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A cold-active heat-labile t-RNA modification GTPase from a psychrophilic bacterium *Pseudomonas syringae* (Lz4W)

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Abstract

A cold-active heat-labile t-RNA modification GTPase (TrmE) from psychrophilic bacterium *Pseudomonas syringae* (Lz4W) has been purified and characterized. The purified TrmE is a 53 kDa protein, has GTPase activity and hydrolyses only the oxy and deoxy forms of GTP but not the other nucleotide triphosphates. The enzyme exhibits optimal activity at 12–18 °C and retains 65% of its optimal activity at 4 °C, indicating that it is a cold-active enzyme. The enzyme is also heat-labile and loses 60% of its activity at 30 °C. This is the first report on the purification and characterization of a TrmE from a psychrophilic bacterium.

Keywords: *Pseudomonas syringae*; Psychrophilic bacterium; t-RNA modification GTPase; TrmE; Cold-active enzyme; Heat-labile enzyme

1. Introduction

TrmE (tRNA modification GTPase) plays an essential role in cell proliferation, signal transduction, protein synthesis and protein targeting [2,9]. The enzyme is unique in that it has a dual function. One of its functions is related to t-RNA modification [12,15,23] as a consequence of which it modifies a specific uridine residue, namely U34 to 5-methyl-aminomethyl-uridine in bacteria, 5-carboxymethyl- amino-methyl-uridine in yeast and 5-taurinomethyl- uridine in human t-RNA. Such modifications may be very crucial, since the specific uridine residue that is modified is in the wobble position and is required for codon-anticodon interaction. In fact, a *trmE* mutant of *Escherichia coli* was found to be lacking the modified nucleotide (5-methylaminomethyl-2-thi-ouridine) and it exhibited reduced read-through at the UAG codon [5]. As yet, however, direct evidence that TrmE helps in tRNA modification is lacking. In fact the role of TrmE may be to drive the reaction by providing energy through GTP hydrolysis without actually being involved in catalysis. In addition, most of the TrmEs possess a very high intrinsic GTP hydrolysis rate [3]. In a recent paper, we implicated TrmE in cold adaptation in psychrophilic *Pseudomonas syringae* [18]. TrmE has thus far been purified and characterized for GTPase activity only from *E. coli*, a mesophilic bacterium [3] and *Thermotoga maritima*, a thermophilic bacterium [22].

In this study, we have cloned and expressed the *trmE* gene from the psychrophilic bacterium *P. syringae* (LZ4W) [16]. The purified TrmE of *P. syringae* (LZ4W) shows very high intrinsic GTPase activity and the unique features of this enzyme are that it is cold-active and heat-labile.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Psychrophilic *P. syringae* (LZ4W) [16] and *E. coli* BL21 (λDE3) [20] were grown at the required temperature in an incubator shaker with continuous shaking at 250 rpm. *P. syringae* (LZ4W) was grown in Antarctic bacterial medium (ABM) containing peptone (0.5%, w/v) and yeast extract (0.2%, w/v) [16] at 22 °C. *E. coli* BL21 (λDE3) was routinely grown in Luria–Bertani (LB) medium at 37 °C. Ampicillin

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and kanamycin were used at a concentration of 100 μg/ml and 25 μg/ml respectively.

2.2. Cloning of trmE of P. syringae (Lz4W)

To express and purify the native form of *trmE* in its own parental strain (*P. syringae* LZ4W), the complete open reading frame of *trmE* was amplified by the polymerase chain reaction (PCR) using genomic DNA of *P. syringae* (Lz4W) [8] as the template and the intrinsic restriction site containing primers Ash 44 (5′ CCG GAA TTC ATG AGT GTT GCT GCT GAA 3′) and Ash 45 (5′ CCC AAG CTT CTA TTT ACC GAT ACA GAA 3′) designed based on the nucleotide sequence of the *trmE* gene from the same organism (AM944531) [18]. PCR was carried out under the following conditions: initial denaturation at 94 °C for 4 min, denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1.5 min for 35 cycles and final extension at 72 °C for 5 min. The PCR-amplified fragment was cloned into pET28b (Merck KGaA, Darmstadt, Germany) in frame with Histag to generate pET28b::Histag-*trmE* in *E. coli* BL21 (λDE3). However, pET28b acts as a suicidal vector in *P. syringae* (Lz4W). Therefore, Histag-*trmE* from pET28b::Histag-*trmE* construct was amplified using primers Ash49 (GGA TAA CAA TTC CCC TCT AGA AAT) and Ash50 (5' CCC TCT AGA CTA TTT ACC GAT ACA GAA 3') under the following conditions: initial denaturation at 94 °C for 4 min, denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 1.5 min for 35 cycles and the final extension was at 72 °C for 5 min. The resulting PCR product was then cloned in pGL10 [1]. The construct pGL10::Histag-*trmE* was isolated from positive clones and about 4–5 μg of the pure plasmid was transformed into electro-competent cells of psychrophilic *P. syringae* (Lz4W) [14]. The transformants of *P. syringae* (Lz4W) containing the construct pGL10::Histag-*trmE* were selected on ABM plates containing kanamycin (25 μg/ml) at 22 °C [14].

2.3. Purification of TrmE of P. syringae (Lz4W)

One of the transformants of *P. syringae* (Lz4W) containing the construct pGL10::Histag-*trmE* was inoculated into 10 ml of ABM broth overnight and used to inoculate 1000 ml of ABM broth which was cultured at 22 °C in an incubator shaker at 250 rpm. This culture was then used for purification of His-tagged TrmE using a Ni-NTA (nickel-nitrilotriacetic acid) agarose column according to the procedure recommended by Qiagen (Hilden, Germany). Briefly, when the culture reached an OD_{600} ~1.5, it was pelleted, resuspended in 10 ml of buffer A (50 mM NaH_2PO_4 .H_2O, 300 mM NaCl and 10 mM imidazole, pH 8.0), lysed by sonication, cell debris was removed by centrifugation at 14,000 rpm for 20 min and the supernatant was applied to an Ni-nitrilotriacetic acid agarose resin (Qiagen, Hilden, Germany) column equilibrated with buffer A. The column was then washed with buffer B (50 mM NaH_2PO_4 .H_2O, 300 mM NaCl and 20 mM Imidazole, pH 8.0), followed by washing with buffer C (50 mM NaH_2PO_4 .H_2O, 300 mM NaCl and 50 mM Imidazole, pH 8.0) and finally, TrmE was eluted using buffer D (50 mM NaH_2PO_4 .H_2O, 300 mM NaCl and 250 mM Imidazole, pH 8.0). The eluted fractions were dialyzed against 0.1 M Tris (pH 9.0) buffer containing 20% glycerol.

Purity of TrmE was checked by SDS-PAGE on a 10% resolving gel and a 5% stacking gel essentially according to the method of Laemmli [11] using a Hoefer Mighty Small apparatus (San Francisco, CA, USA). Electrophoresis was terminated when the bromophenol blue dye in the samples reached the bottom edge of the resolving gel and was further processed for staining overnight in 0.5% Coomassie brilliant blue solution.

2.4. Western blot analysis

The electrotransfer of proteins from the polyacrylamide gel to the nitrocellulose membrane (Hybond C) was essentially done following the semi-dry method of Towbin [21]. After electrotransfer of proteins, the membrane was washed and probed with the primary antibodies followed by the appropriate secondary antibody. Finally, the blot with alkaline-phosphatase-conjugated secondary antibody was developed using NBT and BCIP as substrates [10].

2.5. Assay of GTPase activity

GTPase activity was assayed at 15 °C for 30 min using 10 μg of the purified protein in a 50 μl reaction mixture of 50 mM Tris–HCl (pH 9.0) containing 200 mM KCl, 5 mM $MgCl_2$, 1 mM DTT and 10 μM GTP. The reaction was stopped by addition of 200 μl of 12% SDS and then 100 μl of H_20 was added. Hydrolyzed GTP was quantitated by monitoring the release of PO_4^{-3} using a spectrophotometer according to an established procedure [4,7]. Na_2HPO_4 was used to obtain a standard curve for phosphate estimation.

The K_m and V_{max} of TrmE of *P. syringae* (Lz4W) were determined. In addition, the substrate specificity of TrmE, its optimal temperature for activity, its thermal stability and the effect of metal ions on its activity were also studied. All these experiments were run in triplicate.

3. Results

3.1. Expression and purification of TrmE from P. syringae (Lz4W)

Preliminary experiments to express *trmE* in *E. coli* BL21 (DE3) using pET28b as the expression vector were not successful, since the protein was expressed but accumulated in the inclusion body. Several attempts were also made to express the protein in cultures grown at lower temperatures such as 15, 18 and 22 °C, but it consistently accumulated in the inclusion body. Thus, to produce the protein in the biologically active form, *P. syringae* was then used as the host.

P. syringae (Lz4W), in which the construct pGL10::Histag-*trmE* was cloned, expressed TrmE as a soluble protein of 53 kDa and was not detectable in the insoluble pellet fraction.