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Cloning, expression, purification and characterization of the stress kinase YeaG from *Escherichia coli*

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Abstract

We cloned, overexpressed and purified the *Escherichia coli yeaG* gene product, whose amino acid sequence displays homology to prokaryotic serine protein kinases. The gene coding for YeaG was generated by amplifying the *yeaG* gene from *E. coli* by polymerase chain reaction. It was inserted into the expression plasmid pET-21a, under the transcriptional control of the bacteriophage T7 promoter and *lac* operator. A BL21(DE3) *E. coli* strain transformed with the YeaG-expression vector *pET-21a-yeaG* accumulates large amounts of a soluble protein with a molecular mass of 76 kDa in SDS–PAGE, which matches the expected YeaG molecular weight of 74.5 kDa. YeaG, although soluble, has a marked tendency to aggregate in the absence of detergents, so that it was purified in the presence of 0.1% Triton X-100, by ion exchange chromatography and hydroxyapatite chromatography. The purified protein is monomeric and displays autokinase and casein kinase activities which are optimal in the presence of 10 mM Mn^{2+}. The purification of the active protein kinase YeaG described in this study should allow us to characterize its biochemical target(s) in *E. coli* extracts.

Keywords: Protein kinase; Environmental stresses; Bacteria; *Escherichia coli*

Protein phosphorylation is an important mechanism to translate extracellular signals into cellular responses and is carried out by coupled protein kinases and phosphatases [1]. Bacteria exhibit at least three protein phosphorylating systems: (i) serine/threonine and tyrosine protein kinases that phosphorylate more than 130 different endogenous proteins in *Escherichia coli*, including isocitrate dehydrogenase (whose phosphorylation by AceK in response to acetate growth shifts the Krebs cycle to the anabolic glyoxylate shunt), EF-Tu, DnaK, and succinyl-CoA synthetase [1,2]; (ii) two components systems [1], each of which comprises a sensor kinase that autophosphorylates at a histidine residue in response to stimuli such as osmotic stress (sensor kinase EnvZ), phosphate limitation (sensor kinase PhoR) or the quinone redox state (sensor kinase ArcB) and transfers its phosphoryl group to an aspartate residue of a second protein (OmpR, PhoB, and ArcA, respectively, for EnvZ, PhoR, and ArcB) called response regulator (which is most often a transcription factor), changing its activity, and (iii) the phosphoenolpyruvate carbohydrate phosphotransferase system [3] whose primary function consists in the translocation of sugars across the cytoplasmic membrane, with their concomitant phosphorylation, but which also participates in the general regulation of carbon metabolism in Gram-positive bacteria (catabolite repression, catabolite activation, inducer levels), with its Hpr component functioning as a protein kinase substrate.

Although we have known for some time that serine/threonine protein kinases play major regulatory roles in eukaryotes, their discovery in prokaryotes is relatively recent [4]. They are especially involved in the control of metabolism and in the late stages of development, sporulation, secondary metabolite production and pathogenicity

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[5–10]. *E. coli* YeaG is a well-conserved putative serine protein kinase with homologs in many eubacteria and archaea. It is more than 20-fold overexpressed during both stationary phase, and acid and salt stresses, suggesting that it plays an important role in cellular protection against environmental stresses [11]. Its *Bacillus subtilis* homolog PrkA has been purified as a His-tag fusion protein that phosphorylates a 60 kDa protein of unknown function in crude *B. subtilis* extracts *in vitro* [12]. Our work describes the cloning and expression of YeaG, and its purification in the presence of Triton X-100. We also characterized YeaG as an autokinase, endowed with protein kinase activity towards casein, a substrate of serine protein kinases. The purification of the active protein kinase YeaG, described in this study, should allow us to characterize its biochemical target(s) in *E. coli* extracts.

Materials and methods

Bacterial strains, plasmids, and growth conditons

The *E. coli* BL21 (DE3) strain (Novagen, USA) was used for the transformation of the new expression vector construct containing the gene encoding for YeaG. The gene coding for YeaG was generated by amplifying the *yeaG* gene from *E. coli* genomic DNA (from strain MG1655) (this DNA was prepared using the Wizard genomic DNA purification kit from Promega) by polymerase chain reaction (PCR) using the forward primer 5′-GGTGGTTG CTCTTCACATATGAATATATTCGATCAC-3′ containing a Nde1 site and the reverse primer 5′-GGTGGTC TGGGATCCTCATTAAGACGATTTACGTAC-3′ containing a BamH1 site. The PCR was done in a volume of 50 μl, in the presence of 0.5 μg of template DNA, 1 μg of forward and reverse primers and 5 units of Taq polymerase (Applied Biosystems), with 1 cycle (30 s at 98 °C), 5 cycles (10 s at 98 °C, 15 s at 47 °C, 90 s at 72 °C), 30 cycles (10 s at 98 °C, 1 s at 69 °C, 90 s at 72 °C) followed by a last cycle (10 s at 98 °C, 15 s at 69 °C, 10 min at 72 °C). The resulting product was digested with Nde1 and BamH1, ligated to the pET-21a (Novagen, USA) Nde1 and BamH1 backbone fragment, and transformed into strain BL21 (DE3). The sequence of the cloned gene was confirmed by DNA sequencing (not shown).

The *yeaG*-deficient strain was kindly provided by Dr. H. Mori (Nara Institute of Science and Technology, Japan) and contained *yeaG* disrupted by λRed in the *E. coli* strain BW25113 (*lacI*^{q} *rrnB3* Δ*lacZ4787 hsdR514* Δ(*araBAD*)*567* Δ(*rhaBAD*)*568 rph-1*) [13], leading to strain JW1772.

Bacterial extracts' preparation

For the purification of YeaG, the YeaG overproducing strain (BL21 (DE3) pET-21a-*yeaG* was grown at 37 °C in 1 l of Luria–Bertani medium [14] supplemented with ampicillin (50 μg/ml) to an $OD_{600} = 0.5$. YeaG overexpression was induced with 1 mM IPTG and growth was continued until the culture reached an $OD_{600} = 2.5$. Cells were harvested by centrifugation at 4 °C. The cell pellet was resuspended in 10 ml of 30 mM Tris, pH 7.6, 30 mM NaCl, 1 mM DTT[1], 0.5% Triton X-100 and bacteria were disrupted at 0 °C with a Branson sonicator (microtip, level 5, 5 × 30 s). This extract was centrifuged for 2 h (4 °C) at 200,000*g*, and the supernatant was immediately loaded onto a DEAE–Sephacel column.

For the preparation of crude extracts from the *yeaG*-deficient strain JW1772 and from its parental strain BW25113, bacteria (1.6 g wet weight) were grown in LB medium to the end of the exponential phase and lysed at an $OD_{600} = 100$ in 30 mM Tris, pH 7.6, 30 mM KCl, 1 mM dithiothreitol (lysis buffer). The lysate was centrifuged for 5 min at 5000*g* in order to eliminate cell debris, and the supernatant was centrifuged for 2 h at 200,000*g*, giving the 200,000*g* supernatant and 200,000*g* pellet. 200,000*g* supernatants from both strains were dialyzed for 4 h against lysis buffer, and the 200,000*g* pellet was resuspended in 300 μl of lysis buffer. Both fractions were kept at −80 °C.

DEAE–Sephacel ion exchange chromatography

YeaG was loaded onto a DEAE–Sephacel column (5 × 0.8 cm) equilibrated in 30 mM Tris, pH 7.6, 30 mM NaCl, 1 mM DTT, 0.1% Triton X-100 at 20 °C, and it was eluted (around 110 mM NaCl) with a linear gradient of 0.03–0.5 M NaCl in the same buffer. Fractions were analyzed by SDS–polyacrylamide gel electrophoresis and quantified by the Bradford assay, using bovine serum albumin as standard.

Hydroxyapatite chromatography

Purified fractions from the DEAE–Sephacel column were loaded onto a 4 ml hydroxyapatite column (Bio-Gel HTP from Bio-Rad), equilibrated in 30 mM Tris, pH 7.6, 30 mM NaCl, 1 mM DTT, 0.1% Triton X-100 at 20 °C, and eluted (around 40 mM phosphate) with a linear gradient of 0–100 mM sodium phosphate, pH 7.6, in the same buffer. YeaG was dialyzed against 20 mM Tris, pH 7.6, 30 mM NaCl, 1 mM DTT, 0.1% Triton X-100 and stored at −80 °C.

Native molecular weight determination

The molecular weight of native YeaG was determined by gel filtration on a Bio-Gel A-0.5 m column (Bio-Rad Laboratories, 0.2 × 20 cm). The column was equilibrated in 30 mM Tris, pH 7.6, 100 mM NaCl, 1 mM DTT, 1% octylglucoside (column buffer) at 20 °C, loaded with 20 μl of purified YeaG (1.8 mg/ml) (equilibrated in column buffer

[1] *Abbreviations used:* Hsp, heat shock protein; DTT, dithiothreitol; PEP, phosphoenolpyruvate.