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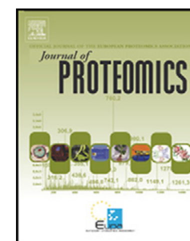

Proteomics of early and late cold shock stress on thermophilic bacterium, *Thermus* sp. GH5

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ABSTRACT

Thermus sp. GH5 is an aerobic thermophilic bacterium with optimal growth at 70–75 °C isolated from a hot spring in Ardabil, North West province of Iran. Due to industrial and biotechnological applications of thermophils, it is very important to know more about their proteomes and metabolomes. Since thermophils live in stressful environments it will be very useful to study their survival mechanisms. There are many reports on stress induced proteins, particularly the well characterized heat shock proteins, but little is known about the functions of proteins induced after a decrease in temperature. In this study, the proteomes of the thermophilic bacterium after a temperature down shift from 75 °C to 45 °C for 2 h and 5 h were investigated. We also compared protein profiles of early and late cold shock processes to that of cells grown at 75 °C and identified a set of proteins, some of which are involved in metabolic processes such as fatty acid synthesis, pentose phosphate pathway, aromatic component degradation and signal transduction. Our data showed this organism could be tolerating the stress conditions by changing its metabolism and physiology.

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1. Introduction

Thermophils are a group of microorganisms that live in the harsh environmental condition. They optimally grow at temperatures between 45 and 80 °C and have been found in both terrestrial and marine environments which are heated by volcanic or geothermal activities. Living in extraordinary temperatures make them an interesting subject of research [1,2]. Thermophils have special metabolic characteristics that are used in biotechnology. They produce biocatalysts, bioremediation and detoxification compounds that are used in pharmaceutics and agriculture [3]. However, thermophils experience a lot of environmental stresses such as osmotic stress, extreme pH, salinity and extreme temperatures. Among these stress factors, temperature is one of the most important factors for life because it influences structural and functional characteristics of cellular components [4–7]. In all organisms a sharp down shift in temperature, which is termed cold shock can change cell functions [8]. Growth measurements show non-lethal cold shock conditions (above the minimal growth temperature) can be divided into three different stages. The first step represents the initial but transient stress response (acclimation phase) that immediately takes place after the temperature down shift and, depending on the organism, may take up to several hours during which a sharp reduction in the growth rate could be observed. In the second stage (recovery phase) the bacteria grow significantly faster and in this step cells adapt to the cold environment and in the third stage they reach stationary phase faster than the previous stages. Bacteria most likely stay in stationary phase [9–13]. The study of protein profiles by two-dimensional gel electrophoresis can help to identify a large number of proteins whose expression levels are modulated by cold shock. The principal aim of present study was to

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examine the response of the thermophilic bacterium *Thermus* sp. GH5 to a down shift in temperature from 70 °C to 45 °C and to use proteomics methods to analyze their protein profiles under different cold shock conditions.

2. Materials and methods

2.1. Materials

IPG strips and IPG buffer were obtained from Bio-Rad. Analytical grade chemicals were purchased from Bio-Rad (France), Merck (Darmstadt, Germany), and Sigma–Aldrich (UK). Antibodies were purchased from "Santa Cruz Biotechnology, Inc (USA) and Enzo Life Sciences, Inc. (USA).

2.2. Methods

2.2.1. Bacterial culture and stress experiment

Thermus sp.GH5 was isolated from a hot spring in Ardabil, North West of Iran. A colony was picked from an agar plate and inoculated into 50 ml of sterile *Thermus* medium in a 250 ml flask as a preculture. The preculture was incubated at 75 °C with shaking at 200 rpm. After 16 h preculture, 1 ml of cell suspension was transferred to each of nine 100 ml flasks of fresh *Thermus* medium [these nine flasks divided to three groups, three flasks for control group, three flasks for early cold shock and another three flasks for late cold shock group). Bacteria were cultured at 75 °C until mid-log phase was reached ($OD_{620\,nm}$ ~ 0.6) and subsequently three flasks was put aside as a control and the others were transferred to a water bath at 45 °C with shaking at 200 rpm and stopped after 2 h and 5 h, respectively. The bacteria were harvested immediately with centrifugation at 4000 ×*g* at 4 °C for 15 min and then washed three times with preparation buffer containing 10 mM Tris–HCl, 1 mM EDTA, 0.1 mM PMSF and 250 mM sucrose (pH 7.8).

Thermus medium is made of 100 ml mineral solution 1, 10 ml mineral solution 2, 0.17 mM $FeCl_3 \times 6H_2O$, 1 g tripton, 1 g yeast extract, dissolve ingredients in the distills water and adjust to pH 7.8 with 1 M NaOH. Autoclave at 121 °C for 15 min. Mineral solution1 is consisted of 1 g nitrilotriacetic acid ($C_6H_9NO_6$), 0.6 g $CaSO_4 \times 2\ H_2O$, 1 g $MgSO_4 \times 7H_2O$, 0.08 g NaCl, 1.03 g KNO3, 6.89 g NaNO3, 1.11 g Na_2HPO_4 per 1000 ml distilled water. Mineral solution 2 is consisted of 0.22 g $MnSO_4 \times H_2O$, 0.05 g $ZnSO_4 \times 7H_2O$, 0.05 g H_3BO_3, 0.0025 g $CuSO_4 \times 5\ H_2O$, 0.0025 g $Na_2MoO_4 \times 2\ H_2O$, 0.0046 g $CoCl_2 \times 6H_2O$ per 1000 ml distilled water.

2.2.2. Sample preparation

Cell pellets were resuspended in TE buffer, containing 20 mM Tris–HCl (pH 7.8) and 1 mM PMSF, and disrupted on ice by sonication (5 cycles of 30 s). Cell debris was removed by centrifugation at 18,000 ×*g* at 4 °C for 20 min. The sample solution was precipitated by adding 10% TCA and 0.1% DTT and stored overnight at −20 °C to precipitate proteins and to remove salts, nucleic acids and other compounds. The suspension was centrifuged at 18,000 ×*g* for 30 min. Protein pellets were resuspended in ice-cold acetone containing 0.1% DTT and stored at −20 °C for 30 min. The protein suspension was centrifuged again at 18,000 ×*g* for 30 min and the pellet was resuspended in ice-cold acetone (without DTT). The protein suspension was stored at −20 °C for 30 min and then centrifuged at 18,000 ×*g* for 30 min. Again protein pellets were resuspended in ice-cold acetone containing 0.1% DTT and stored at −20 °C for 30 min. The obtained pellets were immediately dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% IPG buffer pH 3–10 and 50 mM DTT) [14,15]. Each sample was sonicated and centrifuged, and the protein concentration was determined by Bradford assay [16].

2.2.3. Two-dimensional gel electrophoresis

Each sample was applied to an IPG strip (17 cm, pH 4–7 nonlinear; Bio-Rad) with a final concentration of 1.4 mg of proteins in 350 μl rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% IPG buffer pH 3–10 and 50 mM DTT) and rehydrated for 16 h. Then isofocusing (Bio-Rad) was performed under following conditions: linear ramping to 300 V for 30 min, slow ramping to 300 V for 1 h, linear ramping to 500 V for 30 min, slow ramping to 500 V for 1 h, linear ramping to 1000 V for 30 min, slow ramping to 1000 V for 1 h, linear ramping to 3500 V for 2 h, linear ramping to 10,000 V for 1.5 h and finally the IEF was terminated after 60,000 Vh was reached. Prior to putting onto SDS-PAGE, the focused IPG strips were placed in equilibration buffer (50 mM Tris–HCl, pH 8.8, 6 M Urea, 30% v/v glycerol and 2% w/v SDS) containing 1% w/v DTT for 15 min and soaked again in equilibration buffer containing 2.5% w/v iodoacetamide for 15 min. The strips were then placed on a 14% acrylamide: bis-acrylamide SDS-PAGE and sealed with agarose sealing solution (0.5 w/v agarose, 25 mM Tris, 192 mM glycine, 0.1 w/v SDS and a trace of bromophenol blue). The second-dimension SDS-PAGE was performed at 16 mA/gel for 30 min and 24 mA/gel at 20 °C until the bromophenol blue dye front reached the bottom of the gel. The gels were fixed in fixing buffer (45% v/v methanol and 10% v/v acetic acid) for 30 min and stained in coomassie brilliant blue solution (17% w/v ammonium sulfate, 3% v/v phosphoric acid, 34% v/v methanol and 0.1% w/v coomassie brilliant blue G-250) overnight. Finally the gels were destained in water to clean the background and scanned on a GS-800 Densitometer (Bio-Rad).

2.2.4. Comparative image analysis

The statistical data (spot detection, spot editing, pattern matching, and quantification) were analyzed using the Progenesis PG240 software package (Nonlinear Dynamics, UK) with high image quality TIF format. Statistical analyses were performed on triplicate for each experimental growth condition. For comparison, the 2-D image of cells at 75 °C (control group) was set as the reference gel image. Before matching the images, background was subtracted and normalization was performed to correct for the differences in protein spot intensity. The reference gel image was matched to other gel images and percentage volume (%vol) was used for spot identification. We defined a protein spot that increase or decrease their abundance if the spot %vol changed more than twofold with *p*-value less than 0.05. Three biological repeats were performed for each condition [17–19].

2.2.5. In-gel tryptic digestion and MS analysis

The protein spots of interest were manually excised from preparative 2-D gels. Gel pieces were washed in 100 mM ammonium bicarbonate for 1 h at room temperature followed

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