Proteomic analysis of free-living *Bradyrhizobium diazoefficiens*: highlighting potential determinants of a successful symbiosis

Gomes *et al.*
Proteomic analysis of free-living *Bradyrhizobium diazoefficiens*: highlighting potential determinants of a successful symbiosis

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**Abstract**

**Background:** Strain CPAC 7 (=SEMIA 5080) was recently reclassified into the new species *Bradyrhizobium diazoefficiens*; due to its outstanding efficiency in fixing nitrogen, it has been used in commercial inoculants for application to crops of soybean [*Glycine max* (L.) Merr.] in Brazil and other South American countries. Although the efficiency of *B. diazoefficiens* inoculant strains is well recognized, few data on their protein expression are available.

**Results:** We provided a two-dimensional proteomic reference map of CPAC 7 obtained under free-living conditions, with the successful identification of 115 spots, representing 95 different proteins. The results highlighted the expression of molecular determinants potentially related to symbiosis establishment (e.g. inositol monophosphatase, IMPase), fixation of atmospheric nitrogen (*N₂*) (e.g. *NifH*) and defenses against stresses (e.g. chaperones). By using bioinformatic tools, it was possible to attribute probable functions to ten hypothetical proteins. For another ten proteins classified as "NO related COG" group, we analyzed by RT-qPCR the relative expression of their coding-genes in response to the nodulation-gene inducer genistein. Six of these genes were up-regulated, including *blr0227*, which may be related to polyhydroxybutyrate (PHB) biosynthesis and competitiveness for nodulation.

**Conclusions:** The proteomic map contributed to the identification of several proteins of *B. diazoefficiens* under free-living conditions and our approach—combining bioinformatics and gene-expression assays—resulted in new information about unknown genes that might play important roles in the establishment of the symbiosis with soybean.

**Keywords:** Symbiosis, Nitrogen fixation, Two-dimensional proteomics, RT-qPCR, *Bradyrhizobium*

**Background**

Biological *N₂* fixation (BNF) is a fundamental component of the global nitrogen (N) cycle, both in natural and agricultural environments. The symbiosis of legumes with soil-borne symbiotic *N₂*-fixing bacteria, which are frequently referred to as rhizobia, can often provide more than 60% of the plant’s N requirements [1,2]. Regarding the concept of agriculture sustainability, BNF contributes to the improvement of food production without cultivation of new lands, to lowering input costs for the farmers and to mitigating environmental degradation. Such benefits occur when BNF replaces chemical N-fertilizers, which are expensive, and, among other harmful environment impacts, foment greenhouse-gas emissions [3,4].

Cultivation of soybean [*Glycine max* (L.) Merr.] has increased globally, mainly due to its high protein and oil contents, and plant breeding has resulted in increasing yields [5]. Certainly, efficient BNF is a major contributor to the achievement of high yields with low input costs [6]. An important example is the contribution of BNF to soybean cropping in Brazil, associated with application to the seeds at sowing of inoculants containing elite strains of *Bradyrhizobium*, including CPAC 15 (=SEMIA 5079) and CPAC 7 (=SEMIA 5080) [7,8]. The combination of these strains can fulfill much of the crop’s N needs, resulting in estimated savings of about US $15 billion in N-fertilizers per cropping season [9].
Bradyrhizobium diazoefficiens was recently reclassified as a novel species on the bases of morpho-physiological, genotypic and genomic differences from Bradyrhizobium japonicum [10]. Strain CPAC 7 (=SEMIA 5080) has outstanding efficiency in fixing N₂ with soybean and good adaptation to the often-stressful edaphoclimatic conditions of the tropics [11,12]. These features are responsible for the inclusion of this strain in inoculants applied to soybean in Brazil since 1992 [7,9].

The type strain of B. diazoefficiens, USDA 110 T has had its genome elucidated; however, of the 8,317 potential protein-encoding genes, 30% were assigned as hypothetical and 18% showed no similarity to any known gene [13]. Later, the expression of several predicted protein-coding genes in USDA 110 T was reported in transcriptional and proteomic studies [14-19]. Nevertheless, despite the economic importance of B. diazoefficiens as a component of soybean inoculants worldwide [9,10], few data are available on the proteins it synthesizes in the free-living state. It is well known that major attributes of successful elite strains, such as saprophytic competence, adaptation to stressful conditions and nodulation competitiveness must be expressed when free-living.

Our research group has just completed the genome sequencing of strain CPAC 7 [20] and, as occurred with USDA 110 [13], it was not possible to attribute functions to about 50% of the genes. Therefore, the establishment of a proteomic reference map for this strain in the free-living state can both add valuable protein-expression data to the genomic-annotation process [21-23] and help to attribute probable functions to hypothetical proteins [21,23].

Here we present the first two-dimensional proteomic reference map for free-living B. diazoefficiens strain CPAC 7, emphasizing molecular determinants of symbiosis-establishment and of tolerance of environmental stresses. Additionally, we ascribe putative functions to some hypothetical proteins detected at the proteomic level. For other hypothetical proteins without available information, we analyzed the relative expression of their coding-genes in response to the main soybean-nodulation-inducing molecule, the flavonoid genistein.

Results and discussion

Two-dimensional gel electrophoresis and protein identification

In studies with two-dimensional gels, it is necessary to optimize the resolution of the protein maps as a function of the nature and characteristics of the samples studied. With this goal, a previous experiment to obtain an overview of the protein distribution of B. diazoefficiens strain CPAC 7 was carried out with a broad-range IPG strip (pH 3–10) for the first-dimensional protein separation. After SDS-PAGE, the results showed that most of protein spots remained clustered in the pl range of pH 5–7 (data not shown). To improve the separation of the proteins, we then employed in the first-dimension electrophoresis IPG strips with a narrower pH range (pH 4–7), that confirmed, in triplicated gels, better resolution than the strips with pH 3–10.

Using computer-assisted gel-image analysis software, well-defined spots were detected and the majority of their molecular weights ranged between 14 kDa and 97 kDa (Figure 1). Among these, 150 spots were selected and analyzed by MALDI-TOF MS or, when necessary, by MS/MS. Mass spectra of peptide fragments were compared with database entries, regarding the statistical requirements, and 115 spots were successfully identified, representing 95 different proteins (Tables 1 and 2). Information on the spectrometry data set is available in Additional file 1: Table S1. The presence of distinct spots for the same protein may be due to posttranslational modifications.

Protein functional classification and cellular location

According to the functional classification in COG, proteins were distributed in 16 categories, belonging to four functional groups (Figure 2). In the metabolism-related functional group, there were eight categories, comprising the greatest number of experimentally identified proteins (40%). Next, 21% of the proteins were clustered in three functional categories related to information storage and processing, while the cellular-processes signaling group encompassed 13% of the proteins distributed in three categories. Finally, seven proteins were pooled in two other categories of a poorly characterized group, and 20 proteins did not fit any of the COG categories, being assigned as “not in COG” (Figure 2).

The high percentage of proteins with metabolic functions in CPAC 7 (Figure 2) is consistent with rhizobia’s ability to adapt to varied edaphoclimatic conditions. Several of these proteins are related to energy metabolism, e.g. succinate dehydrogenase flavoprotein subunit and malate dehydrogenase, which participate in the tricarboxylic acid (TCA) cycle, the main pathway for obtaining energy and also important in the synthesis of precursors of the biosynthesis of amino acids, purines, pyrimidines and vitamins [23].

Proteins associated with amino-acid and lipid metabolism may be particularly important for free-living cells, and several of those proteins identified in CPAC 7 have been previously reported in B. japonicum CPAC 15 [23]. Beyond their main functions, proteins in these two categories may also play important roles at various stages of the symbiotic interaction, since auxotrophic mutants in both of them are defective in both nodulation and in N₂-fixation abilities [24].

The second largest functional group—information storage and processing (Figure 2)—encompassed several transcriptional and translational factors (Table 1). These proteins have fundamental roles in controlling metabolic pathways because they regulate and ensure the accuracy.
of gene expression [25]; furthermore, under stress conditions they can also perform as chaperonins, helping in de novo protein folding and preventing damaged proteins from forming aggregates [26,27].

Most proteins belonging to the cellular-processes-and-signaling functional category were correlated with defense against stressful conditions (Table 1). Mechanisms of response to stresses are usually conserved among bacterial species, and required for rapid adaptation to environmental and metabolic changes. One of these responses comprises the expression of molecular chaperones, such as DnaK, GrpE and, GroEL [28], all of which were detected in our study (Table 1). Also related to the mediation of adaptive responses to adverse conditions [29,30], two Clp proteases, ClpB and ClpX, were expressed in B. diazoefficiens CPAC 7. Finally, there was a cytoplasmic protein member of the two component response regulator OmpR family; proteins of this family are amongst the best characterized bacterial positive regulators, improving the transcriptional capacity of RNA polymerase, with reported effects in osmoregulation in Escherichia coli [31].

The cellular locations of all 115 identified proteins, predicted by PSORT-B and PSLpred, are listed in Tables 1 and 2. Although the majority of the proteins extracted in our study are located in cell cytoplasm and periplasm, two inner-membrane and one extracellular protein were obtained. Similar results have been reported in previous rhizobial proteomic studies by our group [23,32].

**Symbiosis establishment and N₂-fixation-related proteins**

In the establishment of the legume-rhizobia symbiosis, an exchange of molecular signals starts with the host plant’s release of molecules, mainly flavonoids, that induce expression of rhizobial nodulation (nod) genes. The products of nod-genese, the Nod factors (lipo-chitin oligosaccharides, LCOs), play critical roles in root nodulation [33]. Host specificity is also determined by the Nod factors, by means of the incorporation of α,β-unsaturated acyl chains in the backbone structure [34]. In our proteomic reference map, we identified one acyl carrier protein, 3-oxoacyl-ACP synthase, and one ACP S-malonyl transferase (Table 2), both required for the synthesis of essential fatty acyl chains.
Table 1 Identified proteins of *Bradyrhizobium diazoefficiens* CPAC 7 whole cell extract and classification according to COG

| Spot ID | Gene            | Product                                      | NCBI ID          | Cellular location | *T/*/E pl       | *T/*/E MW       | Organism            |
|---------|-----------------|----------------------------------------------|------------------|-------------------|----------------|----------------|---------------------|
|         | Metabolism      |                                              |                  |                   |                |                |                     |
|         | Energy production and conversion |                                              |                  |                   |                |                |                     |
| 1       | *nuoC*          | NADH dehydrogenase subunit C                | gi|27380028        | Cytoplasmic       | 4.88/4.94      | 23201/27000    | *B. diazoefficiens* USDA 110 |
| 2       | *sdhA*          | Succinate dehydrogenase flavoprotein subunit | gi|27375625        | Periplasmic       | 5.91/6.34      | 66903/70000    | *B. diazoefficiens* USDA 110 |
| 3       | *pdhB*          | Pyruvate dehydrogenase subunit beta         | gi|27379893        | Cytoplasmic       | 4.81/4.90      | 48906/65000    | *B. diazoefficiens* USDA 110 |
| 4       | *pdhB*          | Pyruvate dehydrogenase subunit beta         | gi|27379893        | Cytoplasmic       | 4.81/4.95      | 48906/65000    | *B. diazoefficiens* USDA 110 |
| 5       | *atpD*          | ATP synthase FO1 subunit beta               | gi|27375551        | Cytoplasmic       | 5.13/5.42      | 50987/62000    | *B. diazoefficiens* USDA 110 |
| 6       | *atpD*          | ATP synthase FO1 subunit beta               | gi|27375551        | Cytoplasmic       | 5.13/5.35      | 50987/57000    | *B. diazoefficiens* USDA 110 |
| 7       | *atpD*          | ATP synthase FO1 subunit beta               | gi|27375551        | Cytoplasmic       | 5.13/5.35      | 50987/62000    | *B. diazoefficiens* USDA 110 |
| 8       | *Aldehyde dehydrogenase* |                                              | gi|27379895        | Cytoplasmic       | 6.04/5.57      | 55297/60000    | *B. diazoefficiens* USDA 110 |
| 9       | *Succinate-semialdehyde dehydrogenase* |                                              | gi|27379109        | Cytoplasmic       | 5.3/5.86       | 50087/51000    | *B. diazoefficiens* USDA 110 |
| 10      | *eno*           | Phosphopyruvate hydratase                   | gi|27379905        | Cytoplasmic       | 5.08/5.36      | 45314/53000    | *B. diazoefficiens* USDA 110 |
| 11      | *eno*           | Phosphopyruvate hydratase                   | gi|27379905        | Cytoplasmic       | 5.08/5.28      | 45314/55000    | *B. diazoefficiens* USDA 110 |
| 12      | *mdh*           | Malate dehydrogenase                        | gi|38421418        | Cytoplasmic       | 5.88/6.09      | 34259/41000    | *B. japonicum* USDA 6 |
| 13      | *mdh*           | Malate dehydrogenase                        | gi|38421418        | Cytoplasmic       | 5.88/6.09      | 34259/41000    | *B. japonicum* USDA 6 |
| 14      | *Rieske iron-sulfur protein* |                                              | gi|354959910       | Cytoplasmic       | 5.9/6.52       | 15192/22000    | *B. japonicum* USDA 6 |
| 15      | *etfL*          | Electron transfer flavoprotein large subunit| gi|27376489        | Cytoplasmic       | 5.14/5.33      | 32186/34000    | *B. diazoefficiens* USDA 110 |
| 16      | *Ferredoxin NAD+ reductase* |                                              | gi|27375211        | Cytoplasmic       | 5.54/5.64      | 31764/29000    | *B. diazoefficiens* USDA 110 |
|         | Carbohydrate transport and metabolism |                                              |                  |                   |                |                |                     |
| 17      | *Inositol monophosphatase* |                                              | gi|27382842        | Cytoplasmic       | 5.61/6.02      | 28290/31000    | *B. diazoefficiens* USDA 110 |
| 18      | *Sugar kinase*  |                                              | gi|27375915        | Cytoplasmic       | 5.06/5.20      | 35336/38000    | *B. diazoefficiens* USDA 110 |
| 19      | *Sugar ABC transporter substrate-binding protein* |                                              | gi|27378319        | Periplasmic       | 5.63/5.68      | 38378/38000    | *B. diazoefficiens* USDA 110 |
| 20      | 6-Phosphogluconate dehydrogenase |                                              | gi|27381870        | Cytoplasmic       | 5.88/6.35      | 35880/36000    | *B. diazoefficiens* USDA 110 |
|         | Amino acid transport and metabolism |                                              |                  |                   |                |                |                     |
| 21      | *trpF*          | N-(5'-phosphoribosyl)anthranilate isomerase  | gi|27375855        | Cytoplasmic       | 5.82/6.36      | 23966/27000    | *B. diazoefficiens* USDA 110 |
| 22      | *leuD*         | Isopropylmalate isomerase small subunit      | gi|27375606        | Cytoplasmic       | 5.52/5.74      | 22781/27000    | *B. diazoefficiens* USDA 110 |
| 23      | *Amino acid ABC transporter substrate-binding protein* |                                              | gi|27379557        | Periplasmic       | 6.21/6.60      | 36860/30000    | *B. diazoefficiens* USDA 110 |
| 24      | *dapF*         | Diaminopimelate epimerase                    | gi|27375588        | Cytoplasmic       | 5.09/5.30      | 31803/33000    | *B. diazoefficiens* USDA 110 |
| 26      | *glnA*         | Glutamine synthetase                         | gi|27380060        | Cytoplasmic       | 5.44/5.47      | 52623/66000    | *B. diazoefficiens* USDA 110 |
| 27      | *L-asparaginase* |                                              | gi|27380061        | Periplasmic       | 5.93/5.93      | 39549/40000    | *B. diazoefficiens* USDA 110 |
| 28      | *serB*         | Phosphoserine phosphatase                    | gi|27381616        | Cytoplasmic       | 4.83/4.93      | 32322/39000    | *B. diazoefficiens* USDA 110 |
| 29      | *argB*         | Acetylglutamate kinase                       | gi|27383212        | Cytoplasmic       | 5.33/5.60      | 31294/32000    | *B. diazoefficiens* USDA 110 |
Table 1 Identified proteins of *Bradyrhizobium diazoefficiens* CPAC 7 whole cell extract and classification according to COG (Continued)

| Coenzyme transport and metabolism | 31 | thiE | Thiamine-phosphate pyrophosphorylase | gi|27381769 | Cytoplasmic | 4.99/5.06 | 22484/26000 | B. diazoefficiens USDA 110 |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 32 | ahcY | S-Adenosyl-L-homocysteine hydrolase | gi|27381055 | Cytoplasmic | 6.00/6.55 | 52318/45000 | B. diazoefficiens USDA 110 |
| 33 | metK | S-Adenosylmethionine synthetase | gi|27381056 | Cytoplasmic | 5.88/6.25 | 43613/42000 | B. diazoefficiens USDA 110 |
| 34 | metK | S-Adenosylmethionine synthetase | gi|27381056 | Cytoplasmic | 5.88/6.38 | 43613/41000 | B. diazoefficiens USDA 110 |
| 35 | hemB | Delta-aminolevulinic acid dehydratase/Porphobilinogen synthase | gi|1170210 | Cytoplasmic | 4.99/5.11 | 38843/42000 | B. diazoefficiens USDA 110 |
| Nucleotide transport and metabolism | 38 | hemH | Phosphoribosylaminoimidazole-succinocarboxamide synthase | gi|27375923 | Cytoplasmic | 5.35/5.64 | 33736/40000 | B. diazoefficiens USDA 110 |
| 39 | hemH | Phosphoribosylaminoimidazole-succinocarboxamide synthase | gi|27375923 | Cytoplasmic | 5.35/5.90 | 33736/34000 | B. diazoefficiens USDA 110 |
| Lipid transport and metabolism | 40 | 3-Oxoacyl-ACP synthase | gi|27378919 | Cytoplasmic | 5.56/5.71 | 44874/45000 | B. diazoefficiens USDA 110 |
| 41 | ispH | 4-Hydroxy-3-methylbut-2-enyl diphosphate reductase | gi|27376425 | Cytoplasmic | 5.75/6.3 | 35239/37000 | B. diazoefficiens USDA 110 |
| 42 | fabD | Nitrogenase iron protein ACP S-malonyl transferase | gi|27379193 | Cytoplasmic | 5.57/5.87 | 32462/32000 | B. diazoefficiens USDA 110 |
| 43 | fabD | Nitrogenase iron protein ACP S-malonyl transferase | gi|27378147 | Cytoplasmic | 5.44/5.73 | 27829/30000 | B. diazoefficiens USDA 110 |
| 44 | ipk | 4-Diphosphocytidyl-2-C-methyl-D-erythritol kinase | gi|27377637 | Cytoplasmic | 5.03/5.19 | 31076/31000 | B. diazoefficiens USDA 110 |
| Inorganic ion transport and metabolism | 45 | modA | ABC transporter molybdenum-binding protein | gi|27383271 | Periplasmic | 6.62/6.46 | 27290/27000 | B. diazoefficiens USDA 110 |
| 46 | nilH | Nitrogenase iron protein | gi|128264 | Cytoplasmic | 5.03/5.36 | 31902/35000 | B. diazoefficiens USDA 110 |
| Secondary metabolites biosynthesis, transport and catabolism | 47 | Thiol oxidoreductase FrnE | gi|27380891 | Cytoplasmic | 5.1/5.33 | 24357/28000 | B. diazoefficiens USDA 110 |
| Information storage and processing | 48 | fusA | Elongation factor G | gi|27380514 | Cytoplasmic | 5.32/5.52 | 76067/84000 | B. diazoefficiens USDA 110 |
| 49 | fusA | Elongation factor G | gi|27380514 | Cytoplasmic | 5.32/5.60 | 76067/81000 | B. diazoefficiens USDA 110 |
| 50 | typA | GTP-binding tyrosin phosphorylated protein | gi|27375651 | Cytoplasmic | 5.49/5.82 | 67131/74000 | B. diazoefficiens USDA 110 |
| 51 | typA | GTP-binding tyrosin phosphorylated protein | gi|27375651 | Cytoplasmic | 5.49/5.26 | 67131/72000 | B. diazoefficiens USDA 110 |
| 52 | aspS | Aspartyl-tRNA synthetase | gi|27379254 | Cytoplasmic | 5.71/6.00 | 66989/73000 | B. diazoefficiens USDA 110 |
| 53 | Transporter ATP-binding protein | gi|27381796 | Inner-membrane | 5.58/6.00 | 61850/67000 | B. diazoefficiens USDA 110 |
| 54 | gatA | Aspartyl-glutamyl-tRNA amidotransferase subunit A | gi|13470863 | Cytoplasmic | 5.66/5.95 | 55801/59000 | Mesorhizobium loti MAFF303099 |
| 55 | tuf | Elongation factor Tu | gi|27380513 | Cytoplasmic | 5.78/5.99 | 43569/44000 | B. diazoefficiens USDA 110 |
| 56 | tuf | Elongation factor Tu | gi|27380513 | Cytoplasmic | 5.79/6.12 | 43569/43000 | B. diazoefficiens USDA 110 |
| 57 | tuf | Elongation factor Tu | gi|27380513 | Cytoplasmic | 5.78/5.00 | 43569/42000 | B. diazoefficiens USDA 110 |
| 58 | tuf | Elongation factor Tu | gi|27380513 | Cytoplasmic | 5.79/6.05 | 43569/43000 | B. diazoefficiens USDA 110 |
| No. | Gene | Description | Accession Number | Location | Mw (kDa) | pI | Source |
|-----|------|-------------|----------------|----------|----------|-----|--------|
| 59  | hisZ | ATP phosphoribosyltransferase | gi|27382635 | Cytoplasmic | 5.1/5.22 | 41078/41000 | B. diazoefficiens USDA 110 |
| 60  | Elongation factor Ts | | gi|27379971 | Cytoplasmic | 6.17/6.77 | 32175/33000 | B. diazoefficiens USDA 110 |
| 61  | Sigma-54 modulation protein | | gi|27375835 | Cytoplasmic | 5.62/5.97 | 21727/29000 | B. diazoefficiens USDA 110 |
| 62  | rplI | 50S ribosomal protein L9 | gi|27379187 | Cytoplasmic | 5.08/5.22 | 21886/26000 | B. diazoefficiens USDA 110 |
| 63  | rpsF | ACP-S-malonyltransferase | gi|27379190 | Cytoplasmic | 5.46/5.64 | 18616/20000 | B. diazoefficiens USDA 110 |

RNA processing and modification

64 | rpsA | 30S ribosomal protein S1 | gi|384214454 | Cytoplasmic | 5.27/5.49 | 62737/71000 | B. japonicum USDA 6 |
65 | rpsA | 30S ribosomal protein S1 | gi|27375851 | Cytoplasmic | 5.27/5.38 | 64213/70000 | B. diazoefficiens USDA 110 |
66 | rpsA | 30S ribosomal protein S1 | gi|27375851 | Cytoplasmic | 5.27/5.31 | 64213/71000 | B. diazoefficiens USDA 110 |
67 | rpsA | 30S ribosomal protein S1 | gi|27375851 | Cytoplasmic | 5.27/5.42 | 64213/64000 | B. diazoefficiens USDA 110 |

Transcription

68 | greA | Transcription elongation factor GreA | gi|27382489 | Cytoplasmic | 5.67/6.18 | 17169/20000 | B. diazoefficiens USDA 110 |
69 | rho | Transcription termination factor Rho | gi|27375746 | Cytoplasmic | 6.08/6.6 | 47121/45000 | B. diazoefficiens USDA 110 |
70 | dnaA | DNA-directed RNA polymerase alpha subunit | gi|354957001 | Cytoplasmic | 4.91/4.97 | 38082/41000 | B. diazoefficiens USDA 110 |
71 | dnaA | DNA-directed RNA polymerase subunit alpha | gi|27380487 | Cytoplasmic | 4.9/5.5 | 38035/35000 | B. diazoefficiens USDA 110 |

Cellular processes and signaling

Posttranslational modification, protein turnover, chaperones

73 | clpB | ATP-dependent protease ATP-binding subunit | gi|27376515 | Cytoplasmic | 5.7/6.10 | 96620/90000 | B. diazoefficiens USDA 110 |
74 | dnaK | Heat shock protein | gi|12642164 | Cytoplasmic | 5.08/5.16 | 69004/72000 | B. diazoefficiens USDA 110 |
75 | hspG | Heat shock protein 90 | gi|27382900 | Cytoplasmic | 5.08/5.19 | 69004/71000 | B. diazoefficiens USDA 110 |
76 | hspG | Heat shock protein 90 | gi|27382900 | Cytoplasmic | 5.08/5.19 | 69004/71000 | B. diazoefficiens USDA 110 |
77 | groEL | Molecular chaperone GroEL | gi|27377170 | Cytoplasmic | 5.19/5.32 | 57749/65000 | B. diazoefficiens USDA 110 |
78 | groEL | Molecular chaperone GroEL | gi|27380737 | Cytoplasmic | 5.45/5.75 | 57716/58000 | B. diazoefficiens USDA 110 |
79 | sig | Trigger factor | gi|27380056 | Cytoplasmic | 4.97/5.06 | 50061/57000 | B. diazoefficiens USDA 110 |
80 | sig | Trigger factor | gi|27380056 | Cytoplasmic | 4.97/5.06 | 50061/57000 | B. diazoefficiens USDA 110 |
81 | clpX | ATP-dependent protease ATP-binding subunit ClpX | gi|27380054 | Cytoplasmic | 5.57/5.80 | 46932/44000 | B. diazoefficiens USDA 110 |
82 | grpe | Heat shock protein GrpE | gi|27380419 | Cytoplasmic | 6.1/6.66 | 24420/29000 | B. diazoefficiens USDA 110 |
84 | grpe | Heat shock protein GrpE | gi|27377587 | Cytoplasmic | 4.84/4.84 | 21642/27000 | B. diazoefficiens USDA 110 |
85 | pcm | Protein-L-isoaspartate O-methyltransferase | gi|27379583 | Cytoplasmic | 5.95/5.93 | 25182/27000 | B. diazoefficiens USDA 110 |

Cell cycle control, cell division, chromosome partitioning

86 | ftsZ | Cell division protein FtsZ | gi|27381707 | Periplasmic | 5.21/5.40 | 62990/72000 | B. diazoefficiens USDA 110 |

Signal transduction mechanisms

88 | Two-component response regulator OmpR | gi|27377311 | Cytoplasmic | 5.69/6.07 | 26233/29000 | B. diazoefficiens USDA 110 |
| No. | Protein            | Accession   | Location     | MW (kDa) | pI     | %Identity | Source             |
|-----|--------------------|-------------|--------------|----------|--------|-----------|--------------------|
| 89  | ychF               | gi|27382550     | Cytoplasmic | 5.13/5.22| 39493/42000 | B. diazoefficiens USDA 110 |
| 90  | cobS               | gi|383768898    | Cytoplasmic | 5.62/5.80| 37304/39000 | Bradyrhizobium sp. 523321 |
| 91  | Dehydrogenase      | gi|27378316     | Cytoplasmic | 5.92/6.32| 33290/38000 | B. diazoefficiens USDA 110 |
| 93  | cinA               | gi|27380722     | Cytoplasmic | 5.29/5.51| 26451/28000 | B. diazoefficiens USDA 110 |
|     |                    |             |              |          |        |           | **NO related COG**   |
| 96  | nusA               | gi|27375896     | Cytoplasmic | 4.70/4.77| 59333/67000 | B. diazoefficiens USDA 110 |
| 97  | ATP-dependent phoshoenolpyruvate carboxykinase | gi|398824719 | Cytoplasmic | 6.01/6.34| 59235/62000 | Bradyrhizobium sp. 523321 |
| 98  | ABC transporter substrate-binding protein | gi|27380707 | Periplasmic | 6.93/6.00| 59019/58000 | B. diazoefficiens USDA 110 |
| 100 | tldD               | gi|27376279     | Cytoplasmic | 5.516.00| 51101/51000 | B. diazoefficiens USDA 110 |
| 102 | ABC transporter substrate-binding protein | gi|27382938 | Periplasmic | 5.57/5.66| 47774/42000 | B. diazoefficiens USDA 110 |
| 104 | glpX               | gi|27379474     | Cytoplasmic | 5.68/6.06| 35603/4000 | B. diazoefficiens USDA 110 |
| 104 | Substrate-binding protein | gi|27382959 | Periplasmic | 6.93/5.35| 40839/41000 | B. diazoefficiens USDA 110 |
| 105 | ilvC               | gi|384216635    | Cytoplasmic | 6.09/6.70| 37076/38000 | B. diazoefficiens USDA 110 |
| 106 | Dioxygenase        | gi|27377910     | Cytoplasmic | 5.35/5.58| 34110/35000 | B. diazoefficiens USDA 110 |
| 111 | ATP-dependent Clp protease proteolytic subunit | gi|338974245 | Cytoplasmic | 5.97/6.32| 22404/26000 | B. diazoefficiens USDA 110 |

*Theoretical and **Experimental.
Matched peptides masses and MS/MS combined results are available at the Additional file 1: Table S1.
| Spot ID | Hypothetical protein | NCBI ID | Cellular location | *T/**E pl | *T/**E MW | Predicted function |
|---------|----------------------|---------|-------------------|-----------|-----------|-------------------|
| **Metabolism** | | | | | | |
| **Amino acid transport and metabolism** | | | | | | |
| 25 | Blr3064 | gi|27378175 | Cytoplasmic | 5.64/6.00 | 50837/49000 | Succinyl-diaminopimelate desuccinylase (COG/NCBI); Peptidase family M20 - dimerisation domain (Pfam); GO: Hydrolase activity (InterPro); Lysine biosynthesis EC:3.5.1.18 (KEGG). |
| 30 | Blr5678 | gi|27380789 | Periplasmic | 5.84/5.11 | 33744/27000 | L-aminopeptidase/D-esterase (COG); Peptidase family S58 (Pfam); GO:arginine biosynthetic process; DmpA/ArgJ-Like domains (InterPro). |
| **Coenzyme transport and metabolism** | | | | | | |
| 36 | Blr3798 | gi|27378909 | Cytoplasmic | 5.66/6.13 | 27452/29000 | Demethylmenaquinone methyltransferase (COG); Methyltransf_6 (Pfam); GO: methyltransferase activity (UniProtKB); Ribonuclease E inhibitor RraA domain (InterPro). |
| 37 | Bll4565 | gi|27379676 | Cytoplasmic | 5.3/5.59 | 24885/28000 | Demethylmenaquinone methyltransferase (COG); Methyltransf_6 (Pfam); GO: methyltransferase activity (UniProtKB); Ribonuclease E inhibitor RraA domain (InterPro). |
| **Information storage and processing** | | | | | | |
| **Transcription** | | | | | | |
| 72 | Bll4752 | gi|27379863 | Cytoplasmic | 4.41/4.43 | 27960/32000 | Predicted transcriptional regulator containing the HTH domain (COG); Putative transcriptional regulators (Tpuh-like)(Pfam); Winged helix-turn-helix DNA-binding domain (InterPro). |
| **Cellular processes and signaling** | | | | | | |
| **Posttranslational modification, protein turnover, chaperones** | | | | | | |
| 83 | BJ6T_08050*** | gi|354953419 | Cytoplasmic | 4.46/4.43 | 20515/28000 | Thioredoxin-like proteins and domains(COG); Scaffold protein Nfu/NifU N terminal (Pfam); GO: Iron-sulfur cluster binding (InterPro). |
| **Signal transduction mechanisms** | | | | | | |
| 87 | Blr2761 | gi|27377872 | Cytoplasmic | 5.465.70 | 29257/33000 | Universal stress protein UspA (COG); Universal stress protein family (Pfam); GO: response to stress (InterPro). |
| **Poorly characterized** | | | | | | |
| **General function prediction only** | | | | | | |
| 92 | Bll5663 | gi|27380774 | Cytoplasmic | 4.92/5.08 | 33547/34000 | MoxR-like ATPases (COG); ATPases Associated with diverse cellular Activities – AAA proteins (Pfam); GO: ATPase activity (InterProt). |
Table 2 Functional prediction of hypothetical proteins identified in *Bradyrhizobium diazoefficiens* CPAC 7 whole protein extract based on protein sequences, conserved domains and motifs, protein-protein interactions and cellular locations (Continued)

| Function unknown |
|------------------|
| 94               |
| Blr5067          |
| gi|27380178        |
| Cytoplasmic      |
| 5.25/5.29        |
| 24247/29000      |
| Uncharacterized ACR (COG); putative metal binding site - region_name = "LabA (NCBI); NYN domain (Pfam)." |
| 95               |
| Blr5067          |
| gi|27380178        |
| Cytoplasmic      |
| 5.93/5.96        |
| 16852/28000      |
| Uncharacterized ACR (COG); putative metal binding site - region_name = "LabA (NCBI); NYN domain (Pfam)." |

**NO related COG**

| 99               |
| Bll0565         |
| gi|27375676        |
| Periplasmic      |
| 4.98/5.04        |
| 41554/57000      |
| No related data |

| 101              |
| Blr7534         |
| gi|27382645        |
| Periplasmic      |
| 5.86/4.99        |
| 49518/45000      |
| No related data |

| 107              |
| Bll5131         |
| gi|27380242        |
| Extracellular    |
| 7.68/5.80        |
| 34214/32000      |
| Protein of unknown function DUF (Pfam/InterPro). |

| 108              |
| Blr2961         |
| gi|27378072        |
| Cytoplasmic      |
| 4.96/5.06        |
| 25510/29000      |
| GO: catalytic activity/Metabolic process (InterPro); Protein of unknown function (Pfam). |

| 109              |
| Bll5307         |
| gi|27380418        |
| Periplasmic      |
| 5.55/5.70        |
| 14177/25000      |
| No related data |

| 110              |
| Blr2191         |
| gi|27377302        |
| Cytoplasmic      |
| 6.58/6.12        |
| 25491/27000      |
| Uncharacterized protein conserved in bacteria (DUF2328) (Pfam); KO: chpT histidine phosphotransferase (KEGG); Two-component system/His Kinase A (phospho-acceptor) domain (IMG). |

| 112              |
| Bll7551         |
| gi|27382662        |
| Cytoplasmic      |
| 5.95/5.92        |
| 27565/26000      |
| No related data |

| 113              |
| Blr0227         |
| gi|27375338        |
| Periplasmic      |
| 5.17/5.29        |
| 22619/25000      |
| PHB accumulation regulatory domain (Pfam);GO: Regulation of transcription/Transcription repressor activity (NCBI/InterPro). |

| 114              |
| Blr0227         |
| gi|27375338        |
| Periplasmic      |
| 5.17/6.40        |
| 22619/24000      |
| PHB accumulation regulatory domain (Pfam); GO: Regulation of transcription/Transcription repressor activity (NCBI/InterPro). |

| 115              |
| Blr7436         |
| gi|27382547        |
| Cytoplasmic      |
| 4.82/4.90        |
| 15290/18000      |
| No related data |

*Theoretical and Experimental.***Best match with *Bradyrhizobium japonicum* USDA 6.

Matched peptides masses and MS/MS combined results are available at the Additional file 1: Table S1.
Also important to the production of an effective N$_2$-fixing symbiosis, the exopolysaccharides (EPSs) play an essential role in the symbiotic interaction with compatible host plants [35,36]. We found one inositol monophosphatase (IMPase) (Table 1); this protein has been related with the regulation of EPS production, which, when mutated in Rhizobium leguminosarum bv. trifolii, resulted in defective-EPS production and a non-N$_2$-fixing phenotype [37-39].

Several microbial factors, classified either as general or host-specific elicitors, are related to the induction of immune responses in plants [40,41]. General elicitors include flagellins, cold-shock proteins (CSPs), LCOs and LPSs. We identified the elongation factor Tu (EFTu), which also acts as elicitor and, in general, is conserved across multiple groups of bacteria, allowing plants to perceive and respond to an epitope common to many bacteria [42,43].

In response to general bacterial elicitors, plants have basal defense mechanisms that include increases in extracellular pH, ethylene production, and synthesis of reactive oxygen species (ROSs) [40]. Upon an initial “unfriendly” reception from the host plant, rhizobia must avoid host defenses, and elicit a successful environment to establish an effective N$_2$-fixing symbiosis [44-46]. Among the several features presented by the bacteria to overcome plant defenses, GTP-binding protein TypA, which confers resistance to certain antimicrobial peptides and survival under stress conditions, has been recognized as the main contributor to a successful interaction between Sinorhizobium (=Ensifer) meliloti and some Medicago truncatula lines [44]. A probable symbiotic function of this protein was also observed in B. japonicum CPAC 15 in response to the host flavonoid genistein [47], and now its constitutive expression has been detected in B. diazoefficiens CPAC 7.

The reduction of N$_2$ to ammonia by the nitrogenase complex can take place either by the rhizobial bacteroids inside the nodules, or in free-living rhizobia, including some Bradyrhizobium strains. In both cases, a finely balanced regulation of oxygen availability is required, since rhizobia are aerobic and need oxygen, whereas the element can denature nitrogenase. Inside the nodule, the ideal oxygen environment is reached by the participation of multiple factors, including the synthesis of heme compounds by the rhizobia [48,49]. Proteins HemB and HemH, identified in CPAC 7 (Table 1), catalyze two important steps in heme synthesis and are essential for the Bradyrhizobium/soybean symbiosis, since mutants defective in these genes generate a microaerobic condition with poorly developed nodules that are inefficient in fixing N$_2$ [48,50].

A protein related to amino acid metabolism and also key in N$_2$ fixation is the glutamine synthetase I (GS I), which was identified in our proteomic map (Table 1). The role of GS I in the regulation of nitrogenase has been highlighted by studies with Rhizobium sp. mutants, resulting in defective ability to derepress the enzyme, both in vitro [51] and in symbiotic conditions [52].
In our study, we also detected the constitutive expression of a sigma-54 modulation protein, which fitted in the transcription-elongation factors, with roles in cell defense. Among several roles, RpoN is directly involved in the N2-fixation process, being required for control of major N2-fixation genes, as nifHDK, the products of which constitute the nitrogenase complex and accessory proteins [55]. RpoN is also related to free-living metabolic pathways, as demonstrated when rhizobia rpoN-mutants showed, beyond defects in symbiotic N2-fixation [56], alterations in free-living nitrate assimilation [57]. We may suppose that the constitutive sigma-54 modulation protein may be important in mediating adaptations to changing environmental conditions, both in free-living and in symbiotic conditions.

The expression of the key protein NifH (nitrogenase iron protein, or component II) in our study may be related to the presence of sigma-54 modulation protein, which was shown to be a regulator of the sigma-54 expression. To support this, results show that the knockout of sigma-54 transcriptional factor in B. japonicum leads to strong pleiotropic effects, including the absence of NifH [57] and the abolishment of symbiotic N2-fixation ability [55]. The detection of NifH in our proteomic study might correlate with reports of expression of nitrogenase in Bradyrhizobium strains under free-living conditions; however, measurements of nitrogenase activity under these conditions are difficult and require a fine adjustment of the oxygen concentration [58-61].

**Stress-tolerance proteins**

In tropical regions, crops and soil microorganisms are frequently exposed to stressful conditions, in particular high temperatures, salinity and soil acidity [6,62,63]. Therefore, in addition to high efficiency of N2 fixation, commercial rhizobial strains must be tolerant of such adverse factors. Indeed, several soybean bradyrhizobia have been extensively studied to characterize their tolerances of salt [64], desiccation [65], antibiotics [66], acidity [67], among other stresses. Now, in B. diazoefficiens CPAC 7 we produced evidence of several molecular determinants related to the ability to overcome adverse conditions, including chaperonins and other proteins, such as Clp proteases and transcription-elongation factors, with roles in cell defense.

ATP-dependent Clp proteases participate in diverse cell processes, including rapid adaptive responses of bacteria to environmental changes [29] and to stressful conditions [30], and ClpB and ClpX were detected in CPAC 7. These two proteins have the properties associated with molecular chaperones, such as preventing the aggregation of denatured proteins and, in some cases, refolding them [68]. These properties are particularly important under stress conditions that exacerbate the occurrence of protein denaturation, and ClpB and ClpX help to ensure a fast return to the pre-stressed state, maintaining cell homeostasis [69,70].

**Bradyrhizobium** is acid-tolerant, it grows at pH 4.5, over 30% of the strains are capable of growing at pH 4.0 and a few are tolerant of pH 3.5 [71]. The proteome of B. diazoefficiens USDA 110, when studied at pH 4.7 [72], revealed differential expression of several proteins, eight of which—spots 5, 8, 15, 49, 70, 81, 82, and 91—were constitutively expressed in the proteome of CPAC 7 (Table 1).

Another limiting factor for rhizobia, and also for the symbiosis, is high soil temperature, which can often exceed 40°C in the tropics and limit the success of inoculants [6,8,62]. Rhizobia, similar to most organisms [73], make use of molecular chaperones to tolerate high temperatures, including heat-shock proteins (HSPs) DnaK, GrpE, GroEL and HtpG, which were identified in our proteomic reference map (Table 1). DnaK and GrpE comprise a versatile chaperone system [74] that, together with GroEL and HtpG, play a critical role in thermotolerance, routinely rescuing the majority of the proteins denatured [69,75].

Still associated with thermotolerance, it is worth mentioning the identification of three elongation factors (Ef-Tu, Ef-Ts and Ef-G) and two ribosomal proteins (30S and 50S) expressed in CPAC 7. Besides their main function of ensuring gene expression, elongation factors can also act as chaperones [26,27]. This secondary role has been demonstrated recently at the proteomic level in Rhizobium tropici strain PRF 81 (now reclassified as Rhizobium freirei) under high-temperature stress [75]. In B. japonicum, 30S and 50S ribosomal proteins may be involved in heat-stress defense, once they were up-regulated at 43°C [76]. Considering this finding, those authors hypothesized that ribosomes may act as sensors of heat shock in B. japonicum. A similar mechanism has been suggested in E. coli, in which ribosomes seem to be the primary sensor of conditions that evoke heat-shock responses [77].

Oxidative stress is frequently caused by cell exposure to reactive oxygen species (ROSs), such as superoxide anion (O2) and hydrogen peroxide (H2O2). ROSs are byproducts of normal metabolic processes and, if not properly detoxified, they become toxic. Oxidative stress also occurs by cell exposure to external ROSs, which in bacteria such as rhizobia may take place during interactions with other microorganisms or eukaryotic hosts. Therefore, tolerating and overcoming oxidative stress is critical to bacteria viability as well as for the establishment of a successful symbiotic infection [32,78].

Of the proteins identified in CPAC 7, at least five have already been reported as showing antioxidant activity (spots 16, 50, 68, 82 and 83, Table 1). Among them, ferredoxin-NADP+ reductase (FRN) has been reported to
overcome the harmful effects of ROSs on DNA replication [79]. Those authors emphasized the importance of FRN to cell protection against oxidative damage by comparing its role with those of superoxide dismutases, a group of proteins well known for mitigating damage caused by ROSs.

Salinity leads to loss of intracellular water, resulting in osmotic disturbances that can influence a range of metabolic activities [80]. Indeed, several negative effects in rhizobia-plant symbioses have been attributed to salinity; e.g. in growth and survival of rhizobia in soil, in root colonization and in nodule development [81].

Mutational studies with *S. meliloti* allowed the identification of multiple genes involved in salt tolerance, including trigger factor (*tig*) [82]. When this gene was absent, *Sinorhizobium* showed reduced ability to grow in LB with high salt concentrations, as well inability to compete against the wild-type for nodule occupancy [82]. Given these results, we suggest that *tig* may also contribute to competitiveness and to saprophytic competence under environmentally stressful conditions, as reported for CPAC 7 [7, 8, 10, 11, 22].

Classified in the information storage and processing COG category, the transcription elongation factor GreA has been recognized as a general stress protein (Gsp) induced in response to various environmental conditions. Additionally to the transcription elongation activity, its role in acid-, salt-, and cold-stress responses in *Streptococcus mutans* [83], *S. meliloti* [84] and *R. tropici* [85] has been reported. Constitutive expression of GreA was previously reported in a *B. japonicum* CPAC 15 proteomic assay [23]. Mutation of *greA* in *R. tropici* impaired the establishment of an effective symbiosis as a result of the altered ability to adapt to hyperosmotic and salt stress [85], highlighting the importance of this protein in overcoming adverse conditions during symbiosis establishment.

Altogether, these bacterial defense mechanisms are crucial to survival in the soil and to symbiosis establishment in the tropics, where rhizobia are commonly exposed to high soil temperatures, acid pH and saline conditions [3, 8].

**Hypothetical proteins: function prediction with bioinformatics tools**

In several genome projects, portions of the annotated sequences have been classified in “hypothetical”, “conserved hypothetical” or “of unknown function” categories [86, 87]. These denominations are used when the existence of a gene is supported only by prediction of gene-finding software, and they do not show significant homology to any characterized gene [23]. With *B. diazoefficiens* USDA 110, these proteins were abundant, and, of the 8,317 protein-coding genes predicted, 30% showed similarity to hypothetical genes, whereas 18% showed no similarity to any registered gene [13]; in the genome of CPAC 7, both categories comprised 50% of all predicted genes [20]. A still modest improvement in annotation of hypothetical proteins has been achieved with proteomic studies [23, 87]. For example, the reference map of *B. japonicum* CPAC 15 contributed to the assignment of 26 hypothetical proteins by using bioinformatics tools [23].

In our study, 20 proteins were classified as hypothetical/conserved hypothetical or unknown, and by using bioinformatics tools, we were able to attribute probable functions to half of them (Table 2). Two proteins (Blr3064 and Blr5678) were assigned to the amino acid transport and metabolism COG category. The first one shows hydrolase activity and, according to the KEGG database, participates in lysine biosynthesis. Blr5678 is probably related to the arginine biosynthetic process, since it has an ArgI-like domain.

Classified according to the COGnitor in the coenzyme transport and metabolism functional category, proteins Blr3798 and Blr4565, represented by spots 36 and 37 (Figure 1, Table 2) presented similar information in the databanks searched in our study. Both were annotated as demethylmenaquinone methyltransferase by COG and exhibited a ribonuclease E inhibitor RraA domain. The inhibitory activity of this domain was recently described in *E. coli* as having a regulatory function in gene expression, since the interactions of RraA with RNase E affect the composition of the RNA-degradosome, modulating its activity [88, 89].

The cytoplasmic protein Blr4752, which belongs to the transcriptional COG group is predicted as a transcriptional regulator protein that contains a Ypuh-like helix-turn-helix domain (Table 2). This protein probably plays a role in chromosomal partitioning during cell division.

BJET08050, classified in the posttranslational modification, protein turnover, chaperones functional category, shows 97% of similarity with Blr0800 of *B. diazoefficiens* USDA 110. After comparison of databases, this hypothetical protein was assigned as a thioredoxin-like protein involved in iron transport (Table 2); however, it may be related to reactivation of proteins damaged by oxidative stress [90]. Also potentially related to cell defense, Blr2761 is closely similar to universal stress protein UspA, expression of which is known to be enhanced when cells are exposed to adverse conditions [91].

Two other proteins constitutively expressed in strain CPAC 7—Blr5663 and Blr5067—remained annotated as hypothetical in the NCBI-nr database (Table 2). Blr5663 is a member of the MoxR family of AAA + ATPases, widespread among bacteria. Associated with diverse cellular activities, MoxR ATPases display a chaperone-like function and have been found to be important modulators of multiple-stress-response pathways in various organisms, including *R. leguminosarum* [92, 93]. The other protein, Blr5067, was also expressed in bacteroids of *B. diazoefficiens* USDA 110, and is still poorly characterized [14]; it could be a LabA-like protein with a putative
metal-binding site. This family of proteins has been studied in cyanobacteria and reported to affect both gene expression and cellular metabolic state [94,95]. Nevertheless, the biological role of Blr5067 in *Bradyrhizobium* remains to be determined.

**Genistein effect on the expression of hypothetical protein-coding genes**

Ten out of the 20 hypothetical proteins identified in our proteomic study did not fit into any of the functional categories of COG, being assigned as “NO related COG” (Table 2). The lack of homology of these sequences with those of known proteins, combined with their detection at the proteomic level, suggest that they could be interesting subjects of study, possibly providing new information and deeper understanding of the organism. We analyzed the relative expression patterns of nine hypothetical protein-coding genes (two spots—113 and 114—showed similarity with the same protein Blr0227) in response to genistein. The localization of the genes encoding these proteins in the genome of *B. diazoefficiens* CPAC 7 is shown in Additional file 1: Table S1.

The product of nodC directs the synthesis of the backbone of lipochitin oligosaccharides (LCOs), also called Nod factors, which are essential for the nodulation process [97-100]. In our study, the nodC gene of CPAC 7 was used as a positive control in the RT-qPCR analysis, and up-regulation was confirmed; in addition, six of the nine hypothetical protein-encoding genes were significantly up-regulated (Figure 3). Of these, bll0565 and blr2961 protein-coding genes showed the highest genistein-induction effect, and up-regulation was observed also for blr7534, bll5307, blr2191 and blr0227.

Blr2191 is a ChpT histidine phosphotransferase, well characterized in *Caulobacter crescentus*, a model organism in cell-cycle studies [101]; in this bacterium, the protein controls, via phosphorylation, the activity of the master cell-cycle regulator CtrA [102,103]. The role of histidine phosphotransferases in the cell cycle of *B. diazoefficiens* is still to be elucidated; however, with the results from our study—showing its induction by genistein—we may suppose that it affects the growth rate of *B. diazoefficiens* in a genistein-enriched environment, such as the soybean rhizosphere [104].

Protein Blr0227 has also been identified in *B. japonicum* under neutral [23] and acidic conditions [72]. A potential implication of this protein in rhizobial competitiveness might exist, since it is up-regulated in response to the flavonoid daidzein in *B. japonicum* strain 4534, a strain highly competitive for nodulation, but not in the poorly competitive *B. japonicum* strain 4222 [105]; it is noteworthy that CPAC 7 is also competitive. Blr0227 has a polyhydroxybutyrate (PHB)-accumulation regulatory domain and it is known that in *B. japonicum* large amounts of carbon are directed to the synthesis of storage compounds, especially PHB [106]. Interestingly, the PHB biosynthesis also seems to be associated with rhizosphere competitiveness, since *B. japonicum* mutants defective in PHB synthesis show reduced competitiveness [107].

Three out of the nine proteins of CPAC 7 analyzed were not significantly induced by genistein, Bll5131, Bll7551 and Blr7436 (Figure 3, Additional file 2: Figure S1), confirming results reported with *B. japonicum* strain CPAC 15 [47]. They may play other roles, e.g. Bll7551 was one of the up-regulated proteins in bacteroids of *B. japonicum*.

![Figure 3 Expression levels of hypothetical proteins coding genes of *Bradyrhizobium diazoefficiens* strain CPAC 7 after growth to exponential phase in the presence of genistein. Relative expression was determined by REST2009 software.](image-url)
USDA 110 [108], suggesting a role in latter steps in the development of the symbiosis.

**Conclusions**

CPAC 7 is an agronomically important strain used in commercial inoculants for application to soybean crops in Brazil and in other South American countries; it presents high N₂-fixation efficiency, and adaptability to tropical conditions [7,8,11,109]. Here we provide the first proteomic map for this bacterium, revealing molecular determinants of distinct steps in the establishment and functioning of the symbiotic biological N₂-fixation process. We also report the constitutive expression of proteins such as DnaK, ferredoxin-NADP⁺ reductase (FRN) and trigger factor (tif) related to cell protection against heat-, oxidative- and salt stress conditions, that should contribute to bacterial survival and symbiotic functioning under adverse environmental conditions common in the tropics. In general, no function can be attributed to more than one third of the putative genes in bacterial genomes. With the approach taken in our study—including proteomics, use of bioinformatics tools and transcriptomic assays—it was possible to obtain information about several hypothetical genes/proteins of *B. diazoefficiens*, revealing interesting information, with an emphasis on genistein-induced genes, that deserve further study to confirm their roles in the soybean root-nodule symbiosis.

**Methods**

**Strain and culture conditions**

*Bradyrhizobium diazoefficiens* strain CPAC 7 (=SEMIA 5080, =CNPSo 6), a natural variant of CB 1809 (=USDA 136, a subculture of USDA 122) [10,110] is used in commercial inoculants in Brazil since 1992 [7]. Information about morpho-physiologic, genetic and symbiotic properties is available elsewhere [10,11,111-113]. CPAC 7 is deposited at the “Diazotrophic and Plant Growth Promoting Bacteria Culture Collection” of Embrapa Soja (WFCC Collection # 1213, WDCC Collection # 1054).

The strain was pre-cultured in 10-mL aliquots of arabinose-glucanate (AG) medium [114], at 80 rpm and 28°C, in the dark. For the proteomic experiment, pre-cultures were transferred to Erlenmeyer flasks containing 200 mL of the same medium and were grown under the same conditions as for the pre-cultures until the exponential phase (O.D. of 0.6 at 600 nm). Low agitation (80 rpm) was employed to minimize the production of extracellular polysaccharides, which can interfere with the 2-D gel electrophoresis.

For the reverse transcription quantitative PCR (RT-qPCR), pre-cultures were transferred to Erlenmeyer flasks containing 100 mL of AG medium. Bacteria were grown to the exponential phase under two treatment conditions: induced or not with genistein (5 μM, final concentration) dissolved in methanol, added as 50 μL per 100 mL of culture [15]; to the non-induced cultures, the same amount of methanol was added. In both proteomics and qPCR experiments, bacteria were grown in triplicates for each treatment.

**Whole-cell protein extraction**

Cultures were centrifuged at 5,000 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 total protein extraction, aliquots were resuspended in lysis buffer (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 before [115]. The lysates were separated from particulate material by centrifugation at 14,000 g for 90 min, at 4°C. Protein extract was washed with phenol and the concentration was determined by NanoDrop 1000 Spectrophotometer V3.7 (Thermo Scientific).

**Two-dimensional gel electrophoresis and visualization**

For isoelectric focusing (IEF), 300 μg of protein extract were dissolved with DeStreak buffer (GE Healthcare) and 2% v/v IPGphor to a final volume of 250 μL. IPG-strips (pH 4–7, 13 cm, GE Healthcare) were rehydrated overnight with the protein solution and covered with Cover Fluid (GE Healthcare). Loaded strips were submitted to isoelectric focalization in an Etten 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. Prior to second dimension, strips were equilibrated first for 20 min in 5 mL of an equilibration 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 of iodoacetamide, also for 20 min.

The second dimension electrophoresis was performed in a 12% polyacrylamide gel 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) as standard. Similarly to the protein extraction step, both dimensions of gel electrophoresis were run in triplicate. Gels were fixed overnight with an ethanol-acetic acid solution before being stained with Coomassie Blue PhastGel™ R-350 (GE Healthcare) and were then scanned (ImageScanner LabScan v5.0).
Gel image analysis and sample preparation to mass spectrometry

Protein spots were automatically detected in the high-resolution digitized gel images and analyzed by Image Master 2D Platinum v 5.0 software (GE Healthcare). Well defined spots were manually selected, excised and processed as previously described [116]. Digestion was achieved with trypsin (Gold Mass Spectrometry Grade, Promega, Madison, WI) at 37°C, overnight.

Peptides from digested proteins 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) target plate and allowed to crystallize at room temperature. The same procedure was used for the standard peptide calibration mixture I (Bruker Daltonics). For mass spectra acquisition, a MALDI-TOF-TOF (MALDI-time-of-flight in tandem) UltraFlex III mass spectrometer (Bruker Daltonics) was operated in the reflector for MALDI-TOF MS peptide mass fingerprint (PMF) and in the “LIFT™” mode for MALDI-TOF-TOF MS/MS fragmentation experiments, on fully manual mode using FlexControl software v. 2.2. To process the data obtained, Flex Analysis v.3.0 software (Bruker Daltonics, Billerica, MA) was employed.

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 search engine v. 2.3 (http://www.matrixscience.com/). 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. Searches on the Decoy database (Mascot) were done and both decoy score and false discovery rates were considered for the identification. The spectrometry datasets are available at the Additional file 1: Table S1.

In silico protein characterization

A set of bioinformatics tools was used to improve the characterization of the proteins. The proteins were fitted into COG (Clusters of Orthologous Groups) categories according to their functional inference, using the COGnitor program (http://www.ncbi.nlm.nih.gov/COG) [117]. Software packages PSORT-B [118] and PSIPred [119] were used for the prediction of subcellular localization.

To search for putative roles of the hypothetical proteins, a package of bioinformatics tools was applied [87]. SignalP [120] was employed for the prediction of signal peptides. To determine the protein family and domains we used Pfam [121] and InterPro [122]. MicrobesOnline (http://www.microbesonline.org) [123], a suite of web-based comparative tools, and the Integrated Microbial Genomes system (http://img.jgi.doe.gov) [124] were also searched. Finally, the prediction of physical and functional protein interactions was carried out with STRING 9.1 (http://string-db.org/) [125].

RNA extraction and primers design

Cells from 35 mL of the control and the genistein induced bacterial cultures (item 2.1) were centrifuged (8,000 g for 10 min at 4°C) and the pellet was resuspended in 280 μg of lysis buffer, consisted of 250 μL of TE (10 mM Tris, adjusted to pH 8.0 with HCl; 1 mM EDTA), 10 μL of lysozyme (5 mg/mL) and 20 μL of 10% SDS solution (w/v). After resuspended, the mixture was incubated at 37°C for 5 min to achieve an efficient cell disruption. Lysates were then centrifuged (8,000 g for 10 min at 4°C) and the supernatants were transferred to new 2-ml tubes and homogenized with 1 mL of TRizol® reagent (Life Technologies). This new mixture was centrifuged and the superior phase was transferred to another tube. After a wash step with chloroform, RNA was precipitated by adding 500 μL of cold isopropanol, purified with RNeasy Mini Kit (Qiagen) and quantified by NanoDrop ND-1000 (NanoDrop Technologies, Inc.). The RNA was assessed in a 1% (w/v) agarose gel.

Primers were designed using PrimerExpress 3.0 (Applied Biosystems/Life Technologies, Grand Island, NY, USA) targeting an amplicon size of 50–200 bp. The primer sequences were searched against the B. diazoefficiens strain USDA 110 genome (http://www.ncbi.nlm.nih.gov/genome/18384) to verify their specificity. Primer sequences and amplification efficiency rates are shown in Additional file 3: Table S2.

Extracted RNAs were submitted to DNase treatment (Invitrogen/Life Technologies, Grand Island, NY, USA), and high quality total RNA was used to synthesize cDNA strands (Superscript II First Strand Synthesis, Invitrogen/Life Technologies, Grand Island, NY, USA).

Relative gene expression analysis by RT-qPCR

After carrying out the amplification to determine the primers efficiency rate, nine hypothetical genes were amplified by RT-qPCR using a 7500 RT-qPCR Thermocycler (Applied Biosystems/Life Technologies, Grand Island, NY, USA) with the following manufacturer’s instructions: 50°C for 2 min, 95°C for 10 min, 45 cycles at 95°C for 2 min, 60°C for 30 s and 72°C for 30 s, in 45 cycles. The 16S rRNA gene was used as endogenous control (Additional file 3: Table S2).
Rest2009 software package [126] was used to evaluate the data by providing a robust statistical analysis (Additional file 2: Figure S1). The normalization of cycle threshold (Ct) of RT-qPCR amplifications was performed based on the selected endogenous gene (16S rRNA). The genistein responsive gene nodC [127] was used as positive control.

### Additional file

**Additional file 1: Table S1.** Complementary information about protein identifications. All searches were performed with Mascot software v. 2.3 (http://www.matrixscience.com/) against the public database NCBI nr (National Center for Biotechnology Information non-redundant).

*Identified by MS; **Identified by MS/MS.

**Additional file 2: Figure S1.** Localization in the genome of Bradyrhizobium japonicum CPAC 15 of the genes coding hypothetical proteins used in our study.

**Additional file 3: Table S2.** Statistical results of RT-qPCR data provided by Rest2009 software package.

### Competing interests

The authors declare that they have no competing interests.

### Authors’ contributions

Conceived and designed the experiments: MH, LVGT Performed the experiments: DFG, JSSB, AAPR, LPS, CB Analyzed the data: DFG, JSSB, AAPR, LPS, CB, LVGT, MH Contributed reagents/materials/analysis tools: MH, LPS, CB Wrote the paper: DFG, JSSB, AAPR, LPS, CB

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