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Use of Proteomic Tools to Analyze Genes Involved in Thermal- and Alkaline-Tolerance *Rhizobium* Strains Nodulating Egyptian Clover.

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ABSTRACT

Environmental stresses such as salinity, alkalinity and high temperature are main factors that restrict or limit symbiotic nitrogen fixation with legumes. Three of 48 *Rhizobium* strains that were isolated from root nodules of Egyptian winter clover displayed tolerance to different harsh environmental conditions that could prove helpful for the development of new and effective inoculants for this legume crop. *Rhizobium* strain Rhiz1017 was selected to analyze proteins that affect its survival under elevated temperature (42ºC) or alkaline conditions pH9. Both of 2D-DIGE and MALDI-TOF-TOF analyses indicated that changes of up- or down-regulated proteins under alkaline conditions (pH9) were less affected than temperature stress. Analysis of 1,792 protein spots under temperature stress indicated that strain Rhiz 1017 are up-regulated proteins responsible for the biosynthesis of organic solutes such as glycine, betaine, ectoine and mannitol, and polyamine (putrascine,) and energy generation. In addition, while the expression of chaperonin GroEl was enhanced, proteins responsible for storing energy such as poly beta hydroxyl butyrate were down regulated. At pH 9, beta keto-thiolase was significantly up regulated protein. Our results give insights into potential mechanism by which rhizobia respond to thermal and alkaline conditions; this knowledge will be useful in developing improved legume-microbe symbiosis to increase the productivity of legume crops in Egyptian dry ecosystems.

Keywords: Egyptians soils, proteomic analysis, thermal- and alkaline tolerance, *Rhizobium* strains.

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INTRODUCTION

The legume-*Rhizobium* symbiosis is the single most important source of biologically-fixed nitrogen in agricultural systems [1]. Symbiosis of legumes with *Rhizobium* partners is responsible for 45 [2] to 85% [3] of BNF in agricultural areas. Symbiotic nitrogen fixation contributes to ecological and sustainable agriculture, as it reduces the need for chemical N-fertilizers and improves crop productivity. However, BNF can be severely limited by environmental stresses such as salinity [4], high temperature [5], drought [6, 7], acidity [8, 5] and alkalinity [4]. For example, soil salinity affects about 800 Mha of arable lands worldwide [9], and these factors are expanding. Almost 40% of the world's land surface is affected by salinity-related problems [10]. In addition, about 25% of the agricultural lands are impacted by problems associated with soil acidity [11]. These soil environmental factors affect the persistence and survival of rhizobia in the soil, either as saprophytes or symbionts inside nodules.

Extreme environmental conditions lead to reduced efficiency of *Rhizobium* strains and their competitiveness for nodule occupancy [12] and can have dramatic impacts on endogenous soil bacteria leading to problems in nitrogen fixation efficiency. Problems of nodulation and nitrogen fixation under environmental stresses have been described in detail by Zahran [11] and Sadowsky [13]. Microorganisms and plants are reported to accumulate several chemical substances under environmental stresses. These include betaines and polyamines [14], amino acids such as proline, serine, and glutamine [15], organic solutes [16], soluble sugars [16, 17], and inorganic cations as K+ [18]. The goal of the current study was to select *Rhizobium* strains for Clover (*Trifoilum alexandrinum* L.) that are tolerant to a broad range of environmental stresses, and identify factors that give these strains an advantage over sensitive isolates. These results may prove useful in establishing a sustainable agriculture system and improving legume production under harsh-environmental conditions and will help to use these strains as inoculum in Egyptian dry ecosystems.

MATERIALS AND METHODS

Rhizobium isolation

Forty-eight *Rhizobium* strains were isolated from surface sterilized, randomly collected clover nodules as described by Shamseldin et al. [19]. Pure culture isolates grown on YEM agar amended with bromothymol blue (BTB) were selected and preserved in 50% glycerol at -80ºC until further analyzed.

Selection of strains resistant to environmental stresses

To select strains resistant to a broad range of environmental stresses, isolates were grown on YEM agar medium at different concentrations of NaCl ranging from 0.2 to 4%, from pH values of 6.8 to 9, and from temperatures of 28 ºC to 42 ºC as previously described by Shamseldin et al. [19]. To examine the growth of *Rhizobium* strains under high pH the growth medium supplemented with 2-amino-2-methyl- 1,3 propanediol (AMPD) for buffering [4]. To verify and quantify tolerance, the selected resistant strains (Rhiz950 and Rhiz1017) were examined in triplicate and growth rates were measured spectrophotometrically at 600 nm.

Proteomic techniques

Protein extraction and proteome analyses were done as described by Shamseldin et al. [20] and Gomes et al. [21].

Protein extraction

Total cell pellets from 100 ml of YEM broth cultures of each *Rhizobium* strains grown under normal and different stress conditions were harvested by centrifugation at 5,000xg, for 15min at 4 ºC. Pellets were washed twice with phosphate buffered saline (PBS), and re-pelleted. Total soluble protein was obtained by re-suspending the pellets in 900µl of cell lysis buffer (30mM Tris-HCl, 7M urea, 2M thiourea and 4% CHAPS, pH 8.8), proteins released by sonicating (6 rounds of tip probe sonication using Branson Sonifier B250; Darbury CT) at 4 ºC for 30 s for each cycle with 1 min on ice between rounds, and incubating the tubes on a shaker for 30 min at room temperature. The solution was centrifuged for 30 min at 4 ºC at 20,000x g, and the resulting supernatant was collected.

Protein concentration

Total protein concentration was determined using the Bio-Rad protein assay method as described by Bradford [22]. Each 100 ml yielded about 9-12 mg proteins. The lysate samples were diluted with cell lysis buffer to a final protein concentration of 5 mg ml^{-1}. In each experiment three replicates were run with total protein ranging from 1-10 pmol.

Two-dimensional PAGE (2D-PAGE) and dye-labelling in-gel electrophoresis (DIGE)

2D-PAGE and mass spectrometry were performed at Applied Biomics Inc. (Hayward, CA 94545, USA). Experimental design and labeling of proteins obtained from strains growing under different conditions are listed in Table (1). For CyDye labeling, 30 µg of protein lysate was mixed with 1.0 µl of diluted Cy-Dye (1:5 diluted with DMF from 1 nmol $µl^{-1}$ stock), followed by vortexing. The tubes kept on ice in the dark for 30 min and labeling reactions were stopped by adding 1µl of 10 mM lysine to each sample. Samples were vortexed and the mixture was incubated on ice in the dark for an additional 15 min. For DIGE, the labeled samples (Cy2, Cy3 and Cy5) were mixed together, and 2X 2D sample buffer (8M urea, 4% CHAPS, 20 mg ml^{-1} DTT, 2% pharmalytes, pH 8.5), 100µl of de-streak solution (GE Healthcare) and rehydration buffer (7M urea, 2M thiourea, 4% CHAPS, 20 mg ml^{-1} DTT, 1% pharmalytes, pH 4-10) and bromophenol blue were added to give a final volume of 350 µl for the 18 cm IPG strip. The samples were mixed well and spin down before being loaded onto a strip holder.

Isoelectric focusing (IEF) and SDS-PAGE

Samples for IEF were handled as described by Gomes et al. (2012). Labeled samples were loaded into the strip holder, overlaid with an 18cm immobilized pH gradient strip (pH 4 to 9 Amersham), and 1.5 ml mineral oil was added on the top of the strip. For IEF samples were rehydrated for 12h in rehydration buffer in the dark at 20 ºC. Loaded strips were subjected to focalization in an Ettan IPGphor IEF system (GE Healthcare) for 1h at 200 V, 1h at 500 V, a gradient step to 1,000 V for 1h, a gradient step to 8,000 V for 2.5h, and fixed at 8,000 V for 1.5h. After running of IEF the IPG strips were incubated in freshly made equilibration buffer (50 mM Tris-HCl pH 8.8 containing 6M urea, 30% glycerol, 2% SDS, a trace amount of bromophenol blue, and 10 mg ml^{-1} DTT) for 15 min with gentle shaking. Strips were soaked in freshly made equilibration buffer 2 which contained the same previous components of this buffer plus 45 mg ml^{-1} iodoacetamide for 10 min with gentle shaking. The IPG strips were rinsed once in SDS gel running buffer before being transferred into 12% SDS gels (18 cm by 16 cm), followed by sealing with 0.5% (w/v) agarose solution in SDS-PAGE running buffer. The SDS gels were run at 10 mA for 30 min, followed by 40 mA for 2.5h at 15 ºC.

Image scanning and data analysis

Gel images were scanned immediately after SDS-PAGE using a Typhoon TRIO scanner (GE Healthcare). The scanned images were analyzed by using Image Quant TL software (GE-Healthcare), and then subjected to in and cross-gel analysis using DeCyder software version 6.5 (GE Healthcare). The change ratio of differential expressions of proteins was obtained from the in-gel DeCyder software analysis.

Mass spectrometry and samples preparation

Only spots that exhibited significant changes (>1.5 fold at $p \leq 0.05$) were considered significant. Fifteen spots that showed highly significant increases or decreases in expression level were selected for further analysis. Spots of selected proteins were picked by using an Ettan Spot Picker (GE Healthcare) based on the in-gel analysis and spot picking design by DeCyder software. The spots were de-stained by incubating at 37 ºC for 20 min with shaking in 200 µl of washing buffer (200 mM NH_4HCO_3, 50% CH_3CN v/v), and dried under vacuum for 30 min. For tryptic digestion, 10 µl each of NH_4HCO_3 (pH 8.95) and DTT were added to each sample, and heated to 60 ºC for 10 min in a preheated digital dry bath, and allowed to cool at room temperature. One micro liter aliquots of modified porcine trypsin protease (Trypsin Gold) Promega, (Madison, WI, USA) at a stock concentration of 50µg $µl^{-1}$ in 50 mM acetic acid was added to each sample and samples incubated for 4h at 37 ºC with gentle agitation. After digestion, samples were treated with 2% trifluoroacetic acid (TFA) to stop the digestion and the resulting peptides were extracted and purified using C18 Zip Tips (Millipore) as recommended by manufacturer. Purified peptides from the previous step were extracted using a pre-cleaned

Zip-tip, washed with water containing 0.1% TFA, re-extracted with acetonitrile:water (50:50) and 0.1% TFA. Peptides were eluted from the Zip-tip with 0.5 µl of matrix solution (α-cyano-4-hydroxycinnamic acid, 5 mg ml^{-1} in 50% acetonitrile, 0.1% TFA, 25 mM ammonium bicarbonate) and spotted onto MALDI gold plates.

Mass spectrometry and protein identification

MALDI-TOF (MS) and TOF/TOF (tandem MS/MS) were done using 5800 mass spectrometer (AB Sciex, San Francisco, USA). MALDI-TOF mass spectra were acquired in reflection positive ion mode, avg. 2000 laser shots per spectrum. The TOF/TOF tandem MS fragmentation spectra were acquired for each sample, avg. 2000 laser shots per fragmentation spectrum on each of the 10 most abundant ions present in each sample (excluding trypsin autolytic peptides and other known background ions such as labeling dyes, and matches to matrix). MS peak lists were generated by the peak-to-mascot script of 4700 Explorer™ program. In addition, peak lists were evaluated by the program MS-Screener (Version 1.01) to remove common contaminant. Both the resulting peptide masses and the associated fragmentation spectra were submitted to GPS Explorer Version 3.5 equipped with MASCOT v2.2.06 search engine (Matrix Science Ltd; http://www.matrixscience.com) to search the database of National Center for Biotechnology Information non-redundant (NCBInr). Proteins searched against the Proteobacteria taxonomic group and parameters that searched on MASCOT website (http://proteomicresource.washington.edu/mascot/serach_from_select.html) were (1) peptides with molecular masses ranging from 800-4000 Da; (2) mass tolerance of the fragment ion 0.25Da; (3) a minimum of one matching peptides; one missed cleavage allowed and (4) carbamidomethylation of cysteine required, acetylation of N-terminus and methionine oxidation allowed. Candidates with either protein score C.I. % or Ion C.I. % greater than 95% were considered highly significance matches. The best matches were selected based on C.I% and PI/MW location of the spot in the gel. Each protein ID was reviewed based on molecular weight and PI to pick up the top hits. For protein identification at least five peptides must be matched and sequence coverage must be greater than 10%, and the error distribution around zero. The differences between probability and score distribution for the first and all other candidates, and matches with >90% sequence identity and maximum e-value of 10^{-10}.

Statistical analysis

Each experiment was replicated three times, and protein abundance or differentiation for *Rhizobium* strain Rhiz1017 is shown the mean ± SD. Statistical significance between spots of proteins was evaluated by using Student's t test on SPSS PASW Statistics version 18 Multilingual (SPSS Inc., USA). A p-value ≤ 0.05 was considered statistically significant.

RESULTS

Selection of *Rhizobium* strains resistant to environmental stresses

The selection of *Rhizobium* strains more adapted to a wide range of environmental stresses may be a useful tool to increase their symbiotic efficiency under these conditions, therefore forty-eight strains were examined for their ability to grow on salt concentrations from 0.2 to 4% NaCl, different pH values from 6 to 9, and in temperatures from 28 ºC to 42 ºC. This was done by replica plating on YEM agar medium amended with BTB indicator (Figure 1). All the examined strains grew well on 0.3% NaCl, and at a temperature 28 ºC and pH 6.8. There was marked reduction in the number of rhizobial strains growing on this medium with increasing salt concentration, pH and temperature. Thirty percent of the strains grew on 0.5% NaCl, four strains could grow on 1% NaCl (Rhiz950, Rhiz956, Rhiz982 and Rhiz984) while only two strains (Rhiz950 and Rhiz956) were able to grow on 3% NaCl. None of the examined strains could grow at 4% NaCl. Fifteen strains were resistant to alkaline pH 9, and strain Rhiz1017 looks like extremophiles because the diameter of its colony was greater than other strains at pH 9. Ninety eight percent of the strains grew well at 37 ºC, while strain Rhiz1017 is the only one which survived at 42 ºC. The salt tolerant strain Rhiz950 and strain Rhiz1017 resistant to alkaline and high temperature were selected to study their growth in liquid culture by measuring the optical density (Figure 2A-C) at different time intervals. There was a lag period of about 84h in the growth of strain Rhiz 950 at 3% NaCl. This retardation in growth was less with strain Rhiz 1017 when grown under high pH9 (48 h) and high temperature (36h).

Analysis of thermos-and alkaline-tolerant *Rhizobium* strains using proteomic approaches

Strain Rhiz1017 was selected as a model strain to identify gene products associated with acquiring tolerance to temperature 42 ºC or alkaline stress pH9. Approximately 1,792 protein spots were included for comparison. Results in Figure 3A show the comparison between proteins from strain Rhiz1017 grown on YEM broth without stress (optimum conditions pH 6.8 and 30 ºC) labeled with Cy2 (green spots) with those from the same strain grown at pH 9 and 30 ºC which labeled with Cy3 (red spots). Figure 3B shows the comparison between proteins from growing strain Rhiz1017 under temperature stress 42 ºC and pH 6.8 which labeled with Cy3 (red spots) and those from the same strain grown under optimum conditions (green spots cy2). Protein spots with either a protein score C.I. % or ion C.I. % of > 95% confidence were considered for further analysis. Table 2 summarizes the percentage similarity between proteins from strain Rhiz1017 under normal conditions and those obtained under high temperature or high pH stress.

Under temperature stress, 81.8% of the spots were similar, with 151 up and 176 down-regulated proteins, while under the pH9 stress 93% of spots were similar with 52 proteins up-regulated and 74 down regulated. The fifteen proteins that showed a highly significant increase or decrease due to stress of temperature or high pH were selected for proteome analysis.

Ten and three proteins that were highly up regulated under temperature stress and high pH stress on respectively and two proteins of the down-regulated under both kind of stresses were analyzed by MALD-TOF-TOF mass spectrum MS/MS. Table 3 contains the proteins and their characteristics, including molecular weight, pI, peptide counts, protein score, sequence coverage, total ion score, E value and closest matched microorganism. Most of the identified proteins had adequate percent of sequence coverage with the known proteins from Data Bank. Fifteen proteins were identified based on the chromosomal genome of *Rhizobium etli* CFN42T and symbiotic genes that were present in Gene-Bank under accession number CP000133.1 for chromosome, and NC_004041.2; NC_007761.1 for symbiotic genes localized on plasmids. The up- regulated proteins under heat stress were aconitate hydratase (8.07±5.91), aldehyde dehydrogenase (19.3±5.91), argininosuccinate dehydrogenase (10.52±5.91), aspartate aminotransferase (20.74±5.91), glucose-6-phospahte dehydrogenase (19.21±5.91), mannitol dehydrogenase (12.77±5.91), transketolase (42±5.91), acetyl CoA acetyltransferase (42.85±5.91), 3-oxoacid CoA transferase subunit A (11.74±5.91), and chaperonin groEL (8.71±5.91). The three induced proteins under alkaline pH (9) were Beta ketothiolase (5.36±3.92), a putative ABC transporter substrate binding protein (11.47±3.92) and a hypothetical protein (11.7±3.92). The two down-regulated proteins were 5-nucleotidase (-11.23±5.91) and phasin family protein (-8.94±5.91).

Potential mechanism of thermal tolerance in *Rhizobium* strain Rhiz1017 grown under stress of high temperature 42 ºC

Results in Figure 4 show that the thermo-tolerant strain Rhiz1017 used different pathways to survive under temperature stresses. It is up-regulated different genes involved in energy biosynthesis, up-regulated six genes involved in the biosynthesis of different organic solutes, up-regulated genes involved in the regulation of chaperonin GroEL, produce water to protect itself from hydration by up-regulated aconitase gene, Keep the balance between the K^+/Na^+ ratio by inducing glucose-6-phosphate dehydrogenase, and on the other side it is down-regulated genes necessary for synthesizing macro molecules such as polyhydroxyalkonates, because *Rhizobium* cells already need energy to overcome these harsh conditions.

DISCUSSION

Forty-eight *Rhizobium* strains which previously reported by Shamseldin et al. [19] and nodulated Egyptian winter clover (*Trifolium alexandrinum* L.) were screened to select strains resistant to one or more of environmental stresses. The strains were chosen because they were likely to be well adapted to the environments from which they had been isolated i.e., hot, dry, high pH, and salt-affected Egyptian ecosystems. The thermo- and alkaline tolerant strain Rhiz1017 was isolated from New Aswan in the South of Egypt which characterized by high temperature area (Shamseldin et al., [19]. Strain Rhiz 950 gave promising results concerning its tolerance to high concentrations of salt up to 3% NaCl. Previous research had shown significant positive correlations between the presence of high salt tolerance and adaptation of rhizobia to alkaline soils [4].

Here we interested in analyzing genes involved in thermos- and alkaline-tolerance among Egyptian Clover-nodulating *Rhizobium* strains, as main factors contributing to failure of rhizobial inoculation in many environments. Strain Rhiz1017 was selected to analyze the genes associated with survival at 42 ºC and at pH9 using proteomic tools. Preliminary proteomic data in Table 2 indicated that heat stress had a greater effect on the protein profiles, than pH9, where 18.2% of the proteins were differentially regulated under temperature stress compared to 7% under high pH9. This is consistent with our earlier results [4] where we demonstrated that high temperature had a greater detrimental effect on nodulation of common bean by rhizobia than did high pH.

Ten of up-regulated proteins found under elevated temperature conditions, belonged to five different categories of clusters of orthologous groups (COGs). The COGs-C (Energy production and conversion) contained two identified proteins, aconitase hydratase (Aco) and aldehyde dehydrogenase (ALDH). The Aco uses a dehydration-hydration mechanism for releasing water to protect from drying through using citrate [23]. Our results were consistent with those obtained by Gomes et al. (2012) who reported the over expression of aconitase under heat stress in *Rhizobium tropici* strain PRF81. The ALDH enzyme is found in thermophilies and alkaliphilies [24]. Likewise, Rodrigues et al. [25] reported that the ALDH gene is associated with enhanced tolerance to drought, salinity, and oxidative stress in soybean plants. The protective function of *ald*H gene is due to increase accumulation of osmolytes (e.g. glycine betaine), and/or to detoxify aldehydes [26].

Two proteins, arginino succinate synthase (ASS) and aspartate aminotransferase (AspAT) were localized to the COGs-E (amino acid metabolism and transport). The ASS enzyme can be used to produce arginine and fumarate [27]. Arginine diverted to produce ornithine by the enzyme arginase, which it can be used to produce putrescine (spermidine) that it is the major synthon for the biosynthesis of polyamines [28]. These results confirmed our previous notes of over expression of carboxysperimidine decarboxylase under 3% of NaCl by *Sinorhizobium meliloti* strain 2011 [20].

AspAT enzyme is a pyridoxal phosphate (PLP) which is responsible for transfer of an amino group between aspartate and glutamate. This enzyme is important in amino acid metabolism [29]. Additionally, Moshe et al. [30] detected the over expression of this enzyme in tomato leaf curl virus infected plants, suggesting its role in protection from plant diseases. Gomes et al. [21] observed the up-regulation of a protein belonging to the same protein family called aspartate-B-semi aldehyde dehydrogenase (ASADH) in *Rhizobium tropici* growing under high temperature. ASADH along with acetyl CoA can be used to synthesis ectoine [31], which is involved in protection of salt stress.

The COGs-G (Carbohydrate metabolism and transport) included three proteins such as glucose-6-phosphate dehydrogenase (G6PD), mannitol dehydrogenase enzyme (Mtd) and Transketolase (Tkt). G6PD is an important enzyme in the pentose phosphate pathway (PPP) and it plays an essential role in cellular redox balance [32]. Our results indicated that G6PD was over expressed under heat stress and this was compatible with results obtained by Liu et al. [33]. Letterrier et al. [34] confirmed the role of G6PD as a second barrier in protection of nitro-oxidative stress generated by salinity. Mtd belongs to the oxido-reductase family acting on CH-OH group of donors with NAD± or NADP± as acceptors. It is oxidized mannitol to mannose [35] and the latter is the main sugar for biosynthesis of polysaccharides that are reported previously to play an essential role in resistance of *Rhizobium* strains under stress conditions [36, 37]. Furthermore, mannitol is considered as osmo-protectants [38, 39].

COGs-I (lipid metabolism) contained two proteins acetyl CoA acetyltransferase (ACAT1) and 3-oxoacid CoA transferase (OXCT1). The ACAT1 belongs to the transferases family and it is known as thiolases which are known to play a prominent role in breaking down proteins and fats to produce energy [40]. Our results were confirmed by Gomes et al. [21] who also noted the induction of acyl CoA dehydrogenase in *Rhizobium tropici* strain PRF 81 grown under heat stress.

Only one protein chaperonin (GroEL) belonged to COGs-O group which responsible for post transitional modification and proteins turn over. GroEL is a heat shock protein (HSP). *Rhizobium leguminosarum* is known to have different copies of chaperonins [41]. Several investigators [21, 42, 43, 44] have also reported the over expression of chaperones under heat stress.

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Three proteins were highly up-regulated at alkaline pH9; each one belonged to a unique COGs group. COGs-I (lipid metabolism) represented by beta ketothiolase (Bkt). The Bkt is involved in production of poly beta-hydroxybutyrate (PHB), which has been shown to be involved in *Rhizobium* survival [45]. COGs-H is represented by a putative ABC transporter substrate binding protein. Our results were similar with results obtained by Lin et al. [40] who found that an ABC transporter was necessary under alkaline stress in *Sinorhizobium meliloti* strains and under desiccation stress in *Rhizobium leguminosarum* sv. viciae [46].

Two proteins, 50-nucleotidase and phasin family protein (PhaP) were highly down-regulated protein under heat or alkaline stress, and they belong to COGs-F (nucleotide transport and metabolism) and non-classified COGs proteins on respectively. PhaP is known to be responsible for the synthesis of polyester polyhydroxyalkonates that accumulated in many bacterial cells as energy reserve material [47]. Yoshida et al. [48] noted that PhaP plays a principal role in the accumulation of Poly-B-hydroxybutyrate in *Bradyrhizobium japonicum*. As well as Shamseldin et al. [20] who found that a protein coding for 3-hydroxybutyrate dehydrogenase was down-regulated in *Sinorhizobium meliloti* strain 2011 grown under salt stress.

Table 1: Experimental design of the labeling treatments.

Gel No.	Cy2 (green)	Cy3 (red)
1	Control (Optimum conditions)	pH 9
2	Control (Optimum conditions)	Temperature 42°C

Table 2: Changes in total soluble proteins of *Rhizobium* strain Rhiz1017 growing in YEM broth medium (normal condition) and exposed to stress of temperature 42ºC or pH 9.

Characteristics	Control/ high temperature 42°C	Control/ high pH pH 9
Spots included for comparison	1792	1792
Number of similar proteins	1465	1666
Percent of similar spots	81.8%	93%
Spots decreased [a]	176	74
Spots increased [a]	151	52
2 S.D value	5.91	3.92

[a]Cut-off setting: 5 fold

Figure 1: Preliminary selection of *Rhizobium* strains resistant to broad range of environmental stresses by growing strains on YEM agar medium amended with bromothymol blue using replica plating technique.

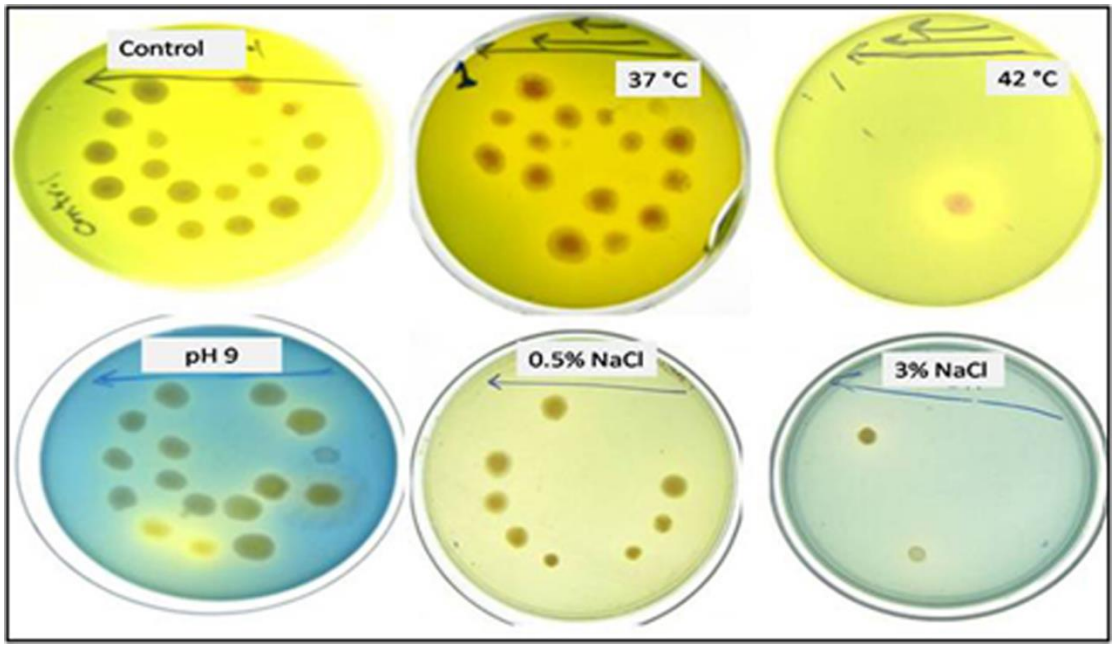

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Table 3: Identification of selected up or down regulated proteins in *Rhizobium* strain Rhiz1017 growing under high temperature 42ºC or pH9 relative to normal conditions.

Spot No.	Accession number	Protein identification	Mw (KDa)	pI	Peptide count	Sequence Coverage	Protein score	Total ion score	Fold change	E-value	Matched organism
Up regulated proteins under heat stress[a]											
C- Energy production and Conversion											
1	gi|190893792	Aconitate hydratase	96,786	5.43	18	31%	152	71	8.07±5.9	5.4E-09	*R. etli* CIAT652
14	gi|241205869	Aldehyde dehydrogenase	51,019	6.05	17	40%	496	384	19.3±5.9	1.4E-06	*R. leguminosraum* sv. *trifolii* WSM1325
E- Amino acid metabolism and transport											
15	gi|218681025	Argininosuccinate synthase	45,186	5.76	20	61%	411	248	10.5±5.9	2.2E-61	*R. etli* CIAT894
16	gi|86358600	Aspartate aminotransferase	43,500	5.8	15	45%	482	380	20.7±5.9	4.3E-32	*R. etli* CFN42
G- Carbohydrate metabolism and transport											
13	gi|190890410	Glucose -6- phosphate 1-dehydrogenase	55,193	6.13	24	58%	790	596	19.2±5.9	8.6E-73	*R. etli* CIAT652
11	gi|209551022	Mannitol dehydrogenase	53,518	5.50	13	30%	383	316	12.8±5.9	2.2E-43	*R. leguminosraum* sv. *trifolii* WSM2304
5	gi|86359086	Transketolase	69,713	6.07	19	26%	580	468	42.3±5.9	6.8E-35	*R. etli* CFN42
I- Lipid metabolism											
22	gi|218672569	Acetyl CoA acteyltransferase	37,942	6.09	15	65%	621	513	42.9±5.9	5.4E-42	*R. etli* GR56
29	gi|103487604	3-Oxoacid CoA-transferase sbunit A	25,565	5.67	6	33%	333	306	11.7±5.9	6.8E-56	*Sphingopyxis alaskensis* RB2256
O- Post-translational modification and protein turn over											
7	gi|190890943	Chaperonin GroEL	57,709	5.17	16	35%	676	557	8.7±5.9	4.3E-27	*R. etli* CIAT652
Up regulated proteins under high pH[a]											
I- Lipid metabolism											
21	gi|13398624	Beta –ketothiolase	40,956	7.10	9	35%	347	285	5.36±3.9	2.7E-24	*R. etli*
H- Coenzyme metabolites											
20	gi|190894446	Putative ABC transporter substrate binding protein	37,611	4.71	5	16%	116	98	11.7±3.9	3.4E-51	*R. etli* CIAT652
S-Proteins of unknown function											
35	gi|218460694	Hypothetical protein Retlk5_14851	17,477	5.48	10	58%	574	483	11.7±3.9	2.2E-05	*R. etli* Kim5
Down regulated proteins under heat stress or high pH[a]											
F- nucleotide transport and metabolism											
2	gi|116253812	5- nucleotidase	68,219	4.89	14	28%	*281*	216	-11.2±5.9	6.8E-22	*R. leguminosraum* sv. *viciae* 3841
Non-classified COGs proteins											
38	gi|209549169	Phasin family protein	13,434	5.39	6	27%	308	248	-8.9±5.9	1.4E-24	*R. leguminosraum* sv. *trifolii* WSM2304

[a]All indicated proteins had C.I. and Total Ion C.I. values of 100%. All proteins are cytoplasmic, except the putative ABC transporter.

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Figure 2: A: Growth curves of salt tolerant *Rhizobium* strain Rhiz950 at 3% NaCl compared to optimum conditions, B: thermotolerant strain Rhiz1017 at 42ºC and C: alkaline tolerant strain Rhiz1017 at pH 9, results± mean of SD.

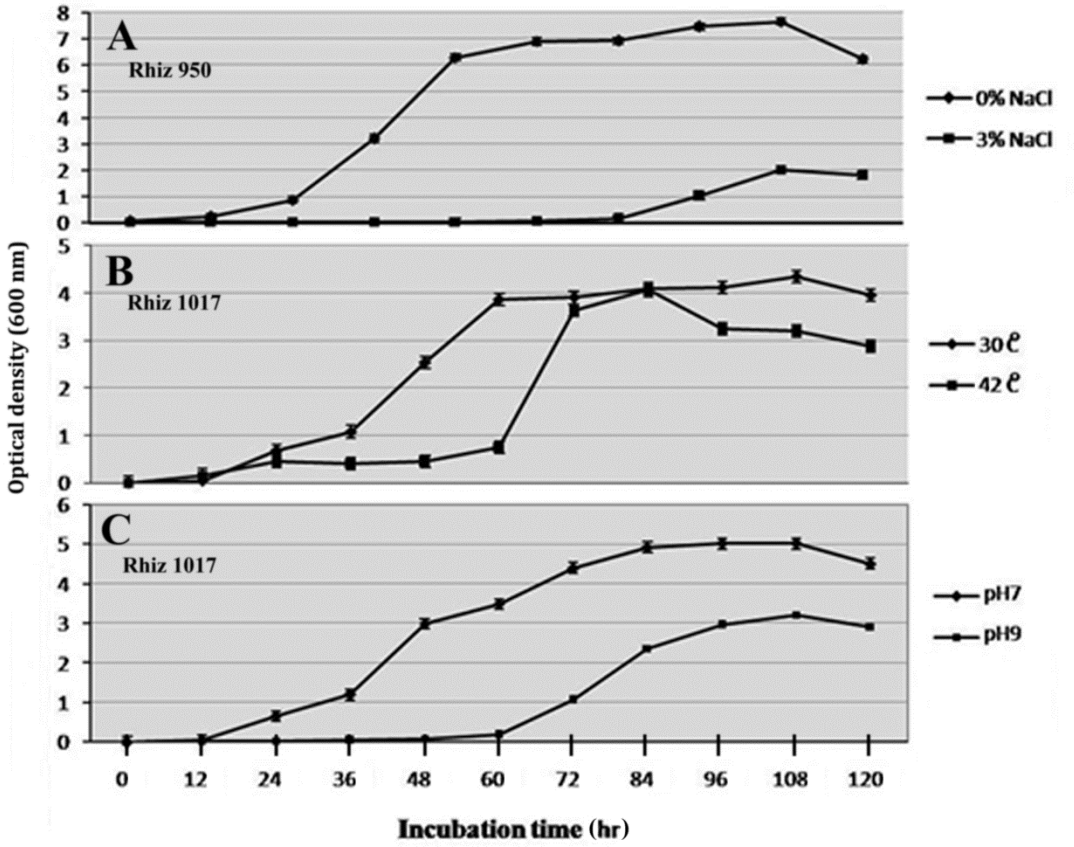

Figure 3: A: 2DE of total soluble protein gel profiles of *Rhizobium* strain Rhiz1017 under optimal condition green spots (labeled with Cy 2) and stress of pH9 red spots (labeled with Cy 3); B: total soluble proteins of strain Rhiz1017 growing at optimum conditions green spots (labeled with Cy 2) compared with stress of high temperature 42ºC red spots (labeled with Cy3). Spots were scanned by Typhoon scanner and proteins that highly up or down regulated were circled and analyzed by MALDI-TOF-TOF after digestion with trypsin

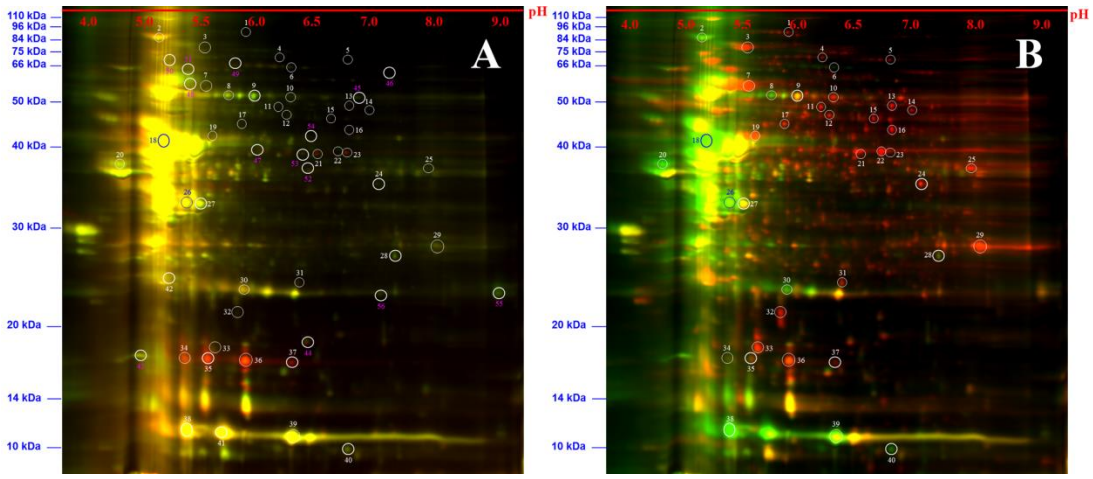

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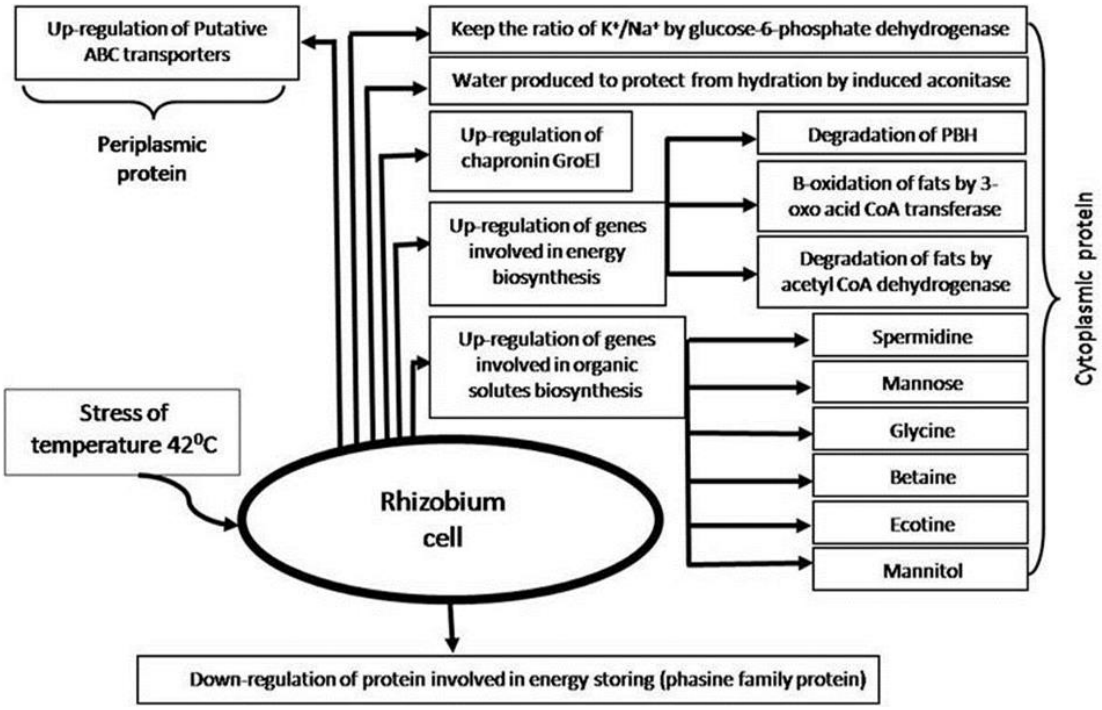
Figure 4: Expected mechanism of thermotolerance in *Rhizobium* strain Rhiz1017 under stress of high temperature 42ºC.



CONCLUSION

Our results indicated that temperature stress affected the regulation of a greater number of proteins in *Rhizobium* strain Rhiz1017 than alkaline stress did at pH9. The thermos-tolerant *Rhizobium* strain Rhiz1017 appeared to use multiple pathways to survive under heat stress. It reduced the synthesis of macromolecules that consume energy such as polyester polyhydroxyalkonates. In addition it is synthesized the energy via the degradation of PHB to generate energy and it induced genes responsible for degradation of fats as actyl CoA dehydrognease. Similarly, strain Rhiz 1017 induced some genes that simultaneously produced organic solutes such as glycine, betaine, mannitol and ectoine, which previously shown to be involved in salt stress. At the same time, it induced the aconitase enzyme to produce water to protect itself from drying and it induced chaperonin too. The results of this analysis of *Rhizobium* strains resistant to heat, or pH9 can be used to improve the response to rhizobial inoculants in dry Egyptian ecosystems. In addition these results will help to understand how some rhizobial strains can survive under environmental stresses and of course will aid to establish a specific strategy to improve the productivity of legume crops in dry areas through gene transfer. Finally, the proteomic analysis of *Rhizobium* strains resistant to different environmental stresses provides information regarding protein functions relevant to the ongoing genome sequencing of these strains, and contributes to our knowledge of molecular mechanisms acquired by these strains to exist in these harsh conditions.

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REFERENCES

[1] Graham PH, Vance CP. F Crops Res 2000; 65:93-106.
[2] Danso SKA. Fert Res 1995; 42:33-41.
[3] Vance CP, Heichel GH, Phillips DA. 1988; Nodulation and symbiotic nitrogen fixation. In: Hanson, A. A., Barnes D. K. and Hill, R. R. (Eds), Alfalfa and Alfalfa Improvement. Madison: Am. Society Agron., p. 229-257.

[4] Shamseldin A, Werner D. Current Microbiol 2005; 50: 11-16.
[5] Shamseldin A. Biotechnol Lett 2007; 29: 37-44.
[6] Hatimi A. Plant Soil 1999; 216: 93-101.
[7] Cytryn EJ, Sangurdekar DP, Streeter JG, Franck WL, Chang WS, Stacey G, Emerich DW, Joshi T, Xu D, Sadowsky MJ. J Bacteriol 2007; 189 (19): 6751-6762.
[8] Carter JM, Gardner WK, Gibson AH. Aust J Agri Res 1994; 45: 613-623.
[9] Munns R, Tester M, Ann Rev Plant Biol 2008; 59: 651-681.
[10] Vriezen JAC, de Bruijn FJ, Nüsslein K. Appl Environ Microbiol 2007; 73: 3451-3459.
[11] Zahran HH. Microbiol Mol Biol Reviews 1999; 63:968-989.
[12] Dowling DN, Broughton WJ. Ann Review Microbiol 1986; 40: 131-157.
[13] Sadowsky JM. 2005; Soil stress factors influencing symbiont Nitrogen fixation, edited in (Nitrogen fixation in agriculture, forestry, ecology and the environment. Edited by Werener D and W. E. Newton, Springer Verlag.
[14] Kasinathan V, Wingler A. Physiol Plant 2004; 121: 101-107.
[15] Chien CT, Maundu J, Cavaness J, Dandurand LM, Orser CS. FEMS Microbiol Lett 1992; 90: 135-140.
[16] Saira, RK, Tyagi A. Current Sci 2004; 86: 407-421.
[17] Hu Y, Schmidhalter U. Func Plant Biol 1998; 25: 591-597.
[18] Asch F, Dingkuhn M, Wittstock C, Doerffling K. Plant Soil 1999; 207: 133-145.
[19] Shamseldin A, Moawad H, Abd El-Rahim WM, Sadowsky MJ. Syst Appl Microbiol 2014; 37: 121-128.
[20] Shamseldin A, Nyalwidhe J, Werner D. Current Microbiol 2006; 52: 333-339.
[21] Gomes DF, da Silva Batista JS, Schiavon AL, Andrade DS, Hungria M. BMC Microbiol 2012; 12: 84.
[22] Bradford M. Anal Biochem 1976; 72: 248-254.
[23] Beinert H, Kennedy MC, Stout CD. Chemical Rev 1996; **7**: 2335-2374.
[24] Yang H, Zhang D, Wang J, Wood AJ, Zhang Y. Mol Biol Reports 2011; 39: 2645-2652.
[25] Rodrigues SM, Andrade MO, Gomes AP, Damatta FM, Baracat-Pereira MC, Fontes EP. J Exp Botany 2006; 57: 1909-1918.
[26] Kotchoni SO, Bartels D. Bul J Plant Physiol 2003; Special issue, 37-51.
[27] Haines RJ, Pendleton LC, Eichler DC. Mol Biol 2001; 2: 8-23.
[28] Davis PK, Wu G. Comp Biochem Physiol Part B: Biochem Mol Biol 1998; 119; 527-537.
[29] Berg JM, Tymoczko JL, Stryer L. Biochemistry. 2006; W.H. Freeman. pp. 656-660. ISBN 978-0-7167-8724-2.
[30] Moshe A, Pfannstiel J, Brotman Y, Kolot M, Henryk C, Rena G. 2012; Metabolomics S:1 http://dx.doi.org/10.4172/2153-0769.S1-006.
[31] Peters P, Galinski EA, Truper HG. FEMS Microbiol Lett 1990; 71: 157-162.
[32] Corpas FJ, Barroso JB, Sandalio LM, Distefano S, Palma JM, Lupiáñez JA, Del Río LA. Biochem J 1998; 330: 777-784.
[33] Liu Y, Wu R, Wan Q, Xie G, Bi Y. Plant Cell Physiol 2007; 48: 511-522.
[34] Leterrier M, Barroso JB, Valderrama R, Palma JM, Corpas FJ. The Scient World J 2012; Article ID 694740, 9 pages doi:10.1100/694740.
[35] Rodrigues C, Laranjo M, Oliveira S, Current Microbiol 2006; 53: 1-7.
[36] Breedveld MW, Zevenhuizen LPTM, Zehnder AJB. J Gen Microbiol 1991; 136: 2511-2519.
[37] Nandal K, Sehrawat AR, Yadav AS, Vashishat RK, Boora KS. Microbiol Res 2005; 160: 367-373.
[38] Clark AJ, Blissett KJ, Oliver RP. Planta 2003; 216: 614-619.
[39] Jennings DH. Adv Microb Physiol 1994; 25: 149-193.
[40] Lin DX, Tang H, Wang ET, Chen WX. FEMS Microbiol Lett 2009; 293: 35-41.
[41] Wallington EJ, Lund PA. Microbiol 1994; 140: 113-122.
[42] Liang WD, Bi YT, Wang H, Dong S, Li KS, Li JS. 2013; BioMed Res Int http://dx.doi.org/10.1155/760904.
[43] Rajaram H, Apte KS. Microbiol 2008; 154: 317-325.
[44] Laranjo M, Oliveira S. Ant Van Leeuwen 2011; 99: 651-662.
[45] Ratcliff WC, Kadam SV, Dension RF. FEMS Microbiol Ecol 2008; 65: 391-399.
[46] Vanderlinde EM, Harrison JJ, Muszyński A, Carlson RW, Turner RJ, Yost CK. FEMS Microbiol Ecol 2010; 71: 327-340.
[47] Prieto MA, de Eugenio LI, Galan B, Luengo JM, Witholt B. 2007; Synthesis and degradation of polyhydroxyalkanoates. In: Ramos JL, Filloux A, editors. Pseudomonas: a model system in biology. New York Springer, pp. 397-428.
[48] Yoshida K, Takemoto Y, Sotsuka T, Tanaka K, Takenaka S. BMC Microbiol 2013; 13: 290.