Proteomic profiling of *Rhizobium tropici* PRF 81: identification of conserved and specific responses to heat stress

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Abstract

**Background:** *Rhizobium tropici* strain PRF 81 (= SEMIA 4080) has been used in commercial inoculants for application to common-bean crops in Brazil since 1998, due to its high efficiency in fixing nitrogen, competitiveness against indigenous rhizobial populations and capacity to adapt to stressful tropical conditions, representing a key alternative to application of N-fertilizers. The objective of our study was to obtain an overview of adaptive responses to heat stress of strain PRF 81, by analyzing differentially expressed proteins when the bacterium is grown at 28°C and 35°C.

**Results:** Two-dimensional gel electrophoresis (2DE) revealed up-regulation of fifty-nine spots that were identified by MALDI-TOF/TOF-TOF. Differentially expressed proteins were associated with the functional COG categories of metabolism, cellular processes and signaling, information storage and processing. Among the up-regulated proteins, we found some related to conserved heat responses, such as molecular chaperones DnaK and GroEL, and other related proteins, such as translation factors EF-Tu, EF-G, EF-Ts and IF2. Interestingly, several oxidative stress-responsive proteins were also up-regulated, and these results reveal the diversity of adaptation mechanisms presented by this thermotolerant strain, suggesting a cross-talk between heat and oxidative stresses.

**Conclusions:** Our data provide valuable protein-expression information relevant to the ongoing genome sequencing of strain PRF 81, and contributes to our still-poor knowledge of the molecular determinants of the thermotolerance exhibited by *R. tropici* species.

Background

In most agricultural soils, nitrogen (N) is the main limiting nutrient and, accordingly, it is often supplied to crops as chemical fertilizers. Significant losses of N-fertilizers occur either by leaching—resulting in eutrophication of rivers, lakes, aquifers— or by denitrification, contributing to global warming [1]. However, estimates indicate that up to 60% of the N needs of legume crops may be obtained from the biological nitrogen fixation (BNF) process [2,3], with significant economic benefits to farmers while mitigating environmental impacts.

Common bean (*Phaseolus vulgaris* L.) is the most important food legume in South and Central America and in East Africa. It can establish symbiotic relationships with a variety of described and still-to-be-described rhizobial species [4]. An important limitation to the BNF process involving common bean is the high genetic instability of the symbiotic plasmid of the rhizobial strains, as reported for *Rhizobium phaseoli* and *Rhizobium etli*. This instability has been attributed to genomic rearrangements, plasmid deletions and mutations, which are intensified under stressful conditions [5,6]. Abiotic stresses such as high soil temperatures, in addition to water deficit, salinity and soil acidity comprise the main factors causing genetic instability [7,8].

Among common-bean rhizobia, *Rhizobium tropici* is recognized for its tolerance of environmental stresses, including high temperatures [7-9]. Within this species,
strain PRF 81 (= SEMIA 4080) is known for the high capacity in fixing N₂, competitiveness against other rhizobia, and tolerance of environmental stresses; it has been used in commercial inoculants in Brazil since 1998 [10,11]. More information about the strain, including genetic characterization, is given elsewhere [10,12,13]. The strain is deposited at the “Diazotrophic and Plant Growth Promoting Bacteria Culture Collection” at Embrapa Soja (http://www.bmrc.lncc.br).

Mechanisms of response to stresses are usually highly conserved among bacterial species, and designed for rapid adaptation to environmental and metabolic changes. These conserved responses comprise the expression of molecular chaperones, such as DnaK (and its assistants DnaJ and GrpE), GroEL (and its assistant GroES), and also of small heat-shock proteins [14]. All are polypeptide-binding proteins implicated in protein folding, protein targeting to membranes, renaturation, and in the control of protein-protein interactions. In addition to conserved responses, some bacterial species also possess specific metabolic adaptations to stressful conditions.

Recently, a draft genome of R. tropici strain PRF 81 revealed several probable genes that may be related to its outstanding symbiotic and saprophytic abilities and also its adaptability to environmental stresses [12]; elucidation of the whole genome of the strain is now in progress (http://www.bnf.lncc.br). However, elucidating biological implications of a given genome requires understanding of gene expression; therefore, proteomic studies, complementary to the structural genome, are critical. Despite the ecological, evolutionary and economic importance of R. tropici, proteomic information about the species is scarce. In addition, the intriguing tolerance to high temperature of R. tropici strains is far from being understood. In this context, our objective with this study was to report a proteomic study of R. tropici strain PRF 81, focusing on the determination of adaptive responses to heat stress.

**Methods**

**Bacterial growth conditions**

*R. tropici* strain PRF 81 was pre-cultured in 10-mL aliquots of tryptone-yeast extract medium (TY), at 80 rpm and 28°C, in the dark. The pre-cultures were then transferred to Erlenmeyer flasks containing 200 mL of TY medium and bacteria were grown under two treatment conditions: control (28°C) and with heat stress (35°C). Cells were incubated until the exponential phase of growth was reached (optical density of 0.6 at 600 nm), what took approximately 18 h, with low agitation (80 rpm) to minimize the production of extra-cellular polysaccharides, which can interfere in 2-D gel electrophoresis.

**Total protein extraction**

 Cultures were centrifuged at 5,000 x g, at 4°C and cells were carefully washed with a solution containing 3 mM KCl; 1.5 mM KH₂PO₄; 68 mM NaCl; and 9 mM NaH₂PO₄. Washed cells were resuspended in 600 µL of a buffer containing 10 mM Tris–HCl pH 8.0; 1.5 mM MgCl₂; 10 mM KCl; 0.5 mM DTT; and 0.5 mM PMSF. Aliquots of 150 µL were stored in ultrafreezer (−80°C) until the analyses.

For whole-cell protein extraction, aliquots were resuspended in lysis buffer containing 9.5 M urea; 2% CHAPS; 0.8% v/v Pharmalyte 4–7; and 1% DTT, and submitted to forty cycles of freezing in liquid N₂ and thawing at 37°C, as described by Lery et al. [15]. The lysates were separated from particulate material by centrifugation at 14,000 x g for 90 min, at 4°C.

An additional step of concentration with phenol was done, increasing significantly the quality and reproducibility of the 2-D gels (data not shown). Aliquots (500 µL) of the lysates were homogenized with a solution containing 0.8 mL of Tris-buffered phenol pH 8.0, and 0.8 mL of SDS buffer (0.1 M Tris–HCl pH 8.0; 2% SDS; 5% β-mercaptoethanol; 30% sucrose; 1 mM phenylmethylsulfonyl fluoride, PMSF). The samples were homogenized for 5 min and centrifuged at 16,000 x g for 15 min at 4°C, and the top phenol layer (500 µL) was transferred to a new tube. Proteins were precipitated for 1 h at −20°C with three volumes of pre-cooled 0.1 M ammonium acetate in absolute methanol and then centrifuged (16,000 x g for 15 min at 4°C). The pellet was washed once with pre-cooled methanol and once with pre-cooled 80% v/v acetone, followed by drying.

The pellet was resuspended with the lysis buffer and concentration was determined by Bradford’s method [16].

**2-D electrophoresis and visualization**

For IEF, lysates were dissolved with DeStreak buffer (GE Healthcare) to a final concentration of 300 µg of protein and 2% v/v IPGphor in 250 µL of solution. IPG-strips (pH 4–7, 13 cm, GE Healthcare) were rehydrated with the protein solution and covered with cover fluid (GE Healthcare). Loaded strips were submitted to focalization in an Ettan IPGphor IEF system (GE Healthcare) for 1 h at 200 V, 1 h at 500 V, a gradient step to 1,000 V for 1 h, a gradient step to 8,000 V for 2 h 30 min, and fixed at 8,000 V for 1 h 30 min. The final Vh was fixed at 24,800. After focusing, strips were equilibrated first for 20 min in 5 mL of TE buffer (50 mM Tris–HCl pH 8.8; 6 M urea; 30% v/v glycerol; 2% w/v SDS; and 0.2% v/v of a 1% solution of bromophenol blue) supplemented with 50 mg DTT and then in TE buffer with 175 mg iodoacetamide, also for 20 min.

**2-D electrophoresis** was performed on a 12% polyacrylamide gel (18 x 16 cm) in a Ruby SE 600 vertical
electrophoresis system (GE Healthcare). The run was carried out for 30 min at 15 mA/gel and 240 min at 30 mA/gel, using the Low Molecular Weight Calibration Kit for SDS Electrophoresis (Amersham Biosciences) to provide standards. For each strain, the extraction procedure and gel electrophoresis were run in triplicates. Gels were fixed overnight with an ethanol-acetic acid solution before being stained with Coomassie Blue PhastGel™ R-350 (GE Healthcare) and scanned (ImageScanner LabScan v5.0).

Gel image analysis and spot selection
Spots were strictly identified in the high-resolution digitalized gel images and analyzed by Image Master 2D Platinum v 5.0 software (GE Healthcare). After background subtraction, ratios of mean normalized spot volumes were calculated and values of related spots were compared between both conditions. All selected spots exhibiting a higher volume in the heat stress condition were statistically evaluated \( p \leq 0.05 \) upon Student’s t-test, using XLSTAT (Addinsoft, France, add-in to Microsoft Excel).

Sample preparation and MALDI-TOF mass spectrometry
Protein spots showing significant changes in mean normalized volume were excised and processed as described by Chaves et al. [17]. Digestion was achieved with trypsin (Gold Mass Spectrometry Grade, Promega, Madison, WI), at 37°C, overnight.

Tryptic peptides (1 μL) were mixed with saturated solution of α-cyano-4-hydroxy-cinnamic acid (HCCA) in 50% acetonitrile, 0.1% trifluoroacetic acid (TFA). The mixture was spotted onto a MALDI (matrix assisted laser desorption ionization) sample plate and allowed to crystallize at room temperature. The same procedure was used for the standard peptide calibration mix (Bruker Daltonics). For mass spectra acquisition, a MALDI-TOF-MS (MALDI-time-of-flight in tandem) Autoflex Spectrometer (Bruker Daltonics) was operated in the reflector for MALDI-TOF peptide mass fingerprint (PMF) and in the “LIFT” mode for MALDI-TOF/TOF in the fully manual mode, using FlexControl 3.0 software.

In silico protein identification
PMFs and MS/MS ion spectra generated were searched against the public database NCBI (National Center for Biotechnology Information non-redundant), using Mascot software v. 2.3 (Matrix Science). For protein searches, performed in the Proteobacteria taxonomic group, monoisotopic masses were used, considering a peptide tolerance of 150 ppm and allowance of one missed cleavage. When MS/MS was carried out, a tolerance of 0.3 Da was acceptable. Carbamidomethylation of cysteine and oxidation of methionine were considered fixed and variable modifications, respectively.

Identifications were validated only when the Mowse (molecular weight search) score was significant, above the recommended cutoff of 52 for PMFs. Searches on the Decoy database were done in the automated mode in the Mascot software, using a random database (NCBI/Proteobacteria) strategy. Both decoy score and false discovery rates were considered for the validation of the searches of MS and MS/MS data and to measure the quality of the matches \( p \leq 0.05 \); using this approach false discovery rates were always less than 1%. The spectrometry datasets are available at PRIDE (http://pride.ebi.ac.uk/pride/) with the experiment accession number 14817.

Protein characterization
A set of bioinformatics tools was used for improved characterization of identified proteins. The proteins were fitted into COG (Clusters of Orthologous Groups) categories according to their functional inference, using the COGnitor program (http://www.ncbi.nih.gov/COG) [18]. Software packages PSORT-B [19] and PSLpred [20] were used for prediction of subcellular localization.

Results and discussion
2-D electrophoresis and differential spots selection
Several compounds, such as salts, nucleic acids and polysaccharides, may interfere with electrophoretic separation, resulting in streaky 2-D patterns, and thus should be removed. R. tropici PRF 81 produces high amounts of exopolysaccharides (EPS) in vitro and interference with electrophoretic resolution was overcome with a final wash step of the whole protein extract with phenol. In addition, to improve separation resolution, we employed IPG strips with a pH range of 4.0 to 7.0 in the first-dimension electrophoresis, to achieve better protein resolution than with broader-range (pH 3.0 to 10.0) strips (data not shown), in which the proteins remained concentrated in the central part of the gel (pH 5.0 to 7.0).

Using the computer-assisted gel-image analysis software, the majority of the molecular masses associated with the spots ranged between 14 and 97 kDa (Figure 1). The volume of each spot was normalized as a percent of the total volume of all detected spots in the gel. This procedure was followed for all gels and the values generated for each spot were compared between the control (28°C) and the experimental (35°C) treatment, and only well-defined spots present in the three replicates and showing statistically significant differences \( p \leq 0.05 \) were selected. A significant difference in several relative spot volumes indicated that the elevated temperature led to changes in the proteome of R. tropici PRF 81.
General proteome response to heat stress

Maximum soil temperatures in tropical soils can often exceed 40°C. Optimal temperature of growth of *R. tropici* species is around 28°C, and although there are reports of tolerance of PRF 81 to 40°C [9,10], our preliminary tests have shown that 35°C was the highest temperature that did not affect substantially growth; under higher temperatures, the slower growth rate had critical effects on the proteomic profile (data not shown). Joszefczuk *et al.* [21] also reported, in a heat stress response experiment with *Escherichia coli*, that one of the most striking features was the strong influence of high temperatures on the bacterium growth. In addition, contrasting with the majority of the studies about heat stress only with a short period of growth at high temperatures, our study considered a heat stress for the whole period of PRF 81 growth.

In comparison to other common-bean rhizobial species, *R. tropici* is known for its genetic stability and adaptation to stressful conditions [8,9], and, although PRF 81 is an outstanding strain in terms of these properties [10,11,13], little is known of the molecular determinants of its heat tolerance. In order to obtain an overview of the heat responses, we analyzed the cytoplasmic and periplasmic contents and identified the whole-cell protein expression changes when the cells were grown at 35°C. Fifty-nine significantly induced proteins were identified by mass spectrometry, and twenty-six of them were detected exclusively under heat stress conditions. All identified proteins were distributed across fifteen COG functional categories; six fit into the category of general prediction (R), one was classified in the category of unknown function (S) and only one was assigned as “not in COG” (Table 1).

Among the differentially expressed proteins, twenty-five were related to metabolic functions, the majority of them associated with amino acid transport and metabolism (group E) (Table 1), corroborating the proteomic reference map of *Bradyrhizobium japonicum* strain CPAC 15, a microsymbiont of soybean [22], and indicating high metabolic activity even under stressful conditions.

Also within this category, it is worth mentioning that NocP, an opine permease ATP-binding protein, was differentially expressed under high temperature. Opine is a compound released by crown-gall tumors produced by *Agrobacterium (=Rhizobium*) [23], and genes related to its metabolism were detected in the draft genome of PRF 81 and now confirmed at the translational level in our study. Putative genes related to rhizopine metabolism (an opine-like compound) were reported in *R. tropici* for the first time by our research group [12]. The ability to catabolize rhizopine appears to enhance the rate at which a strain is able to form nodules when it is in competition with a strain that is unable to catabolize a rhizopine. The mechanism responsible for this enhanced symbiotic ability is still unclear [24]. Moreover, we were unable to establish an exact association between stressful conditions and opine-like compounds metabolism, but our results presented an indicative of this correlation.

In relation to cellular processes and signaling, thirteen proteins were identified in categories D, T, O, M and N.
| Spot ID | NCBI ID | Gene   | Protein description                           | Organism (best match)                  | T/E 1 | T/E 1 mass (Da) | Fold change ratio | p-value | Cellular location          |
|---------|---------|--------|---------------------------------------------|----------------------------------------|--------|----------------|--------------------|---------|---------------------------|
| 1       | gi|46909738 | icd   | Isocitrate dehydrogenase                    | Rhizobium leguminosarum                | 5.9/5.96 | 45320/49000    | 1.10               | -       | Cytoplasmic               |
| 2       | gi|222087461 | sucC  | Succinyl-coa synthetase beta subunit protein | Agrobacterium radiobacter              | 4.98/4.96 | 42028/46000    | 3.27 ± 0.12        | 0.001   | Cytoplasmic               |
| 3       | gi|8639524   | acnA  | Acconitate hydratase                        | Rhizobium etli                         | 5.48/5.69 | 97180/98000    | 1.65 ± 0.06        | 0.001   | Cytoplasmic               |
| 4       | gi|116254139 | atpD  | F0F1 ATP synthase beta subunit              | Rhizobium leguminosarum                | 5.03/4.88 | 50885/56000    | 2.68 ± 0.03        | 0.001   | Cytoplasmic               |
| 5       | gi|1245379   | glnA  | Glutamine synthetase I                      | Sinorhizobium meliloti                 | 5.2/5.33  | 52287/61000    | 2.92 ± 0.08        | 0.001   | Cytoplasmic               |
| 6       | gi|15887731  | argB  | Acetylglutamate kinase                      | Agrobacterium tumaeicis               | 5.16/5.41  | 31083/90000    | 2.19 ± 0.09        | 0.001   | Cytoplasmic               |
| 7       | gi|89258357  | Putative periplasmic substrate binding protein | Ochrobacterium anthropi              | 5.84/5.78  | 28188/24000    | 1.00               | -       | Periplasmic               |
| 8       | gi|222109054 | nocP  | Opine permease ATP-binding protein          | Agrobacterium radiobacter              | 6.98/5.22  | 28288/20000    | 1.10               | -       | Inner Membrane             |
| 9       | gi|222087066 | pepF  | Oligoendopeptidase F protein                | Agrobacterium radiobacter              | 5.32/5.33  | 69899/76000    | 1.10               | -       | Cytoplasmic               |
| 10      | gi|222087908 | asd   | Aspartate-B-semialdehyde dehydrogenase protein | Agrobacterium radiobacter              | 5.46/5.59  | 37925/45000    | 1.38 ± 0.043       | 0.001   | Cytoplasmic               |
| 11      | gi|222084726 | argD  | Diaminobutyrate–pyruvate aminotransferase protein | Agrobacterium radiobacter              | 5.63/6.35  | 42909/43000    | 1.10               | -       | Cytoplasmic               |
| 12      | gi|114765810 | lVE   | Branched-chain amino acid aminotransferase | Pelagibaca bermudensis                 | 5.31/5.68  | 32142/35000    | 1.10               | -       | Cytoplasmic               |
| 13      | gi|861468888 | pyrH  | Uridylate Kinase                            | Vibrio sp.                             | 5.08/5.82  | 26284/33000    | 1.38 ± 0.13        | 0.008   | Cytoplasmic               |
| 14      | gi|222088374 | eno   | Phosphopyruvate hydratase                   | Agrobacterium radiobacter              | 4.84/4.95  | 45120/53000    | 2.88 ± 0.37        | 0.005   | Cytoplasmic               |
| 15      | gi|282887091 | Alpha amyrase catalytic region              | Burkholderia sp.                      | 6.26/5.03  | 64245/34000    | 1.10               | -       | Cytoplasmic               |
| 16      | gi|24120646 | Transaldolase                              | Rhizobium leguminosarum                | 5.32/6.12  | 35091/29000    | 1.10               | -       | Cytoplasmic               |
| 17      | gi|11493200  | pgm   | Phosphoglucomutase                          | Rhizobium tropici                      | 5.16/5.38  | 58641/72000    | 1.10               | -       | Cytoplasmic               |
| 18      | gi|222084905 | aglA  | Alpha-glucosidase protein                   | Agrobacterium radiobacter              | 4.84/4.86  | 62592/65000    | 1.10               | -       | Cytoplasmic               |
| 19      | gi|222086485 | ABC transporter                            | Agrobacterium radiobacter              | 5.23/5.21  | 38975/42000    | 1.70 ± 0.09        | 0.001   | Periplasmic               |
| 20      | gi|296105270 | Biotin protein ligase                      | Enterobacter cloacae                   | 5.23/5.42  | 35255/28000    | 3.98 ± 0.24        | 0.001   | Cytoplasmic               |
| 21      | gi|299768808 | AcyC   | Acyl-Coa dehydrogenase                      | Agrobacterium tumaeicis               | 5.37/4.66  | 65994/40000    | 1.10               | -       | Cytoplasmic               |
| 22      | gi|282888281 | 3-Oxoacyl-(acyl-carrier-protein (ACP)) synthase III domain protein | Burkholderia sp.                      | 6.27/5.74  | 38552/35000    | 1.10               | -       | Cytoplasmic               |
| 23      | gi|159186213 | pcaf  | Beta-ketoacyl-CoA thiolase                  | Agrobacterium tumaeicis               | 5.51/6.37  | 41850/46000    | 2.95 ± 0.07        | 0.001   | Cytoplasmic               |
| 24      | gi|222087891 | bfr   | Bacterioferritin                           | Agrobacterium radiobacter              | 4.81/4.94  | 16860/19000    | 2.27 ± 0.07        | 0.001   | Cytoplasmic               |
| 25      | gi|87199081 | Tnb-dependent receptor                     | Novosphingobium aromaticivorans       | 5.82/5.01  | 87810/75000    | 1.10               | -       | Extra Cellular            |
| Cellular processes and signaling | Information storage and processing |
|--------------------------------|----------------------------------|
| **D - Cell cycle control, cell division, chromosome partitioning** | **J - Translation, ribosomal structure and biogenesis** |
| 26 gi|222086436  ftzZ2  Cell division protein  Agrobacterium radiobacter  5.21/5.39  63014/81000  2.42 ± 0.26  0.003  Cytoplasmic | 39 gi|222085858  tsf  Translation elongation factor Ts  Agrobacterium radiobacter  5.15/5.14  32268/40000  1.86 ± 0.02  0.001  Cytoplasmic |
| 27 gi|50121473  kicB  Condesin subunit F  Pectobacterium atrosepticum  4.7/4.78  50717/57000  11.00  -  Cytoplasmic | 40 gi|227821753  fusA  Elongation factor G  Rhizobium sp.  5.17/5.3  77968/89000  1.98 ± 0.13  0.001  Cytoplasmic |
| 28 gl|17926246  Protein tyrosine phosphatase  Magnetococcus sp.  6.29/5.28  18731/19000  11.00  -  Cytoplasmic | 41 gi|86355771  pnp  Polynucleotide phosphorylase/polyadenylase  Rhizobium etli  5.26/5.66  84589/90000  11.00  -  Cytoplasmic |
| 29 gl|222087232  prkA  Serine protein kinase protein  Agrobacterium radiobacter  5.42/5.69  74417/84000  2.41 ± 0.19  0.001  Cytoplasmic | 42 gi|218672404  tufB1  Elongation factor EF-Tu protein  Xanthomonas fuscans  5.89/5.79  83626/75000  1.29 ± 0.09  0.003  Cytoplasmic |
| 30 gi|116252038  ntrX  Nitrogen assimilation regulatory protein  Rhizobium leguminosarum  9.15/5.66  30427/34000  11.00  -  Cytoplasmic | 43 gi|218672404  tufB1  Elongation factor EF-Tu protein  Xanthomonas fuscans  5.89/5.79  83626/75000  1.29 ± 0.09  0.003  Cytoplasmic |
| 31 gi|159184131  chyl  Two component response regulator  Agrobacterium tumefaciens  5.56/5.85  27253/30000  1.35 ± 0.10  0.003  Cytoplasmic | 44 gi|89056301  LysR family transcriptional regulator  Jannaschia sp.  5.57/4.58  32077/28000  11.00  -  Cytoplasmic |
| 32 gi|222087564  trxA  Thioredoxin  Agrobacterium radiobacter  4.83/4.85  34469/39000  11.00  -  Cytoplasmic | 45 gi|159184760  AraC family transcriptional regulator  Agrobacterium tumefaciens  7.11/5.67  27409/25000  11.00  -  Cytoplasmic |
| 33 gi|118590060  bsp  Bacterioferritin comigratory protein  Stappia aggregata  5.63/5.3  16749/22000  3.40 ± 0.26  0.001  Cytoplasmic | 46 gi|222081230  Transcriptional regulator protein  Agrobacterium radiobacter  6.38/5.6  98220/98000  4.71 ± 0.09  0.001  Cytoplasmic |
| 34 gi|58826564  dnaK  Dnak  Rhizobium tropici  4.91/5.3  68393/74000  11.00  -  Cytoplasmic | 47 gi|190895600  Probable transcriptional  Rhizobium etli  6.91/5.42  42937/85000  11.00  -  Cytoplasmic |
| 35 gi|222085003  groEL  Chaperonin GroEL  Agrobacterium radiobacter  5.03/5.11  57836/69000  1.36 ± 0.19  0.012  Cytoplasmic | 48 gi|222106418  Transcriptional regulator GntR family  Agrobacterium vitis  5.82/5.78  26596/49000  11.00  -  Cytoplasmic |
| 36 gi|86359655  Putative metalloendopeptidase protein  Rhizobium etli  5.36/4.89  49514/29000  1.31 ± 0.22  0.02  Periplasmic | 49 gi|222106466  Transcriptional regulator ROK family  Agrobacterium vitis  7.03/5.14  41156/42000  11.00  -  Cytoplasmic |
| 37 gi|222085864  omp1  Outer membrane lipoprotein  Agrobacterium radiobacter  5.26/5.66  84589/90000  11.00  -  Cytoplasmic | 50 gi|222082875  Transcriptional regulator, MarR family  Agrobacterium radiobacter  5.46/5.57  18141/20000  11.00  -  Cytoplasmic |
Table 1 Identified proteins of *Rhizobium tropici* PRF 81 whole cell extracts up-regulated after growth at high temperature (35°C) (Continued)

|   | Description | Organism | Experimental/ Theoretical Value | Matched Peptides Masses | Fold Change | p-value | Location |
|---|-------------|----------|---------------------------------|--------------------------|-------------|---------|----------|
| L - Replication, recombination and repair | ATP-dependent RNA helicase protein | *Agrobacterium radiobacter* | 9.17/5.36 | 69955/67000 | 2.29 ± 0.14 | 0.001 | Cytoplasmic |
| Poorly characterized | FeS assembly ATPase SufC | *Agrobacterium radiobacter* | 5.08/4.95 | 27375/32000 | 11.00 | - | Inner Membrane |
| R - General function prediction only | Chloride peroxidase protein | *Agrobacterium radiobacter* | 7.88/6.37 | 34965/32000 | 1.59 ± 0.02 | 0.001 | Periplasmic |
| | Flavoprotein WrbA | *Burkholderia phymatum* | 6.19/5.91 | 20930/26000 | 2.58 ± 0.14 | 0.001 | Cytoplasmic |
| | NADPH-dependent FMN reductase | *Burkholderia ambibiana* | 6.71/6.31 | 8539/17000 | 2.03 ± 0.19 | 0.002 | Periplasmic |
| | 2,5-diketo-D-gluconic acid reductase A | *Shigella dysenteriae* | 6.22/5.15 | 19399/23000 | 1.34 ± 0.21 | 0.002 | Cytoplasmic |
| | Ferredoxin reductase protein | *Agrobacterium radiobacter* | 5.88/5.65 | 43777/53000 | 1.48 ± 0.12 | 0.003 | Cytoplasmic |
| S - Function Unknown | Hypothetical protein Avl_3814 | *Agrobacterium vitis* | 5.03/5.01 | 24632/29000 | 1.42 ± 0.34 | 0.033 | Periplasmic |
| NO related COG | Hypothetical protein Rlrg2_5527 | *Rhizobium leguminosarum* | 6.02/5.89 | 33584/44000 | 1.57 ± 0.13 | 0.002 | Cytoplasmic |

1 Theoretical/Experimental values. Da: Daltons.
2 1.00 in the fold change ratio means that the protein was only identified in the experimental condition (35°C).
Matched peptides masses and MS/MS combined results are available in PRIDE (http://ebi.ac.uk/pride/) under the experiment accession number 14817.
(Table 1). Two of these proteins are known to be correlated with heat tolerance, DnaK and GroEL. Molecular chaperones [12,25]. Two proteins also found in this group were thioredoxin TrxA and bacterioferritin comigratory proteins (Bcp), which have been characterized as oxidative-stress responsive.

Still considering the COG classification, thirteen induced proteins comprised a set related to information storage and processing (Table 1), including transcription regulators and translation factors. The translation factors can act as chaperones in response to heat stress, and more details of this function are discussed below.

Interesting was also the differential expression of VirD4, a TraG-like protein that plays an important role in conjugal transfer showing high similarity to Agrobacterium, and also reported in the draft genome of strain PRF 81 [13]. The transcription of the vir regulon in Agrobacterium tumefaciens is induced by specific plant-phenolic compounds, but also by several other abiotic stimuli, such as low pH and temperatures below 30°C [26]. VirD4 acts in the translocation of effector proteins and has been associated with different plant-bacterium interactions, both pathogenic and symbiotic. Also, VirD4 acts in couple DNA processing and transference by conjugation mechanism. Therefore, this protein has a broader role than the action in type IV secretion system. An association between heat stress and type IV secretion system components was described by Zahri et al. [27], since the expression of type IV secretion system in a modified E. coli induced heat shock genes.

Differential expression of the two-component response regulators (NtrX and ChvI)

Two-component systems are composed by a sensor kinase protein that transmits the environmental stimulus to a response regulator protein via phosphorylation. The phosphorylated regulator modulates the expression of the target genes required for the appropriate changes, mediating rapid metabolic responses for adaptation to new conditions [28]. Interestingly, these two up-regulated proteins in our study (NtrX and ChvI) are the response-regulator components.

NtrX has also been found to be expressed in Gluconacetobacter diazotrophicus [29], Sinorhizobium (=Ensifer) meliloti [30], and Mesorhizobium loti [31]. This protein is recognized to be involved in N metabolism and nitrogen fixation, probably acting as a transcriptional activator of genes related to nitrate metabolism [32,33].

The second two-component system, ChvI, characterized in several bacteria such as S. meliloti [34] and A. tumefaciens [35], acts in translation regulation of enzymes related to the biosynthesis of the succinoglycan exopolysaccharide (EPSI). In addition to this role, this two-component system signaling is critical for the viability of free-living S. meliloti strains [36], by acting in biofilm formation, motility, nutrient utilization and cell protection [37-39]. It has been reported that the succinoglycan may form a diffusion barrier, protecting against oxidative stress [40], suggesting that, in R. tropici PRF 81, in addition to participating in symbiosis signaling, the succinoglycan EPSI plays an important role in heat-stress protection.

Induced molecular chaperones DnaK and GroEL

Temperature is especially harmful to cells because it can damage the structure of macromolecules. Many of the molecular chaperones—such as DnaK and GroEL—are highly conserved in evolution [41], preventing and repairing harmful effects. As reported in other proteomic studies [42-44], DnaK and GroEL were significantly induced in PRF 81 at high temperature.

DnaK is classified according to its molecular weight in the Hsp70 chaperone group, the most versatile chaperone system. In addition to a main role in de novo folding, DnaK has various other functions, including protein transport [45], and in the increased stability of RNA polymerase σ32 factor (RpoH), an important component of the heat-shock response in several organisms [46-49].

At optimal temperature, σ32 factor is rapidly degraded, but if temperature is raised, σ32 stability increases due to its interaction with DnaK chaperone [50]. Therefore, in response to a sudden increase in temperature, the levels of σ32 in the cell rise, leading to the regulation of transcription of genes encoding other heat-shock proteins, which also contribute to heat tolerance [51].

As described for E. coli [52], Bacillus cereus [53] and Acinetobacter baumannii [54], in R. tropici PRF 81 the molecular chaperone GroEL was up-regulated under high temperature. The differential expression of GroEL is critical to thermotolerance, since the chaperone can routinely rescue more than 80% of a denatured protein population [55]. Essentially, GroEL modulates its affinity for folding intermediates through the binding and hydrolysis of ATP; and the highly coordinated binding and releasing of substrate proteins may lead to recovery of the functional state of the proteins [56].

Induction of chaperone-like proteins: Translation factors

Besides the main function of ensuring gene expression accuracy by transporting the correct codons in the translation process, elongation and initiation factors can also act as chaperones in response to heat stress [57,58]. In our study, three elongation factors (EF-Tu, EF-G and EF-1α) and one initiation factor (IF-2) were up-regulated when R. tropici PRF 81 was grown at 35°C (Table 1), indicating the probable involvement of these factors in
protein folding and protection, contributing to the thermostolerance of PRF 81.

EF-Tu is highly homologous to cellular GTP-proteins, occupying a key position in translation [59]. EF-Tu interacts with GTP, aminoacyl-tRNA, ribosomes, and a second factor, EF-Ts, which mediates GDP/GTP exchange on EF-Tu. In addition, Hendrick, and Hartl [60] observed that EF-Tu protein may also act as a molecular chaperone, protecting proteins against thermal damage. Studying heat responses, Jacobson and Rosenbuch [61] reported that large quantities of EF-Tu molecules in cells might constitute a reservoir of chaperone-like molecules that prevent the aggregation of non-native proteins until permissive renaturation conditions are restored. The shift of the activities of transport of aminoacyl-tRNA to the aminoacyl ribosome site and as chaperone of EF-Tu is dependent on the binding of this factor with GTP or GDP.

Considering the efficiency of chaperone activity, [57] showed that the elongation factor EF-Tu when bonded with GDP had greater capacity of stimulating renaturation of enzymes than when interacting with GTP. In contrast, Kudlicki and collaborators [62] found that EF-Tu bonded with GDP is less active than when it is bonded with GTP in catalyzing protein renaturation. Still, in that study, the authors reported that the EF-Ts elongation factor plays a similar role as GTP, suggesting that in the presence of these cofactors—EF-Ts or GTP—EF-Tu can perform several rounds of protein renaturation. These divergent studies indicate that the EF-Tu chaperonin activity is dependent on the specific protein in which the protection will be promoted. Interestingly, in our study, both elongation factors—EF-Tu and EF-Ts—were up-regulated under heat stress.

Both the elongation factor EF-G and the initiation factor IF2 were also found to act as chaperone proteins [58]. These factors are involved in the translocation of ribosomes on mRNA and in the binding of initiator tRNA to the 30 S ribosomal subunit, respectively [63]. EF-G bound to GDP, instead of to GTP, seems to be more active in the formation of stable complexes with unfolded proteins, assisting in protein folding and renaturation [52]. Finally, the chaperone properties of EF-Tu, EF-G, and IF2 suggest that translation factors are ancestral protein-folding factors that appeared before chaperones and protein-disulfide isomerases [58].

Cross-talk between heat and oxidative stress
Reactive oxygen species (ROS) are by-products of normal metabolic processes, but at high levels may be lethal for cells. However, in both symbiotic and pathogenic relations, transient production of ROS, detected in the early events of plant-microorganism interactions, may be considered as specific signals during the interaction process [64]. Previous studies have reported the accumulation of ROS in early stages of Rhizobium/legumes symbiosis establishment [65-67]. Therefore, the ability of the bacteria to tolerate and overcome the changes in the environment induced by the plant host seems to be important for the establishment of a successful symbiotic interaction [68].

To detoxify ROS, symbiotic bacteria display a multiple antioxidant defense that is required for both the development and the functioning of the symbiosis [69]. Fernando et al. [70] showed participation of thioredoxin (TrxA) in the reactivation of proteins damaged by oxidative stress, or by other conditions that cause the generation of ROS. These compounds cause covalent modifications in proteins, for example the oxidation of free sulfydryl groups (-SH), forming disulfide bonds (S-S). In this case, thioredoxin transfers reducing power to damaged proteins, restoring their reduced state [71]. Finally, thioredoxin was synthesized under high-temperature conditions, confirming its induction as a general response to stress [72]; it is also induced in the early stages of symbiotic interaction in B. japonicum [73] and in the plant interaction with G. diazotrophicus [74].

Both bacterioferritin (Bfr), a protein related to inorganic ion transport, and bacterioferritin comigratory protein (Bcp), a peroxiredoxin protein, were up-regulated in our study. These proteins have been related to oxidative stress responses, similarly to thioredoxin. The former (Bfr) acts indirectly in defense mechanisms against oxidative damage effects inside the cell, since it transports inorganic ions, for example Fe^{2+}, resulting in the decomposition of the peroxides over-produced during the oxidative stress [70]. The latter (Bcp) has a protective role in the defensive response to oxidative stress, possibly via up-regulation of total and reduced glutathione levels [75].

In Salmonella typhimurium, the oxidative stress caused by hydrogen peroxide treatment led to the induction of heat shock proteins such as DnaK, while the heat stress induced proteins related with cell protection against the oxidative stress [76]. Interestingly, when Lenco et al. [77] studied oxidative stress responses from a proteomic perspective, they observed the induction of several heat-responsive proteins, such as GroEL and GroES, as a reflection of regulation of heat-shock protein biosynthesis during bacterial oxidative stress.

We found up-regulation of several proteins responsive to oxidative stress, such as isocitrate dehydrogenase, which plays a key role in NADPH recycling under oxidative stress [78-80], also the flavoprotein WrBA, a quinone oxidoreductase with redox activity [80,81], among others. These results, added to others reporting the expression of heat responsive proteins during the oxidative
stress, suggest a cross-talk between heat stress and oxidative stress responses.

Conclusions
Although most of the proteins involved in responses to heat are highly conserved, the regulatory mechanisms vary among bacterial species. In our study, we have shown differential expression of some conserved heat-responsive proteins, such as DnaK and GroEL. However, we have also reported the up-regulation of proteins involved in a variety of metabolic pathways, including translation factors and oxidative stress-responsive proteins, indicating that the responses of \textit{R. tropici} strain PRF 81 to heat stress go beyond the induction of heat-shock proteins.

Strain PRF 81 is known for its high efficiency in fixing nitrogen, and in our study two component response regulators (NtrX and ChvI) were induced during the heat stress. These proteins act in the regulation of the nitrogen-fixation-gene expression and in the regulation of the succinoglycan exopolysaccharide (EPSI) production, respectively, showing that, even under stress conditions, PRF 81 retains nitrogen-fixing and symbiosis-establishment potential, which are essential characteristics for agricultural inoculants.

Finally, this proteome experiment provides valuable protein-expression information relevant to the ongoing genome sequencing of strain PRF 81 (http://www.bnf.incc.br), and contributes to our still-poor knowledge of the molecular determinants of the thermotolerance exhibited by \textit{R. tropici} species. It is a useful reminder that \textit{R. tropici} is an important species of agronomic interest for its capacity to fix nitrogen under tropical stressful conditions, and also demonstrates high resemblance in many genes, and —now also confirmed in many proteins—to those in pathogenic strains of the genus \textit{Agrobacterium}. 

Additional file

Additional file 1: Table S1. Information about mass spectrometry identification of differentially expressed proteins. All the information contained in Table S1 were obtained for differentially expressed proteins by Mascot (Matrix Science) searches against the public database NCBI nr. These spectrometry datasets are also available at PRIDE (http://pride.mpimp-golm.mpg.de) with the experiment accession number 14817.

Acknowledgments
The work was partially supported by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil) and FAPESP (57/933/2008) and FAP-Repensa (562009/2010-1). MALDI-TOF was acquired with resources from Fundação Araucária, in a common project coordinated by Dr. Fábio Pedroso, at the Federal University of Paraná. D.F. The authors thank Dr. Allan R. J. Eaglesham for suggestions on the manuscript.

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Received: 27 October 2011 Accepted: 30 May 2012
Published: 30 May 2012

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