic amino acid, usually Ile, Leu, or Val. A current BLAST (2) search of the data banks for polypeptides and expressed sequence tags containing Sequence 1, reveals over 1100 open reading frames from more than 250 species ranging from viruses to humans. We have been systematically cloning, expressing, and characterizing members of the superfamily, and without exception, all of the enzymes identified so far hydrolyze nucleoside diphosphate derivatives including nucleotide sugars, dinucleoside polyphosphates, coenzymes, (deoxy)nucleoside triphosphates and ADP-ribose. Since the same amino acid signature sequence, the Nudix box, represents the nucleotide binding and catalytic site for all these proteins (3-5), the different specificities toward the respective substrates must lie in a region or in regions peripheral to this area. By aligning amino acid sequences of those enzymes hydrolyzing a similar spectrum of substrates, we have identified certain landmark amino acids outside of the Nudix box, enabling us to classify some of the members of the superfamily into distinct subfamilies (6). This has been of singular value, because it has allowed us to predict the enzymatic activity of unidentified open reading frames involved in physiological processes, merely by observing specific telltale amino acid patterns peculiar to each subfamily.
In this paper, we describe the cloning, expression, purification, and identification of members of a new subfamily of the Nudix hydrolases, highly active on 5-methyl-UTP (riboTTP) and UTP, and recognizable by characteristic amino acid sequences outside of the Nudix box.
EXPERIMENTAL PROCEDURES

Materials

The expression plasmid pET-24a(+) (Km\(^r\)) and *E. coli* HMS174 (DE3) were from Novagen (Madison WI), *Agrobacterium tumefaciens* strain GV3101 was a gift from Judith Bender, Johns Hopkins School of Public Health, *Pseudomonas aeruginosa*, strain BB3/216/ATCC27853, was a gift from Thomas Cebula of the Food and Drug Administration, and *Caulobacter crescentus* strain CB15 was a gift of Lucy Shapiro, Stanford University and Bert Ely, the University of South Carolina. The plasmid, pTrc99A (Amp\(^r\)), and Sephadex G-100 were from Amersham Pharmacia Biotech (Piscataway, NJ), and the restriction enzymes, PCR kits, calf intestinal alkaline phosphatase and yeast inorganic pyrophosphatase were from Stratagene (La Jolla, CA). The canonical nucleotide substrates and common chemicals were from Sigma-Aldrich Chemical Co. (St. Louis, MO) or Life Technologies Inc. (Rockville, MD), and 5-methyl-UTP was from TriLink Biotechnologies, (San Diego, CA). The PCR primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Protein assay reagent was from Bio-Rad Laboratories (Hercules, CA).

Methods

Cloning of *A. tumefaciens* orf147 and its orthologs

Standard cloning technology was used. Briefly, orf147 was amplified directly from one colony of *A. tumefaciens* strain GV3101 using the DNA polymerase chain reaction. Nde1 and BamH1 restriction sites were incorporated at the start and end of the gene, respectively, and the amplified DNA was purified, digested with Nde1 and BamH1, and ligated into pET24a(+) to place the gene under transcriptional control of the T7 lac promoter. The resultant plasmid, pETORF147 was transformed into *E. coli* DH5\(a\) for storage and into HMS174 (DE3) for protein expression. The orthologous genes from *P. aeruginosa* and *C. crescentus* were
cloned using similar procedures. For complementation studies, the gene was cut out of pETORF147 with Xba1 and HindIII and ligated into the corresponding sites of pTrc99A putting orf147 under control of the trc promoter. The resulting plasmid, pTrcORF147 was transformed into DH5α.

**Growth and Expression of HMS174:pETorf147, ORF147 and its orthologs**  
One colony of the expression strain was inoculated into 40ml of LB medium containing 30 μg/ml of kanamycin, incubated at 37 °C on a rotary shaker over night, transferred to 2 l of the same medium and grown to an A600 of 0.8. The culture was derepressed by the addition of isopropyl-β-D-thio-galactopyranoside to a concentration of 0.5 mM, and grown for an additional 3 hours, after which the cells were harvested by centrifugation and washed in buffered isotonic saline solution.

**Purification of the Enzymes**  
The washed cells were frozen at -80 °C over night and re-suspended in 2.5 volumes of buffer A (50 mM Tris-Cl, pH7.5, 1 mM EDTA, 0.1 mM DTT). The supernatant (Fraction I) containing the expressed protein was collected by centrifugation, and 10 % streptomycin sulfate in buffer A was added to a final concentration of 1.5 % to precipitate nucleic acids. These were removed by centrifugation, and the supernatant, Fraction II, was brought to 60% saturation with ammonium sulfate. The precipitate was collected and dissolved in a minimal volume of buffer A (Fraction III), and chromatographed on a Sephadex G-100 gel-filtration column equilibrated and eluted with buffer A plus 200 mM sodium chloride. The fractions were located by A280 absorbance and identified by their migration on an SDS-PAGE gel. Those containing the purified protein were pooled, assayed, and stored at –80 °C (Fraction IV). All of the orthologs of ORF147, including those from *A. tumefaciens*, *P. aeruginosa*, and *C. crescentus* were purified by similar procedures.
**Enzyme Assays** — The standard reaction contained in 50 μl: 50 mM Tris-Cl pH 9.0, 5 mM Mg$^{2+}$, 2 mM substrate, 0.5 unit of yeast inorganic pyrophosphatase for substrates such as (deoxy)nucleoside triphosphates and their derivatives, or 4 units of alkaline phosphatase for all other substrates, and 0.1-2 milliunits of purified enzyme. The solution was incubated at 37 °C for 15 min, stopped by the addition of 250 μl of 4 mM EDTA, and the liberated inorganic orthophosphate was assayed by the colorimetric procedure of Fiske and SubbaRow (7) as modified by Ames and Dubin (8). A unit of enzyme hydrolyzes one μmol of substrate per min.

**Complementation Test for the mutT Mutator Phenotype** — gene orf147 was cloned into pTrc99A and transferred into *E. coli* strain SB3 lacking a functional *mutT* gene. The mutation frequencies were calculated from the number of colonies resistant to nalidixic acid or streptomycin as described in O’handley *et al.*(9).
RESULTS AND DISCUSSION

Recognition of the Putative Subfamily

Our customary procedure for identifying the activity of a new open reading frame containing the Nudix box involves BLAST searches (10) and CLUSTAL alignments (11) of the amino acid sequence of the unknown polypeptide. When this was done for an open reading frame from *A. tumefaciens*, we identified a group of polypeptides with conserved sequences upstream and downstream of the Nudix box (Fig. 1). Since these regions were different from any previously described landmark amino acids (6,12), we hypothesized that the associated polypeptides represented a new subfamily of Nudix hydrolases. Accordingly, we investigated this possibility by isolating the expressed proteins and identifying the enzymatic activities of some of the members of the group.

Gene Cloning, Expression and Protein Purification

Cloning of the genes from single colonies of the individual organisms proceeded *pro forma*; the inserts were sequenced and were as described in the data banks. All three of the orthologous genes from *A. tumefaciens*, *P. aeruginosa*, and *C. crescentus* expressed proteins in soluble form when introduced into *E. coli*, but with different levels of expression. As with some of the other Nudix hydrolases (6,9,13-15), the expressed proteins were extractable into buffer merely by freezing and thawing the cells. This left the bulk of the cellular proteins behind, thereby considerably simplifying the purification. The procedure described in Methods led to essentially homogeneous proteins (Fig.2).
A unifying property of the Nudix hydrolases is their activity on nucleoside diphosphate derivatives, although these substrates may vary widely for different members of the superfamily. Starting with the purified enzyme expressed from the *A. tumefaciens* gene, we examined a large number of nucleoside diphosphate derivatives as potential substrates and found that UTP was hydrolyzed at the highest rate of all those naturally occurring metabolites tested (Table I). However, we noted an apparent inconsistency in our substrate survey when we observed that dTTP was hydrolyzed at a measurable rate, approximately 18 per cent of UTP, whereas dUTP was not a significant substrate. This seemed surprising since it appeared to us that UTP resembled dUTP more closely than it did dTTP and so it called our attention to the methyl group as a potentially important feature involved in substrate recognition. Accordingly we tested 5-methyl-UTP and found it is hydrolyzed at three times the rate of UTP. This is shown in Table I along with a comparison to the purified orthologous proteins from *P. aeruginosa* and *C. crescentus*. The enzyme from *P. aeruginosa* and *A. tumefaciens* have similar specificities, but the active site of the *C. crescentus* enzyme is more accommodating and has approximately equal activities on UTP and the other pyrimidine ribonucleoside triphosphate, CTP.

A comparison of some of the kinetic properties of the three enzymes is shown in Table II. The combined higher $K_{cat}$ and lower $K_M$ for 5-methyl UTP leads to a 10-fold higher catalytic constant, $K_{cat}/K_M$ over that for UTP for the *A. tumefaciens* and *P. aeruginosa* enzymes. These inequalities are not nearly as
marked for *C. crescentus* resulting in less than a 2-fold difference in the values of the catalytic constants for the two substrates, supporting the notion that its catalytic site is more accommodating than those of the other two enzymes. In this respect, it is interesting to note that all known eukaryotic UMP kinases, enzymes involved in the phosphorylation of UMP to UDP, are equally active on UMP and CMP (16), suggesting that there may be similarities in the active sites of these kinases and the *C. crescentus* enzyme. Work in progress on the X-ray crystal structure of members of the family should be informative in ascertaining the basis of these observations.

*Products of the Reaction*— With one possible exception (17), virtually all of the Nudix hydrolases studied so far catalyze a nucleophilic attack by water on a pyrophosphate linkage. An analysis of the products of UTP hydrolysis with purified *A. tumefaciens* UTPase indicates that this enzyme also catalyzes an attack on the pyrophosphate linkage. Fig. 3A shows that after a 30 min incubation, the UTP peak decreased, a UMP peak appeared, and there was no apparent formation of UDP. This indicates that there was either a direct conversion of UTP to UMP with the release of inorganic pyrophosphate, or that UTP was hydrolyzed to UDP as the rate limiting step and the UDP was then rapidly converted to UMP. The second pathway is ruled out for 2 reasons. First, as shown in Fig. 3B, no inorganic orthophosphate is formed during the course of the reaction, as would be required for conversion of UTP to UDP, unless inorganic pyrophosphatase is present. Second, UDP itself is not a substrate of
the enzyme as shown in Table I. Therefore the following equation describes the stoichiometry of the reaction:

\[ \text{UTP} + H_2O \rightarrow \text{UMP} + PP_i \]

Five other Nudix hydrolase nucleoside triphosphatases are known with major activities on dGTP (18), dATP (19), dCTP (15), 3’-amino-3’-dATP (20) and dTTP (21), all forming the respective mono-phosphates and inorganic pyrophosphate, classifying them in the category EC 3.6.1.19, with the systematic name nucleoside triphosphate pyrophosphohydrolase. Thus, the UTPase described herein is properly designated UTP pyrophosphohydrolase, and it is the first of these enzymes preferring ribo- to deoxyribo nucleotides.

Since, to our knowledge, this gene and enzyme has not been described before, we propose the acronym and mnemonic, \textit{utp} as its designator.

\textbf{Other properties of the Enzyme}—The \textit{A. tumefaciens} UTPase requires \(Mg^{2+}\) for activity. Unlike many of the other Nudix hydrolases, this requirement cannot be met by \(Mn^{2+}\), \(Zn^{2+}\), or \(Co^{2+}\). In respect to pH, however, the \textit{A. tumefaciens} UTPase, like most of the other members of the superfamily, prefers alkaline conditions and is optimally active at pH 9. The protein elutes from a pre-calibrated gel filtration column as expected for an 18 kDa protein and thus probably exists as a monomer in solution.

\textit{Complementation Studies}—The original Nudix hydrolase, MutT, is also a nucleoside pyrophosphohydrolase, although it has a markedly different specificity from the UTPase described here (1,18,21). Nevertheless, we tested a plasmid containing the UTPase gene for its ability to complement a \textit{mutT} strain of \textit{E. coli}. 

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Whereas the mutT strain gave rise to 892 ± 170 streptomycin resistant colonies per 10⁹ cells, the strain transformed with a mutT+ gene produced only 10 colonies, the same number as a mutT+ strain. However, the mutT strain when transformed with the UTPase gene produced 1059 ± 590 colonies. Similar results were seen when nalidixic acid was substituted for streptomycin as the selective antibiotic (data not shown). These results disqualify the UTPase as an orthologue of MutT.

Two issues regarding this newly discovered subfamily of Nudix hydrolases merit consideration: 1- What is the nexus linking the members of the group, or what is the commonality of the properties they share? 2- What, if any, is the physiologic relevance of their preference for ribo TTP? In regard to the first question, this small group of 12 open reading frames (Fig. 1) was culled from over 1100 putative Nudix hydrolases identified by BLAST (10) searches of the data banks. CLUSTAL (11) alignments revealed the “L(VL)VRK” motif upstream and the “AANE” motif downstream of the Nudix box, and these two amino acid cassettes define the signature sequence of the family and demarcate it as a distinct subfamily of proteins. The conserved “PGGK” tetrad, overlapping the Nudix box, is not diagnostic, since it is seen in many other Nudix hydrolases with dissimilar specificities. A unifying feature of these six diverse bacterial genera is their relationship to plants, either directly as symbionts (R.galegae), growth-promoting rhizobacteria living in the soil or rhizosphere (P. fluorescens, P. aeruginosa, P. putida, C. efficiens), plant pathogens (A. tumefaciens, P. syringae), or indirectly as indicated by comparative genomics. For example,
virulence factors in the animal pathogens *B. melitens* and *B. suis*, are related to factors involved in endosymbiosis or pathogenesis in plants, and the erythromycin resistant plasmid containing the Nudix gene in the human pathogen *C. diphtheriae*, is related to the chloramphenicol resistance plasmid in the soil bacterium, *C. glutamicum*. The free-swimming aquatic bacterium, *C. crescentus*, although not found in the same ecosystem as the other members of the subfamily, derives much of its nutrients from plant polysaccharides including cellulose, lignins, xylan, glucan and pectin, and has gene clusters for their catabolism. Finally, *C. smegmatis*, usually associated with humans, was originally isolated from soil.

An exegesis of the enzymes’ preference for riboTTP is more elusive. We are not aware of any reports of riboTTP in the nucleotide pool of any organism, to say nothing of the organisms comprising the subfamily, although we are also not aware of anyone looking for this nucleoside triphosphate. On the other hand, 5-methyl-Uracil is the most common methylated base in t-RNA, and therefore riboTMP could arise from tRNA turnover. Furthermore, it has been reported that thymidylate synthetase, which catalyzes the methylation of dUMP to TMP, can also use UMP as the acceptor with the formation of riboTMP (22). The formation of the corresponding triphosphate would require sequential activities of a nucleotide kinase and nucleoside diphosphokinase. Although not tested directly, studies on the specificity of purified UMP kinase (23) suggest that riboTMP could be phosphorylated to riboTDP, and the next step, the phosphorylation to riboTTP, would almost certainly proceed smoothly because
of the exceedingly broad specificity of nucleoside diphosphokinase. Once formed, riboTTP could be readily incorporated into RNA since it has been demonstrated that both prokaryotic and eukaryotic RNA polymerases can substitute riboTTP for UTP during RNA synthesis (24,25). What effect methyl uracils would have on the physiological functions of messenger and/or ribosomal RNA is conjectural. It has been shown that E.coli mutants completely lacking 5-methyl-Uracil in their t-RNA show no differences in growth rate, codon recognition, protein synthesis, and macromolecular composition. However, there is a distinct advantage of wild-type cells over those lacking 5-methyl-Uracil in mixed population studies (26).

An interesting parallel exists between the riboTTPase described here and Orf135, another Nudix hydrolase from E.coli (15). The latter enzyme also prefers the non-canonical methylated nucleotide, 5-methyl-dCTP instead of the natural substrate, dCTP. As pointed out earlier (1), the Nudix hydrolases may be thought of as surveillance enzymes, hydrolyzing potentially toxic or deleterious compounds arising during metabolism, or preventing the accumulation of anabolic intermediates during the vicissitudes of the cell cycle. The enzymes of this newly discovered subfamily conform to this pattern. If riboTTP is an undesirable metabolite indigenous to these related organisms, then they have a means to deal with it. On the other hand, the role of the enzymes may be to monitor the excessive accumulation of the natural nucleoside triphosphate, UTP, and thus could be related functionally to the dGTPase (21), dATPase (19), dCTPase (15),
and dTTPase$^2$ Nudix hydrolases regulating the composition of the nucleotide pool.
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Footnotes

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1 Work in progress, S. B. Gabelli

2 Unpublished results, C. A. Dunn, D. Smith, and M. J. Bessman
Fig. 1. **The UTPase subfamily of the Nudix hydrolases.** Open reading frames identified in a BLAST (2) search using the *A. tumefaciens* sequence as the query. The checks ( ) indicate UTP pyrophosphohydrolases positively identified and partially characterized in this study. The asterisk (∗) demarcates amino acid identity. The accession numbers identify amino acid sequences deposited in the protein data base of NCBI (National Center for Biotechnology Information) except for the following: a*R. galegae*, a translation of the reverse complement of nucleotides 4256-4651 from accession number AF109172, NCBI; b*M. smegmatis*, a fragment of the unfinished genomic sequence designated gnITIGR_1772lmsmeg_3267 from TIGR (The Institute for Genomic Research).

Fig. 2 **Expression and purification of the proteins from A. tumefaciens, P. aeruginosa, and C. crescentus.** Shown is a gradient polyacrylamide gel (4-20%) containing 1% sodium dodecyl sulfate. Lanes 1 and 9 are protein standards with the indicated molecular masses. Lane 2, crude extract of induced cells containing pET24a(+) with no insert. Lanes 3, 5, 7, induced cells containing pET 24a(+) and the respective inserts for the riboTTPase genes from A. tumefaciens, *A. aeruginosa*, and *C. crescentus*. Lanes 4, 6, and 8; purified enzymes corresponding to lanes 3, 5, and 7. Lanes 1-6 and 7-9 were from two separate gels photographed side by side.

Fig. 3. **Product formation by the A. tumefaciens UTPase.** Panel A. Ion pair HPLC chromatography of aliquots taken at zero time and after a 30 minute incubation of a standard reaction mixture minus inorganic pyrophosphatase. Panel B. The standard assay mixture minus pyrophosphatase was scaled up and incubated at 37 °C. At the indicated times, two aliquots were removed. One (solid circles) was used for the determination of inorganic orthophosphate by the procedure of Fiske and Subbarow (7). The second aliquot was treated with Norit to remove nucleotide derivatives, and the supernatant was incubated with yeast inorganic pyrophosphatase for an additional 15 min after which inorganic orthophosphate was measured as above.
Table I
Assays were done under standard conditions containing 2mM of the indicated substrate.

| Substrate                      | A. tumefaciens | P. aeruginosa | C. crescentus |
|--------------------------------|----------------|---------------|---------------|
|                                | Specific activity | Relative activity | Specific activity | Relative activity | Specific activity | Relative activity |
| 5-methyl UTP                   | 261 (100)       | 227 (100)     | 82 (100)      |
| UTP                            | 90 34           | 83 37         | 61 73         |
| dTTP                           | 16 6            | 11 5          | 6 8           |
| CTP                            | 1 <0.5          | 1 <0.5        | 59 69         |
| ATP,dATP,GTP,dGTP,dCTP,dUTP,UDP,dTDP,FAD,NADH,Ap₅A,UDP-glucose,ADP-ribose | <0.5 <0.2 | <0.5 <0.3 | <0.5 <0.7 |

Ap₅A is adenosine(5’)-pentaphospho-(5’)-adenosine
Table II
Kinetic analysis of the enzymes

Standard assays were as described under “Methods” and $K_m$ and $V_{max}$ were determined from a non-linear regression analysis. $K_{cat}$ was calculated from $V_{max}$, and a unit of activity represents the hydrolysis of 1 µmol of substrate/min.

| Organism     | Substrate | $V_{max}$ (units/mg) | $K_M$ (mM) | $K_{cat}$ (s⁻¹) | $K_{cat}/K_M$ (M⁻¹s⁻¹) |
|--------------|-----------|----------------------|------------|-----------------|-------------------------|
| A. tumefaciens | 5-CH₃UTP  | 261                  | 0.12 ± 0.04| 72              | 6.0 X 10⁵             |
|              | UTP       | 90                   | 0.48 ± 0.08| 26              | 5.1 X 10⁴             |
| P. aeruginosa | 5-CH₃UTP  | 228                  | 0.18 ± 0.05| 63              | 3.5 X 10⁵             |
|              | UTP       | 53                   | 0.49 ± 0.08| 14              | 3.0 X 10⁴             |
| C. crescentus | 5-CH₃UTP  | 82                   | 0.21 ± 0.05| 19              | 9.1 X 10⁴             |
|              | UTP       | 62                   | 0.11 ± 0.04| 14              | 1.3 X 10⁵             |
| Organism                        | Accession No.       | LVVRKRTQFMOPGGK | IDPGETPEQALH | RELAEELGLTLPKNAVRYEGIFREEAANE | PGADV |
|--------------------------------|---------------------|-----------------|--------------|-----------------------------|-------|
| Agrobacterium tumefaciens      | NP_355339           | LLVRKRGTAIFMKPGGKRDAGEDDLTTLA | RELREELGCDL--VSAELLGHFSARA | AANEAGFTV |
| Caulobacter crescentus         | NP_420159           | LLVRKRGTAIFMLPGGKREPGTPTAAQRLREELRLPMGASTFEHLGS | QAPAANE | ANTRV |
| Pseudomonas aeruginosa         | NP_252160           | LLVRKRNTTAFMQPGGKIEAEHELPVHALARELEELGLVIDPVQASFLGCS | APAANE | PGFV |
| Pseudomonas fluorescens        | ZP_00087811         | LLVRKRNTTAFMQPGGKIEAEHELPVHALARELEELGLVIDPVQASFLGCS | APAANE | PGFV |
| Pseudomonas putida             | NP_743223           | LLVRKRNTTAFMQPGGKIDAGETPQVQALVRELQELGLRIDPAAQAMHLGCS | APAANE | PGFV |
| Pseudomonas syringae           | ZP_00126564         | LLVRKRNTTAFMQPGGKIEAEHELPVHALARELEELGLVIDPVQASFLGCS | APAANE | PGFV |
| Brucella melitens              | NP_540557           | LLVRKRNTTAFMQPGGKIEAEHELPVHALARELEELGLVIDPVQASFLGCS | APAANE | PGFV |
| Brucella suis                  | NP_697315           | LLVRKRNTTAFMQPGGKIEAEHELPVHALARELEELGLVIDPVQASFLGCS | APAANE | PGFV |
| Corynebacter efficiens         | NP_737757           | LGVRKANTTAFMQPGGKIEAEHELVIDPQVQALVRELQELGLRIDPAQAMHLGCS | APAANE | PGFV |
| Corynebacter diphtheriae       | AF492560_9          | LGVRKANTTAFMQPGGKIEAEHELVIDPQVQALVRELQELGLRIDPAQAMHLGCS | APAANE | PGFV |
| *Rhizobium galegae             |                     | LVVRKRTQFMOPGGK | IDPGETPEQALH | RELAEELGLTLPKNAVRYEGIFREEA | AANE |
| bMycobacterium smegmatis       |                     | LLVRKRGTAIFMLPGGKREPGTPTAAQRLREELRLPMGASTFEHLGS | QAPAANE | ANTRV |

**Fig.1**

Nudix Box
Fig. 3
A new subfamily of the nudix hydrolase superfamily active on 5-methyl-UTP (riboTTP) and UTP

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