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Molecular Level Stress Response in Rhizobia-Identification of Heat Shock Proteins

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A B S T R A C T

Heat shock proteins (Hsps) are equally well termed stress proteins, and their expression is termed the stress response. Assessing the heat shock protein at different temperature of 35, 40, 45 and 50º at pH 5.5, the four numbers of isolates were taken from each host species of *Rhizobium* (COS1, COG15, CO5, TNAU 14 and CRR6) and specific temperature of 35, 40, 45 and 50ºC respectively and compared with the control (reference culture maintained at 28ºC). Totally 20 number of isolates were taken and subjected for protein studies At 50°C, the protein content (0.92 mg ml^{-1} of cells) was higher but the strain CRR 6 and TNAU 14 had lower protein content of 0.72 and 0.81 mg ml^{-1} of cells respectively. Qualitative and quantitative differences in polypeptide patterns of rhizobial strains were detected after growth at 35, 40, 45 and 50°C when compared to the control (28°C) conditions. Mostly all the 20 temperature and acid tolerant rhizobial isolates revealed the synthesis of heat shock proteins at higher temperature. For example, rhizobial strains CO 5 and COG 15 revealed that the simultaneous overproduction of three polypeptides (60 / 36 / 43 kDa) when submitted to 35, 40 and 45°C but at 50°C only one polypeptide of molecular weight 60 kDa expressed.

Introduction

Molecular chaperones, including the heat-shock proteins (Hsps), are a ubiquitous feature of cells in which these proteins cope with stress-induced denaturation of other proteins. Not all heat shock proteins are stress-inducible, but those that are respond to a variety of stresses, including extremes of temperature, cellular energy depletion, extreme concentrations of ions, other osmolytes, gases, and various toxic substances. Activation of various intracellular signaling pathways results in heat shock protein expression. Lindquist (1986) and Ritossa (1996) reported that heat-shock proteins (Hsps) first achieved notoriety as gene products and its expression is induced by heat and other stresses. Loewen and Hengge - Aronis (1994) suggested that this stationary phase intrinsic resistance is dependent upon protein synthesis and Hsp are preferentially produced in nutrient starved *E.coli* during the first several hours of starvation and DnaK

(Hsp70 – heat inducible) has been reported to have an essential role in the thermotolerance and hydrogen peroxide resistance under these conditions (Rockbrand *et al.,* 1995).

Benov and Fridovich (1995) showed evidence that aerobic heat shock imposes an oxidative stress and it can induce a heat shock response. Gething (1997) reported that newly discovered proteins are now known to play diverse roles, even in unstressed cells, in successful folding, assembly, intracellular localization, secretion, regulation, and degradation of other proteins and failure of these activities is thought to underline numerous and important human diseases.

Arsene *et al.,* (2000) reported that in *E.coli*, the complex control system regulates the expression of heat shock genes where rpoH, which encodes the sigma 32 transcriptional factors and it played a major role.

Echave *et al.,* (2002) reported that the chaperone Dna K acts as a molecular shield of partially oxidatively damaged proteins. Gruber and Gross (2003) found that sigma factors are transcriptional initiation factors and it may recruit RNA polymerase to a particular class of promoters. Nollen and Morimoto (2002) found that heat shock proteins comprise chaperones, proteases and other stress related proteins that are not only important during stress conditions. Agents other than heat such as ethanol, cadmium chloride, antibiotics (such as novobiocin) and hydrogen peroxide induce the synthesis of heat shock proteins.

Guisbert *et al.,* (2004) observed that DnaK (Hsp70) and GroEL (Hsp60) as an additional feedback post translational control of sigma 32, where both Hsp bind to the sigma factor, preventing the transcription of heat shock genes. King and Ferenci (2005) reported divergent of sigma factor in *E. coli* under aerobic and anaerobic conditions. The heat shock response of *E.coli* K12 cells in the presence or absence of oxygen in an exponential or stationary phase of growth and on the oxidative stress response of this bacterium in the absence of oxygen. Winter *et al.,* (2005) studies shown that the oxygen tension with the heat shock response.

Role of heat shock proteins and its thermotolerrance to diazotrophic microorganisms

Parsell and Lindquist (1994) reported that the role of Hsps in the restoration of cellular and homeostasis and thermotolerance. The expression of the heat inducible Hsp70 (DnaK) has been shown to support the growth of *E.coli*, *Saccharomy cescerevisiae* and *Drosophila* and moderately high temperatures (40 - 42°C) although not at extreme temperatures (50°C) and above.

Yura *et al.,* (2000) observed the transient induction of heat shock proteins (Hsps) in response to temperature upshifts and it was seen both in prokaryotes and eukaryotes. Most heat shock proteins are synthesized even under normal growth conditions and play a fundamental role in cell physiology. The most abundance of the Hsps in *Escherichia coli* are either molecular chaperones like Dna K and groEL proteins or proteases like ClpB and Lon.

Hsp-inducing stress in nature and natural induction of heat shock proteins

Terrestrial temperature stress

A terrestrial environment often offer diverse heat sources and sinks which retreats that organisms to avoid thermal stress. Thus, natural thermal stress and accompanying Hsp expression in terrestrial environments typically involve limitations in mitigating

thermal extremes by movement and conflicts between thermoregulation and other needs. Plants should also be prone to natural cold stress (Morris *et al*., 1983), which ought to induce expression of heat shock proteins.

By inference, the entire range of plant heat - shock responses (Nagao *et al.*, 1990) should manifest themselves in nature. Indeed, a small number of case studies document natural Hsp expression (Nguyen *et al.*, 1994), which can be greatest at times of day or in regions of an individual plant at which temperatures are highest (Colombo *et al.*, 1995). Plant species can differ dramatically, however, in both the magnitude and diversity of the particular heat shock proteins that are expressed during days with especially warm weather (Hamilton *et al*., 1996).

Inducing stresses other than temperature

Every nonthermal stress can induce heat shock proteins. The resurrection plant, a desert species, expresses heat shock proteins in vegetative tissues during water stress; this expression is thought to contribute to desiccation tolerance (Alamillo *et al*., 1995). Similarly, rice seedlings express two proteins in the Hsp 90 family upon exposure to water stress and elevated salinity. Examples include variation in the expression of Hsp 70 and ubiquitin in the *Drosophila* central nervous system under anoxia (Ma and Haddad, 1997) and in protein expression during osmotic shock in isolated fish gill cells (Kultz, 1996).

Materials and Methods

Protein extraction

Protein extraction was done by following the method described by Saumya and Hemchick (1983).

To assess the heat shock protein at different temperature of 35, 40, 45 and 50º at pH 5.5, the four numbers of isolates were taken from each host species of *Rhizobium* and specific temperature of 35, 40, 45 and 50ºC respectively and compared with the control (reference culture maintained at 28ºC).Totally 20 number of isolates were taken and subjected for protein studies and the details were given (Table 1) below.

Tryptone yeast extract medium (Annexure I) was prepared at the pH range of 5.5 and sterilized. The specific high temperature (35, 40, 45 and 50ºC) and acid tolerant (pH 5.5) of the above given *Rhizobium* isolates were inoculated in different tubes and kept at room temperature in rotary shaker at 200 rpm. After 36 h growth, isolates were taken in five different test tubes and exposed to heat shock (35, 40, 45 and 50ºC) for a period of 3 hours (Cloutier *et al*., 1992).

Then the treated cultures were harvested by centrifugation (7000 rpm) and washed twice with buffered saline at pH 7.0 and centrifuged. Then the cells were suspended in 10 ml of ice-cold acetone, allowed to stand on ice for 5 min, and collected by centrifugation (7000 rpm). Residual acetone was removed by inverting the tube on tissue paper and the protein was extracted by incubating with 1.0 ml of 10% SDS for 2 min. The extracts were clarified by centrifugation (7000 rpm) and supernatants were used for protein estimation when compared with the reference culture maintained at 28ºC.

Estimation of cell protein

The protein content of the cell culture was determined using Lowry's method (Lowry *et al*., 1951).

Working standard

Dilute 10 ml of stock solution (50 mg BSA in 50 ml of water) to 50 ml with distilled water

in a standard flask. One ml of this solution contains 200 mg protein.

Estimation of protein

One ml of the sample was taken in a test tube and the volume was made up to 4.5 ml with distilled water. To each tube, five ml of reagent C (Annexure II) was added and allowed to stand for 10 min. Then 0.5 ml of reagent D (Annexure II) was added and mixed well. The intensity of blue color developed was read at 620 nm in spectrophotometer (ATIN A 2000z double beam) against appropriate blank. The protein content was calculated by referring to the standard curve prepared with Bovine Serum Albumin (BSA).

SDSPAGE

Solution and stocks

Acrylamide solution

Acrylamide – 29.2 g
Bisacrylamide - 0.8 g
Dissolve in 50 ml water and volume made upto 100ml with distilled water

Tris SDS, pH 8.8

Tris HCl 1.5 M, pH 8.8 with 0.4 per cent SDS. This buffer was used for casting separating gel for SDS PAGE

Tris SDS, pH 6.8

Tris HCl 1.5 M, pH 8.8 with 0.4 per cent SDS. This buffer was used for casting separating gel for SDS PAGE

Tris Glycine - SDS Buffer -10x

This buffer was used as electrode buffer for SDS PAGE. The buffer at 1 x concentration contains 0.025 M Tris, 0.192 M Glycine and 0.1 per cent SDS (approximately pH 8.5).

Sample buffer: 2x concentration

4% SDS
20% Glycerol
10% 2 - Mercaptoethanol
0.04% Bromophenol Blue
0.125 M Tris HCl, pH 8.8

Gel composition	Composition of 10 ml running gel			
	8%	10%	12%	**15%**
30% Acrylamide	2.7	3.3	4.0	**5.0**
1.5 M Tris pH 8.8, 0.4% SDS	2.5	2.5	2.5	**2.5**
De ionized water	9.7	4.1	3.4	**2.4**
10% APS	0.1	0.1	0.1	**0.1**
TEMED	**0.006**	**0.004**	**0.004**	**0.004**

Gel composition	Composition of 5% stacking gel	
	5 ml	**10 ml**
30% Acrylamide	0.83	**1.70**
1.5 M Tris pH 6.8, 0.8% SDS	0.63	**1.25**
De ionized water	3.45	**6.90**
10% APS	0.05	**0.10**
TEMED	**0.005**	**0.01**

Brilliant blue R stain 2 x concentration (staining solution)

0.25% - Brilliant blue
40.0% - Methanol
7.0 % - Acetic acid

Destaining solution

Methanol - 40 ml
Acetic acid -10 ml
Distilled water – 50 ml

The electrophoresis was carried out in a vertical unit in a continuous system using 12 per cent acrylamide gel. The gel plates were cleaned thoroughly with water followed by alcohol and acetone. The plates were sealed at the bottom and the sides. The separating gel was casted as per the details given above. APS and TEMED were used as polymerizing agent and were added in the separating and stacking gel before pouring the gel solution.

Separating gel was overlaid with a few ml of water. After polymerization, the water layer was removed and stacking gel was poured. Then placed the comb carefully on the top of the sandwich. After polymerization, the comb was removed carefully and the slots formation may occur. The slots were rinsed with electrode buffer before loading the samples.

All the samples were mixed with 1x loading buffer and were boiled for two min. and then carefully loaded into the gel slots. Medium molecular weight protein marker (Bangalore Genei Private Ltd.,) with marker sizes of (23 - 97 kda) was used as protein marker. Initially the gel was run at a constant current of 15 mA till the dye front reached the separating gel. Then the current was increased to a constant supply of 30 mA till the dye front reached the bottom of the gel. After the run, the gel unit was disassembled and gel was put immediately for overnight in staining solution. Gel was destained until the background becomes colorless and photographed.

Results and Discussion

Protein content

The protein estimation was done as per Lowry's method. The results revealed that the protein content of *Rhizobium* sp. was found increased, when the temperature enhanced from 35 to 50°C. Incase of *Rhizobium* sp. CO 5 and COG 15, the maximum protein content (0.92 mg ml $^{-1}$ of cells) was found to occur at 50°C. *Rhizobium* sp. (TNAU 14) showed the maximum protein content (0.90 mg ml^{-1} of cells) at 45°C followed by CO 5, COG 15, CRR 6 and COS 1. At 50°C, strain CRR 6 and TNAU 14 had lower protein content of 0.72 and 0.81 mg ml^{-1} of cells respectively (Table 2; Plate 1).

Particulars	SEd	CD (0.05%)
Strain	0.023	**0.046**
Temperature	0.023	**0.046**
Strain x Temperature	**0.051**	**0.103**

Polypeptide profiles of temperature and acid tolerant *Rhizobium* strains by SDS PAGE analysis

The whole cell protein concentration of the rhizobial strains was estimated according to the method of Lowry *et al.*, (1995). Bacteria were grown for 72 h and given heat shock for 3 h at specific temperature of 35, 40, 45 and 50°C, after which the same was for pelleted in Eppendorf tubes by centrifugation (5000 rpm). Polypeptides profile was made with reference to the protein marker ranged from 14.3 to 97 kDa. Polypeptide profiles of 20 temperature tolerant rhizobial isolates were obtained by electrophoresing the protein sample on 12 per cent polyacrylamide gel. Lanes 1- 6 represents medium molecular weight marker and isolates tolerant to 28, 35, 40, 45 and 50°C temperature.

The results revealed that these twenty temperature tolerant rhizobial isolates generated reproducible polypeptides profile. Qualitative and quantitative differences in polypeptide patterns of rhizobial strains were detected after growth at 35, 40, 45 and 50°C, when compared to the control conditions (28°C). However, the detected changes are distinct, depending on the isolates tested and growth conditions.

In different strains, polypeptides with the same molecular weight were overproduced under temperature stress. For example, rhizobial strains CO 5 (Plate 1) and COG 15 (Plate 2) revealed the simultaneous overproduction of three polypeptides (60, 43 and 36 kDa) when subjected to 35, 40 and 45°C, but at 50°C only one polypeptides of molecular weight 60 kDa expressed. Rhizobial strains COS 1 (Plate 1) and CRR 6 (Plate 2) revealed the simultaneous overproduction of two polypeptides (43 and 18 kDa) when subjected to 35, 40 and 45°C, but at 50°C only one polypeptides of molecular weight 43 kDa was recorded and CRR 6 *Rhizobium* strains (Plate 2) expressed 60 kDa polypeptides at 35, 40, and 45°C. *Rhizobium* strains TNAU 14 (Plate 3) expressed three polypeptides of molecular weight 43, 60, and 77 kDa when subjected to 35, 40 and 45°C but 43 kDa, the only polypeptides also expressed at 50°C (Table 3).

Table.1 List of rhizobial isolates taken for protein studies

S.No	Rhizobial isolates	Temperature	pH	No. of isolates	Name designated
1.	COS 1	35	5.5	1	**Sap1**
2		40	5.5	1	**Sbp1**
3.		45	5.5	1	**Scp1**
4.		50	5.5	1	**Sdp1**
5.	COG 15	35	5.5	1	**Gap1**
6.		40	5.5	1	**Gbp1**
S.No	**Rhizobial isolates**	**Temperature**	**pH**	**No. of isolates**	**Name designated**
7.		45	5.5	1	**Gcp1**
8.		50	5.5	1	**Gdp1**
9.	CO 5	35	5.5	1	**Bap1**
10.		40	5.5	1	**Bbp1**
11.		45	5.5	1	**Bcp1**
12.		50	5.5	1	**Bdp1**
13.	TNAU 14	35	5.5	1	**Gnap1**
14.		40	5.5	1	**Gnbp1**
15.		45	5.5	1	**Gncp1**
16.		50	5.5	1	**Gndp1**
17.	CRR 6	35	5.5	1	**Cap1**
18.		40	5.5	1	**Cbp1**
19.		45	5.5	1	**Ccp1**
20.		**50**	**5.5**	**1**	**Cdp1**

Table.2 Protein content of temperature (35 -50°C) and acid (pH 5.5) tolerant rhizobial strains

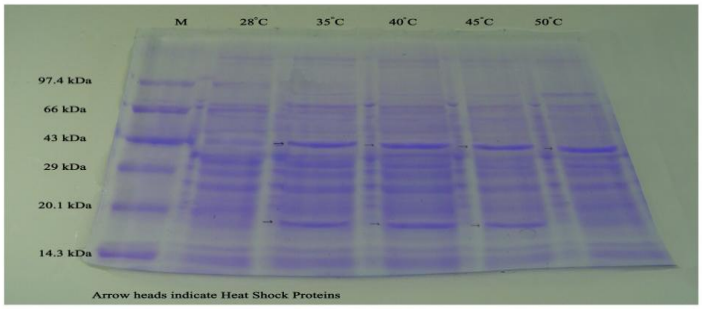
S.No.	Rhizobial strains	Protein content (mg ml^{-1} of cells)				
		28°C	35°C	40°C	45 °C	50°C
1	COS1	0.43	0.52	0.62	0.71	**0.82**
2	CO5	0.45	0.51	0.70	0.83	**0.92**
3.	COG15	0.50	0.63	0.72	0.80	**0.92**
4	TNAU14	0.63	0.74	0.85	0.90	**0.81**
5.	**CRR6**	**0.42**	**0.55**	**0.63**	**0.75**	**0.72**

Table.3 Molecular weight of polypeptides that were over produced under temperature stress (28 - 50°C) and acid (pH 5.5)

S.No	Rhizobial strains	Overproduced protein (kDa)				
		28°C	35°C	40°C	45°C	50°C
1.	CO 5	-	60/ 36/ 43	60/36/43	60/ 36/ 43	**43**
2.	COG 15	-	60/ 36/ 43	60/ 36/ 43	60/ 36/ 43	**43**
3.	TNAU 14	-	60/ 43/ 77	60/ 43/ 77	60/ 43/ 77	**43**
4.	CRR 6	-	60/ 43/ 18	60/ 43/ 18	60/ 43/ 18	**43**
5.	**COS 1**	-	**43/ 18**	**43/ 18**	**43/ 18**	**43**

Plate.1

Polypeptide profiles of temperature and acid tolerant *Rhizobium* sp. (COS 1)



Polypeptide profiles of temperature and acid tolerant *Rhizobium* sp. (CO 5)

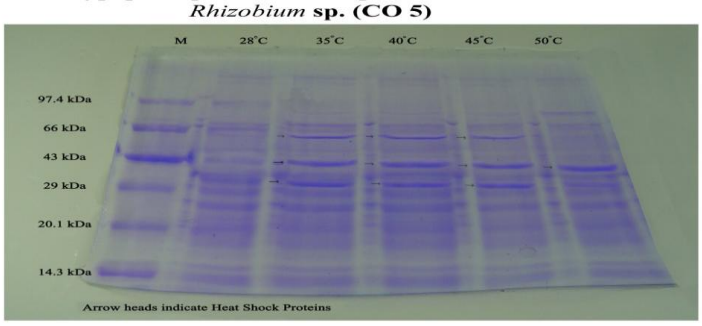
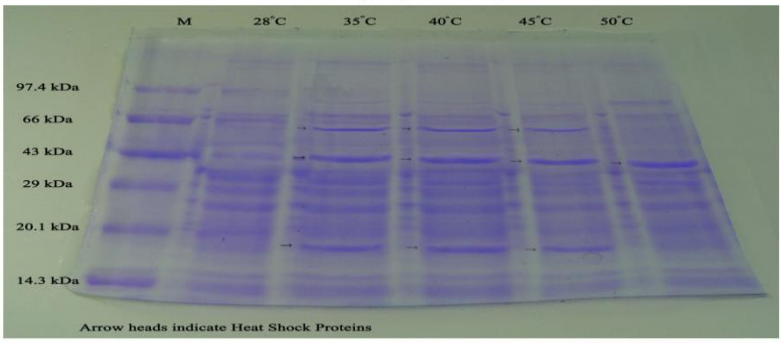


Plate.2

Polypeptide profiles of temperature and acid tolerant *Rhizobium* sp. (CRR 6)



Polypeptide profiles of temperature and acid tolerant *Rhizobium* sp. (COG 15)

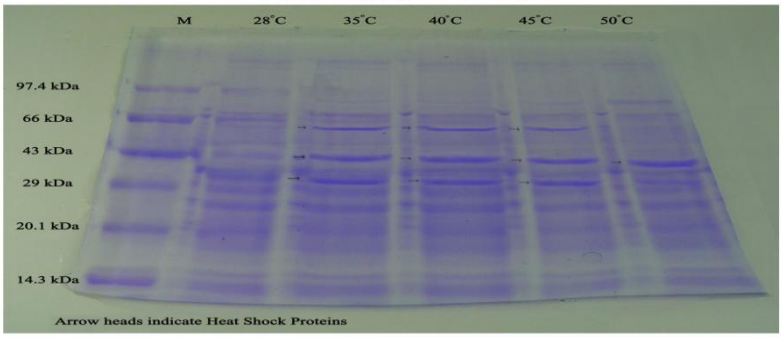
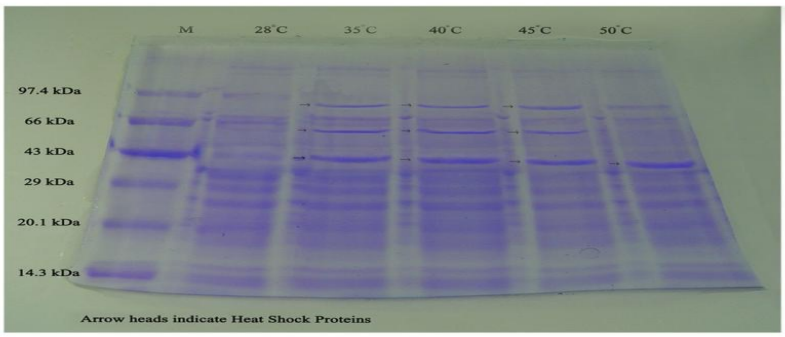


Plate.3

Protein profiles of temperature and acid tolerant *Rhizobium* sp. (TNAU 14)



The protein studies revealed that different species of *Rhizobium* showing different thermal adaptation characteristics to produce Heat Shock Proteins (HSP's) at temperatures outside their normal growth range.

Goldstein *et al.,* (1990), Jones *et al.,* (1987) and Mc Callum *et al.,* (1986) reported heat shock responses of microorganisms for a wide range of growth permissive temperatures and in a few cases, as with *E. coli*, at higher

temperature (Neidhardt *et al.*, 1984). In the present study, when HSP's were synthesized, three major polypeptides with molecular weights (43, 60, 36 kDa) were always present in all the five rhizobial strains, the 60 kDa polypeptides being the most abundant at 45 ^{o}C.

These results are consistent with the observations of Mc Callum *et al.*, (1986) who reported that 59.5 kDa polypeptides being the most abundant at higher temperatures (46.4 ^{o}C), also observed additional shock proteins whose synthesis was dependent upon the severity of the thermal shock.

In the present study, we observed that COG 15, CO 5, COS1, CRR 6 and TNAU 14 strains of *Rhizobium* expressed 60 kDa polypeptides at 35^{o}C to 50^{o}C temperature and pH 5.5 stress conditions. These results agree with the findings of Rodrigues *et al.*, (2006), who reported that tolerance to temperature and pH stress was evaluated by quantification of bacterial growth at 20 – 37^{o}C and pH 5-9, respectively. Tolerance to heat shock was studied by submitting isolates to 46^{o}C and 60^{o}C. They further reported that 60 kDa polypeptides were overproduced by all isolates under heat stress. Qualitative and quantitative differences in polypeptides patterns of rhizobial strains were detected by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis when isolates were subjected to temperature and pH stress.
Zahran *et al.*, (1994) studies revealed that rhizobia isolates subjected to temperature stress promoted the production of polypeptides of 65 kDa. Cloutier *et al.*, (1992) detected the over production of polypeptides with a similar molecular weight (59.5 kDa) for all heat shock treatments tested (29 – 46.4^{o}C), which apparently did not confer a greater tolerance to temperature stress.

Compared to normal growth conditions (28^{o}C), all the isolates synthesized heat shock proteins at 35^{o}C to 50^{o}C. These result confirms with the findings of Cloutier *et al.*, (1992), who suggested that the molecular weight of values of the polypeptides were over produced after the growth at 37^{o}C related with other studies of rhizobia. The results are corroborated with the earlier findings of Michiels *et al.*, (1994) who noted that the synthesis of heat shock proteins were observed in both heat tolerant and heat sensitive bean nodulating *Rhizobium* strains at different temperatures. The results lead to similar conclusion related with Rusanganwa *et al.*, (1992) suggested that the molecular weight of the polypeptides detected in the present study (60 kDa) as well as the observation that it is over produced upon stress conditions might suggest its identification as the heat shock protein GroEL. This protein is involved in *nif* gene regulation in *Bradyrhizobium japonicum* (Fischer *et al.*, 1993) and *Klebsiella pneumoniae* (Govezensky *et al.*, 1991).

Strains of COS1 and CRR 6 were overproduced polypeptides of molecular weight 18 kDa when subjected to 35^{o}C, 40^{o}C and 45^{o}C temperature stress conditions for 3 hours. These results coincide with Kishinevsky *et al.*, (1992) who observed that exposure of the bacteria (*Bradyrhizobium* sp.) to 40^{o}C for 4 hours resulted in the production of two heat shock proteins with molecular weights of approximately 17 kDa and 18 kDa. Krishnan and Pueppke (1999) observed that four heat shock proteins were produced by a strain of *Rhizobium fredii* and two were of similar molecular weights to those observed in this investigation but the other two were much larger (78 and 70 kDa). These findings were correlated with the present data that the strains TNAU 14, *Rhizobium* over expressed 77 kDa and other two polypeptides of molecular weights (43 and 60 kDa) were found. Also correlated with the findings of Nandal *et al.*, (2005) who reported that the heat shock protein (Hsp) of 63 – 74 kDa was

overproduced in all mutant strains of *Rhizobium* sp. (Cajanus) incubated under high temperature (43 ^{o}C) conditions.

Yamamori and Yura (1982) reported that the number of (HSP's) heat shock proteins found in all rhizobial strains under different shock temperatures was not related to their survival, even though there is evidence that heat shock response confers thermal resistance in *E. coli* cells. On the contrary, our experiments, although the survival was less than 1 per cent at 50^{o}C, the strains of rhizobia maintained polypeptides synthesis under this treatment. However we did not determine whether polypeptides synthesis was performed by all cells at the beginning of the treatment or by surviving cells throughout the shock. Usually, the thermostability of proteins in bacteria increases with optimum growth temperature of the species (Kogut and Russell, 1987). On the contrary, in our experiments, polypeptides synthesis is more tolerant to high temperature (37^{o}C to 50^{o}C) in temperate strains of rhizobia. Many hypotheses could explain the lack of induction of heat shock proteins (HSP's) in the temperate strains of rhizobia at 46.4^{o}C.

The present study revealed that all the strains of rhizobia expressed HSP's at 40 and 45^{o}C respectively. The results are postulated by Zahran (1994) that an increased synthesis of 14 heat shock proteins in heat-sensitive strains and of 6 heat shock proteins in heat tolerant strains was observed at 40 and 45^{o}C. They observed sudanese rhizobial protein with relative mobility of 65 kDa appeared during temperature (44.2 ^{o}C) stress.

The temperature stress consistently promoted the production of polypeptides with a relative mobility of 65 kDa in four strains of tree legume rhizobia. The 65 kDa polypeptides that were detected under heat stress were heavily over produced. These polypeptides were not over produced during salt or osmotic stress, which indicates that it is a specific response to heat stress.

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