UNIVERSIDADE FEDERAL DO PARANÁ

DOUGLAS FABIANO GOMES

ANÁLISE PROTEÔMICA DE BACTÉRIAS SIMBIÓTICAS FIXADORAS DE NITROGÊNIO DE INTERESSE AGRONÔMICO

CURITIBA
2014

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Tese apresentada ao Curso de Pós-Graduação em Genética, Setor de Ciências Biológicas, Universidade Federal do Paraná como requisito parcial à obtenção de título de Doutor.

Orientadora: Profª Drª Lygia Vitória Galli-Terasawa

Co-Orientadora: Drª Mariangela Hungria

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Ata da Defesa de Tese de Doutorado de
DOUGLAS FABIANO GOMES

Aos vinte e quatro dias do mês de novembro do ano de dois mil e quatorze, foi realizada na sala sessenta e sete do Departamento de Genética do Setor de Ciências Biológicas da Universidade Federal do Paraná, a Defesa de Tese do doutorando **DOUGLAS FABIANO GOMES**, intitulada "Análise Proteômica de Bactérias Simbióticas Fixadoras de Nitrogênio de Interesse Agronômico". A abertura teve início às quatorze horas pelo Doutor **Ricardo Lehtonen Rodrigues de Souza**, Coordenador do Programa de Pós-Graduação em Genética, que em seguida passou a palavra à Presidente da Banca Examinadora e Orientadora do aluno, Professora Doutora **Lygia Vitória Galli-Terasawa**, do Departamento de Genética da Universidade Federal do Paraná. A Presidente apresentou ao público presente os membros da Banca Examinadora e passou a palavra ao aluno para que fizesse a apresentação sucinta da sua Tese. Após a explanação oral, a Doutora **Lygia Vitória Galli-Terasawa** passou a palavra ao primeiro examinador, Doutor **Javier Ollero**, da Universidade de Sevilla na Espanha. Em seguida, passou a palavra ao segundo examinador, Doutor **Manuel Megías**, da Universidade de Sevilha na Espanha. Na sequência, passou a palavra ao terceiro examinador, Doutor **André Shigueyoshi Nakatani**, da Embrapa Soja. Em seguida, a quarta examinadora, Doutora **Vanessa Kava-Cordeiro**, do Departamento de Genética da Universidade Federal do Paraná, fez suas considerações. Na sequência, a Doutora **Mariângela Hungria da Cunha**, da Embrapa Soja, Coorientadora do aluno fez suas considerações. Findas as arguições pelos membros da banca, a Doutora **Lygia Vitória Galli-Terasawa** fez uma rápida apreciação das conclusões mais importantes dos debates realizados e comunicou que a Banca Examinadora iria proceder à discussão para atribuição dos conceitos, reunindo-se em sessão secreta. Os trabalhos foram interrompidos por cinco minutos. Após, foram proclamados os conceitos atribuídos pela Banca Examinadora, a seguir descritos: Doutor **Javier Ollero**, conceito "A"; Doutor **Manuel Megías**, conceito "A"; Doutor **André Shigueyoshi Nakatani**, conceito "A"; Doutora **Vanessa Kava-Cordeiro**, conceito "A"; Doutora **Mariângela Hungria da Cunha**, conceito "A"; Doutora **Lygia Vitória Galli-Terasawa**, conceito "A", com o conceito médio final "A". Tendo cumprido o que dita o artigo setenta e cinco das Normas Internas do Programa, o candidato cumpriu os requisitos para obtenção do grau de Doutor em Genética. Como não havia nada mais a ser tratado, a Doutora **Lygia Vitória Galli-Terasawa**, após informar ao candidato que ele tem, a partir desta data, até trinta dias para a entrega da versão definitiva de sua Tese, deu por encerrada a sessão. Eu, **Ricardo Lehtonen Rodrigues de Souza**, lavrei a presente ata, a qual assino juntamente com os senhores examinadores. Curitiba, vinte e quatro de novembro de dois mil e quatorze.

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PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA

PARECER

Os abaixo-assinados, membros da Banca Examinadora da Defesa de Tese de Doutorado, a qual se submeteu **DOUGLAS FABIANO GOMES,** para obtenção do título de Doutor em Genética pela Universidade Federal do Paraná, no Programa de Pós-Graduação em Genética, são de parecer que se confira ao candidato o conceito "A".

Secretaria da Coordenação do Programa de Pós-Graduação em Genética do Setor de Ciências Biológicas da Universidade Federal do Paraná.

Curitiba, 24 de novembro de 2014

Doutor Javier Ollero
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Aos Meus pais;
À minha linda esposa por
tornar os meus dias
especiais.
Dedico

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RESUMO

A fixação biológica do nitrogênio (FBN) desempenha um papel fundamental na incorporação do nitrogênio na biosfera, principalmente através da simbiose entre bactérias fixadoras de nitrogênio, comumente denominadas de rizóbios, e plantas leguminosas. Na agricultura, a utilização de bactérias competitivas, tolerantes a condições de estresse e eficientes na fixação do nitrogênio tem promovido redução de custos, aumento da produtividade e benefícios ao meio ambiente, características que fizeram da FBN um dos pilares da sustentabilidade agrícola. Neste contexto, identificar os fatores que conferem tais qualidades a determinadas estirpes de rizóbios é necessário para o melhor entendimento e o aproveitamento do processo simbiótico de fixação do nitrogênio. Com esse objetivo, duas estirpes com elevada eficiência em fixar nitrogênio e capazes de tolerar estresses comuns a regiões tropicais, como altas temperaturas, *Rhizobium freirei* PRF 81 e *Bradyrhizobium diazoefficiens* CPAC 7, foram caracterizadas com o auxílio de ferramentas da genômica funcional. *R. freirei* é simbionte do feijoeiro (*Phaseolus vulgaris* L.) e *B. diazoefficiens* da soja (*Glycine max* (L.) Merr). Através da eletroforese bidimensional (2-DE) seguida da espectrometria de massas foram obtidos os mapas proteômicos, identificando, para cada estirpe, 115 proteínas. Nos dois estudos, as proteínas relacionadas com as atividades metabólicas foram as mais numerosas, refletindo diretamente na elevada competitividade apresentada por essas estirpes. Proteínas importantes para o estabelecimento da simbiose e relacionadas à tolerância a condições de estresse, sobretudo as mais comumente encontradas em regiões tropicais, também foram evidenciadas. Diversas proteínas hipotéticas identificadas no mapa proteômico da estirpe CPAC 7 tiveram suas prováveis funções atribuídas por meio de ferramentas de bioinformática. Outras, com pouca ou nenhuma informação disponível, tiveram a expressão de seus respectivos genes avaliada por PCR quantitativo em tempo real (RT-qPCR) na presença do flavonoide genisteína, um composto liberado pelas raízes das plantas hospedeiras capaz de induzir o estabelecimento da simbiose. Entre os genes analisados, seis apresentaram indução significativa, incluindo o gene para a proteína Blr0227 de *B. diazoefficiens*, que participa da biossíntese de poli-beta-hidroxibutirato (PHB), um composto que pode estar relacionado com a elevada competitividade apresentada por essa estirpe. Os resultados alcançados com a combinação da análise proteômica, bioinformática e o estudo da expressão de genes para proteínas hipotéticas geraram novas informações sobre as propriedades dos microssimbiontes estudados.

Palavras-chave: *Rhizobium freirei*, *Bradyrhizobium diazoefficiens*, proteômica, RT-qPCR, estresses ambientais, proteínas hipotéticas.

ABSTRACT

Biological nitrogen fixation (BNF) has an outstanding role in the nitrogen incorporation on the biosphere, mainly by the symbiosis between N_2-fixing bacteria, which are frequently referred to as rhizobia, and leguminous plants. In agriculture, the use of bacteria highly competitive, tolerant to stressful conditions and efficient in nitrogen fixation contributes to the improvement of food production, to decrease the input costs and to mitigate environmental degradation, features that include BNF as one of the pillars of the sustentability. In this sense, highlighting the factors that confer such qualities to rhizobial strains is required to better understand and maximize the nitrogen fixation process. With this aim, two strains efficient in fixing nitrogen and tolerant to different stressing conditions, *Rhizobium freirei* PRF 81 and *Bradyrhizobium diazoefficiens* CPAC 7, were studied by functional genomic tools. *R. freirei* is a symbiont of common bean (*Phaseolus vulgaris* L.) and *B. diazoefficiens* of soybean (*Glycine max* (L.) Merr.). The proteomic reference maps were obtained by two-dimensional electrophoresis (2-DE) and mass spectrometry, and allowed the identification, for each strain, of 115 proteins. In both strains, proteins related to the metabolism were the most abundant, reflecting the metabolic plasticity showed by these strains. Important determinants to the symbiosis establishment and to the tolerance of adverse conditions, especially those experienced in tropical regions, were also detected. Several hypothetical proteins identified in the CPAC 7 proteomic map had their putative functions attributed by the use of bioinformatics tools. Others, with few or without available information, had their coding-genes analysed by real time quantitative PCR (RT-qPCR) in response to one nodulation-inducing molecule, the flavonoid genistein. Among these genes, six were significantly up-regulated, including the protein codding-gene *blr0227* that is envolvet with poly-beta-hydroxybutirate (PHB) biosynthesis, which might be related to the bacteria competitiveness. The results obtained by the combination of proteomics analysis, bioinformatics and gene-expression assays resulted in new information about the microssymbionts properties.

Keywords: *Rhizobium freirei*, *Bradyrhizobium diazoefficiens*, proteomics, RT-qPCR, environmental stress, hypothetical proteins.

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1 INTRODUÇÃO

1.1 PLANTAS LEGUMINOSAS

Pertencentes à terceira maior família de angiospermas, Fabaceae (=Leguminosae), estima-se que a diversidade das plantas leguminosas compreenda mais de 19.000 espécies distribuídas em três subfamílias: Mimosoideae, Caesalpinioideae e Papilionoideae (LEWIS et al., 2005). Dentro da grande variedade de espécies, o potencial econômico das leguminosas é vasto e inclui variedades alimentícias, medicinais, madeireiras, ornamentais, produtoras de fibras e óleo (WOJCIECHOWSKI et. at, 2004).

Em geral, as plantas leguminosas são lembradas pela importância que têm na alimentação humana. Feijões (*Phaseolus* spp.), ervilha (*Pisum sativum*), lentilha (*Lens culinaris*), grão-de-bico (*Cicer arietinum*) e amendoim (*Arachis hypogaea*) são alguns exemplos de plantas cultivadas devido às propriedades alimentícias de suas sementes, ricas em proteínas, ferro e carboidratos, ou em óleo. Além da utilização na alimentação, algumas leguminosas são cultivadas devido aos efeitos benéficos que promovem no solo. Quando são empregadas com esse objetivo recebem a denominação de “adubos verdes”.

No Brasil, diferentes leguminosas são utilizadas de acordo com seus variados atrativos. O pau-rainha (*Centrolobium paraense* Tul.), por exemplo, é uma espécie bastante conhecida na Região Norte do país, onde sua madeira é utilizada na construção de casas e móveis artesanais (KAMINSKI, 2004). Espécies utilizadas como adubos verdes são amplamente cultivadas para enriquecer o solo, bem como em sistemas de consórcio. Nesse contexto, as leguminosas representam uma alternativa ao suprimento, substituição ou complementação da adubação mineral na recomposição da fertilidade do solo (SCIVITTARO et al., 2000).

Mesmo com as diversas aplicações, as leguminosas de maior destaque no Brasil são as destinadas à alimentação humana e animal. São várias as espécies pertencentes à família Leguminosae empregadas com essa finalidade. Por ser uma importante fonte de proteínas para os brasileiros, o cultivo do feijoeiro (*Phaselus vulgaris* L.) destaca-se no cenário agrícola do país. A soja (*Glycine max* (L.) Merr.), além de ser utilizada na alimentação

humana e na composição de rações para animais, apresenta outras aplicações de interesse comercial, sendo seu cultivo amplamente difundido no Brasil.

1.1.1 Feijoeiro

O feijoeiro é originário do continente americano e foi domesticado separadamente em dois centros distintos de diversidade genética, de modo que os alelos estão distribuídos em dois grupos: o mesoamericano, ou grupo do norte (do México à região norte da América do Sul - México, América Central, Colômbia, norte do Peru) e o andino, ou grupo do sul (do sul do Peru ao norte da Argentina – Equador, Bolívia, Peru, Argentina) (GEPTS e DEBOUCK, 1991; CHACON et al., 2005). Essa leguminosa participa da alimentação de cerca de 500 milhões de pessoas na América latina e África, sendo bastante difundida em todo o território brasileiro, principalmente como cultura de subsistência em pequenas propriedades, muito embora também seja cultivada com o emprego de tecnologias avançadas. Além disso, é considerada uma fonte proteica vegetal e de aminoácidos essenciais importante, representando de 20% a 28% das proteínas ingeridas pela população (VELAZQUEZ, 1988; EMBRAPA, 2005).

Segundo dados da CONAB (2014), o Brasil continua sendo o maior produtor e consumidor mundial de feijão. Cultivado em três períodos; “safra das águas”, “safra da seca” e “safra de outono/inverno”, a produção total de 2013/2014 poderá chegar a 3,4 milhões de toneladas. A produtividade deve crescer, passando de 913 kg ha^{-1} em 2012/2013 para 1.030 kg ha^{-1} na safra atual. Apesar dessa estimativa de aumento, a produtividade média desta leguminosa no Brasil ainda é considerada baixa, sendo que os principais fatores que contribuem para esse cenário são a pequena adoção de tecnologias e o cultivo em solos marginais.

1.1.2 Soja

Originária da região leste da Ásia, a soja foi introduzida no Brasil no ano de 1882. Entretanto, apenas em meados de 1970 essa cultura recebeu maior atenção no Brasil, isso devido à alta do preço no mercado mundial. Atualmente, devido às suas aplicações comerciais, a soja é a principal cultura brasileira para exportação e o sucesso alcançado está diretamente relacionado à

existência de diversas cultivares adaptadas a diferentes regiões brasileiras, fruto do intenso processo de melhoramento genético (SHURTLEFF e AOYAGI, 2009).

Entre suas diversas aplicações, a soja é utilizada, principalmente, na alimentação humana e animal. O óleo de soja é um dos produtos mais utilizados na alimentação humana e a sua participação no mercado mundial de óleos vegetais comestíveis é de 27,5% (SEDIYAMA et al., 2005). A proteína de soja é consumida como complemento alimentar, com a vantagem de ser mais barata que as proteínas de origem animal (MARTIN et al., 2010). Além das funções nutricionais básicas, a soja tem recebido destaque por seus efeitos benéficos à saúde humana (KWON et al., 2010, NAKAHARA et al., 2010). Mesmo com grande importância na alimentação, essa leguminosa também tem sido bastante empregada na produção de biocombustíveis (FAO, 2008).

De acordo com a Companhia Nacional de Abastecimento (CONAB) em2014 o Brasil produzirá cerca de 86 milhões de toneladas na safra atual em uma área plantada que atinge 30,1 milhões de hectares (CONAB, 2014). Grande parte do sucesso que o Brasil vem alcançando no cultivo da soja é devido às tecnologias desenvolvidas e aplicadas nas lavouras. Dentre essas, destacam-se o plantio direto, o melhoramento genético de sementes e a inoculação com bactérias fixadoras de nitrogênio. Essas bactérias compõem inoculantes agrícolas para essa leguminosa e fornecem a grande maioria do N necessário para o desenvolvimento das plantas, garantindo também a elevada produtividade de grãos (HUNGRIA et al., 2006).

1.2 NITROGÊNIO

A importância do nitrogênio para as plantas foi reconhecida por volta do ano 1660. Saussure em 1804 foi além, classificou esse nutriente como um elemento vital aos vegetais e revelou que sua obtenção ocorre principalmente a partir do solo (BARKER e BRAYSON, 2007). Até então, acreditava-se que o nitrogênio obtido pelas plantas era proveniente da atmosfera. Entretanto, mesmo representando 80% dos gases atmosféricos, as plantas não são capazes de utilizá-lo diretamente do ar, isso devido à forte tripla ligação entre os dois átomos que constituem a molécula.

De todo o nitrogênio adquirido pelas plantas, aproximadamente 85%, encontra-se distribuído entre as proteínas. Cerca de 5% está presente nos ácidos nucleicos – ADN e ARN. O restante permanece solúvel em água ou em moléculas de baixo peso molecular (BARKER e BRAYSON, 2006). Assim, por constituir moléculas fundamentais para os processos biológicos, a carência desse nutriente é reconhecida como um dos principais fatores limitantes ao desenvolvimento das plantas e, consequentemente, à agricultura.

1.2.1 Formas de disponibilização às plantas

As plantas fazem uso de diferentes vias para a assimilação do nitrogênio, sendo elas; a decomposição da matéria orgânica presente no solo; o processo de fixação não-biológica, resultante de descargas elétricas, combustão e vulcanismo; os fertilizantes nitrogenados; o processo de fixação biológica do nitrogênio atmosférico (N_2) (BRILL, 1979; POSTGATE e HILL, 1979; POSTGATE, 1982).

A matéria orgânica presente no solo é limitada e, por isso, não fornece a quantidade necessária requerida para o desenvolvimento vegetal, esgotando-se com os sucessivos cultivos. Além disso, deve-se considerar que as condições de temperatura e umidade predominantes nos trópicos aceleram sua decomposição, levando a perdas de nitrogênio. A ocorrência de competição entre plantas e microrganismos do solo por nitrogenio mineral, proveniente da matéria orgânica, também reduz a disponibilidade desse nutriente, sendo que a manutenção desses microrganismos é necessária para a sustentabilidade dos sistemas agrícolas (HUNGRIA et al., 2001).

A fixação não-biológica, decorrente de processos naturais como as descargas elétricas, promove a conversão do nitrogênio atmosférico (N_2) em formas assimiláveis pelas plantas. Entretanto, essa via exerce pouca influência na disponibilização de N às culturas vegetais, contribuindo com aproximadamente 10% da entrada de nitrogênio na biosfera (NEWTON, 2000).

Os fertilizantes nitrogenados, produzidos industrialmente, contribuem com cerca de 25% da fixação anual de nitrogênio (NEWTON, 2000). Um dos compostos nitrogenados utilizados na agricultura é a amônia (NH_3), que, apesar de ser facilmente assimilada pelas plantas, gera o aumento dos custos de produção e provoca impactos negativos no ambiente. O elevado custo dos

fertilizantes está relacionado à demanda energética do processo industrial, onde aproximadamente seis barris de petróleo são requeridos para a obtenção de uma tonelada de NH_3. Os impactos ambientais estão associados com a quantidade de nitrogênio aplicado nas lavouras, sempre superior à capacidade de assimilação das plantas. Assim, o excesso pode contaminar corpos d'água e lençois freáticos devido à lixiviação (HUNGRIA et al., 1994, 1997, 2001, 2006). Através da volatilização e desnitrificação do nitrogênio excedente ocorre a emissão de gases que potencializam o efeito estufa (VANCE, 2001; GRAHAM e VANCE, 2003).

A fixação biológica do nitrogênio (FBN) representa a principal via de incorporação do N_2 à biosfera, cerca de 65%, principalmente através da simbiose entre bactérias fixadoras de nitrogênio, comumente denominadas de rizóbios, e plantas leguminosas (DELWICHE, 1970; BURNS e HARDY, 1975; BRILL, 1979; POSTGATE e HILL, 1979; NEWTON, 2000; GRAHAM e VANCE, 2003). No processo da FBN o N_2 é aproveitado por alguns microrganismos procariotos (arqueobactérias e, principalmente, bactérias), graças à ação de um complexo enzimático denominado dinitrogenase, capaz de romper a tripla ligação da molécula N_2 e reduzi-la a amônia (NH_3), a mesma forma obtida no processo industrial (RUBIO e LUDDEN, 2008). Neste sentido, a utilização da FBN na agricultura representa uma alternativa viável no suprimento de nitrogênio, em substituição à fertilização química, levando à redução dos custos de produção e minimizando os impactos ambientais gerados pelos fertilizantes.

1.3 INTERAÇÃO RIZÓBIO/HOSPEDEIRO

O estabelecimento da simbiose constitui um processo complexo que envolve a troca de sinais moleculares entre o microssimbionte e a planta hospedeira, muitos deles responsáveis pela especificidade da interação. Uma das interações simbióticas mais bem estudadas é a realizada entre microrganismos fixadores de nitrogênio presentes no solo e plantas leguminosas. Neste caso, o início da interação ocorre quando as plantas liberam na rizosfera moléculas específicas, em geral misturas de flavonoides, que são reconhecidas pelos microssimbiontes e atuam como compostos quimiotáticos, além de estimularem a multiplicação dos rizóbios e a expressão de genes responsáveis pela nodulação (REDMOND et al., 1986; ESTEVEZ et

al., 2009). Estes genes codificam fatores espécie-específicos, os fatores Nod, que são oligossacarídeos reconhecidos pelos receptores LysM, da planta hospedeira, que, por sua vez, enviam sinais de transdução requeridos para o processo de invasão da raiz através dos pelos radiculares e posterior desenvolvimento de um novo órgão radicular, o nódulo (RIELY et al., 2004; GEURTS et al., 2005).

Os nódulos são originados pelas modificações no periciclo e nas células corticais, de modo que após seu crescimento e diferenciação o microssimbionte inicia o processo de fixação biológica, onde o N_2 é convertido em amônia e transferido para as plantas hospedeiras, que por sua vez, disponibilizam para as bactérias fontes de carbono. Para que esta troca de nutrientes ocorra se faz necessário um mecanismo de transporte coordenado entre ambas as partes (LODWIG et al., 2003). Esta troca de compostos pode ser interrompida por um mecanismo tipo “feedback”, ou seja, o processo para durante o período em que a planta tem nitrogênio suficiente para a manutenção de suas atividades (CAETANO-ANOLLÉZ e BAUER, 1988). Após o estabelecimento da simbiose, o microrganismo permanece separado do citoplasma da célula vegetal por uma membrana denominada de simbiossoma ou membrana peribacteroidal, cuja função principal é a de regular o intercambio de nutrientes entre os simbiontes.

1.3.1 A Simbiose *Rhizobium*-feijoeiro

Inicialmente, a simbiose com o feijoeiro era considerada bastante restrita, sendo relatado que apenas um grupo de bactérias classificadas, em 1932, como *Rhizobium phaseoli* era capaz de realizar o processo de FBN (FRED et al., 1932). Entretanto, os avanços obtidos com a caracterização fisiológica, bioquímica e genética, somados à coleta de rizóbios em vários locais do mundo, permitiram a reclassificação dessas bactérias (JORDAN, 1984). A partir de então foi constatado que o feijoeiro pode ser bastante promíscuo em suas associações simbióticas (HERNANDEZ-LUCAS et al., 1995; MICHIELS et al., 1998; MARTÍNEZ-ROMERO, 2003).

Entre as diversas espécies que estabelecem simbiose com o feijoeiro, as pertencentes à espécie *Rhizobium tropici* (MARTÌNEZ-ROMERO et al., 1991) tem sido extensivamente estudadas, levando à reclassificação de estirpes em

novas espécies. Em um primeiro momento, baseado em diferenças fenotípicas e genéticas, verificou-se que algumas estirpes classificadas como *R. tropici* deveriam compor uma nova espécie, que foi denominada *Rhizobium leucaenae* (RIBEIRO et al., 2012). Em seguida, a estirpe PRF 81, que havia permanecido como *R. tropici*, foi reclassificada como *R. freirei* espécie nov. (DALL'AGNOL et al., 2013). Seguindo o mesmo roteiro dos estudos anteriores, as estirpes PRF 35^T, PRF 54, CPAO 1135 e H 52, antes pertencentes a espécie *R. tropici*, originaram uma nova espécie, denominada *R. paranaense* (DALL'AGNOL et al., 2014).

Por serem competitivas, eficientes em fixar nitrogênio e tolerantes a condições de estresse, estirpes pertencentes a esse conjunto de espécies têm sua importância reconhecida e são empregadas na formulação de inoculantes agrícolas para a cultura do feijoeiro. Em ensaios conduzidos a campo na Região Sul e no Centro-Oeste, todos com rendimento de grãos superiores a 1.500 kg ha^{-1}, a inoculação do feijão com as estirpes PRF 81, da espécie *Rhizobium freirei*, H12 e CIAT 899 de *R. tropici*, resultou em ganhos no rendimento de 78 a 1.101 kg ha^{-1} (HUNGRIA et al., 2000). Outros seis experimentos realizados em duas cidades do estado do Paraná, Londrina e Ponta Grossa, revelaram incrementos de até 460 kg ha^{-1} na produtividade do feijoeiro inoculado em relação ao tratamento não inoculado (HUNGRIA et al., 2003). Nesse mesmo estudo constatou-se que a inoculação do feijão com estirpes competitivas e eficientes na fixação do nitrogênio gera rendimentos semelhantes aos alcançados com a adição de 60 kg N ha^{-1} (HUNGRIA et al., 2003). Posteriormente, em 2007, no estado de Mato Grosso do Sul, a inoculação das sementes de feijão com a estirpe CIAT 899, em conjunto com a adubação nitrogenada (20 kg ha^{-1}), elevou o rendimento de grãos de forma equivalente ao uso de até 160 kg ha^{-1} de N (PELEGRIN et al., 2009). Todos esses resultados comprovam a eficiência da FBN para a cultura do feijoeiro que, se empregada de acordo com as recomendações, pode elevar consideravelmente a produtividade dessa leguminosa no Brasil.

1.3.2 A simbiose *Bradyrhizobium*-soja

Aproximadamente 40% do peso seco da semente de soja é composto por proteínas, gerando elevada demanda por nitrogênio (HUNGRIA et al., 2006).

Apesar da grande influência para a aplicação de fertilizantes nitrogenados, sabe-se que a fixação biológica do nitrogênio pode suprir a necessidade da soja por esse nutriente, garantindo não apenas o teor proteico dos grãos, como também elevadas produtividades (ALVES et al., 2003; HUNGRIA et al., 2006; KASCHUK et al., 2010; DE LUCA et al., 2014,).

Nos últimos anos, diversos experimentos a campo têm comprovado a eficiência da FBN no fornecimento de nitrogênio à soja (ZILLI et al., 2010). Rendimentos superiores a 3500 kg ha^{-1} de grãos são obtidos, sem a adição de fertilizantes nitogenados, por meio da inoculação de sementes com bactérias fixadoras de N (ZILLI et al., 2010). Como comparação, no mesmo estudo, os tratamentos com a adição de fertilizantes nitrogenados levaram a uma produção de 3200 kg ha^{-1}.

Frente aos estudos realizados acerca da FBN em soja, a utilização de inoculantes agrícolas para essa cultura vem crescendo a cada ano (HUNGRIA et al., 2006; HUNGRIA e MENDES, 2015). Atualmente, quatro estirpes são autorizadas pelo Ministério da Agricultura para a formulação de inoculantes para a soja no Brasil; *Bradyrhizobium japonicum* CPAC 15 (=SEMIA 5079), *B. diazoefficiens* CPAC 7 (= SEMIA 5080), *B. elkanii* SEMIA 587 e SEMIA 5019. Em geral, os inoculantes apresentam uma ou duas estirpes e, independente da formulação, todas as quatro estirpes podem suprir a demanda de nitrogênio das cultivares de soja disponíveis para o plantio no Brasil, levando à produção de até 5000 kg ha^{-1} (HUNGRIA et al., 2009).

Por fim, a utilização de bactérias eficientes na fixação de nitrogênio e tolerantes às condições ambientais adversas pertencentes ao gênero *Bradyrhizobium*, em detrimento à aplicação de adubos nitrogenados, possibilita a economia de aproximadamente 15 bilhões de dólares por safra (HUNGRIA e MENDES, 2015). Além dessa vantagem econômica, a FBN reduz os impactos ambientais gerados pela fertilização nitogenada, previamente reportada, enquadrando-se nos princípios da sustentabilidade (VANCE, 2001; ORMEÑO-ORRILLO et al., 2012).

1.4 FATORES LIMITANTES À FBN

1.4.1 Instabilidade genética e estresses ambientais

Bactérias do gênero *Rhizobium* que estabelecem simbiose com o feijoeiro apresentam elevada instabilidade genética, caracterizada pelos rearranjos genéticos, deleções de plasmídios e mutações. Os rearranjos genéticos ocorrem devido à presença de sequências que atuam como sítios de recombinação no DNA cromossomal (FLORES et al., 1988). A deleção de plasmídeos pode afetar diretamente a FBN, isso porque nas linhagens de *Rhizobium* os genes responsáveis pela nodulação e fixação de nitrogênio estão alocados em um único replicon, denominado de plasmídeo simbiótico *(psym)* (ORMEÑO-ORRILLO et al., 2012). Outro evento genético que também pode alterar as propriedades simbióticas destes microrganismos são as mutações, entretanto, a menos que estas tenham um efeito competitivo, dificilmente assumem dominância em uma colônia.

A instabilidade genética das estirpes simbiontes do feijoeiro é acentuada quando expostas a condições de estresse (ZURKOWSKI et al., 1982; DJORDJEVIC et al. 1983; BERRY e ATHERLY, 1984). Dentre os fatores que contribuem para o aumento da instabilidade genética destes microssimbiontes destacam-se as temperaturas elevadas, o pH ácido e a salinidade dos solos (DUDEJA e KHURANA, 1989; HUNGRIA e FRANCO, 1993).

Diferentemente das bactérias do gênero *Rhizobium,* as classificadas como *Bradyrhizobium*, em geral, não apresentam problemas decorrentes da instabilidade genética. Essa característica está diretamente relacionada com a disposição do conteúdo genético que, nessas bactérias, compõe um único cromossomo onde os genes essenciais para o sucesso da FBN estão inseridos (SIQUEIRA et al., 2014). Mesmo com maior estabilidade genética, as estirpes de *Bradyrhizobium* aplicadas em inoculantes para a soja também sofrem com as condições ambientais desfavoráveis e, por isso, além da elevada capacidade em fixar nitrogênio, são selecionadas de acordo com a tolerância a condições de estresse (HUNGRIA e VARGAS 2000; BATISTA et al., 2006; HUNGRIA et al., 2006; TORRES et al., 2012).

A importância de selecionar bactérias geneticamente estáveis e tolerantes a condições desfavoráveis está diretamente relacionada ao cultivo das leguminosas para as quais a FBN é empregada. Em geral, as lavouras dessas plantas ocorrem em regiões tropicais, onde os solos são lixiviados, ácidos e inférteis, além de conterem concentrações tóxicas de alumínio e

manganês (LAL, 1993). Essas características atingem diretamente a planta, a bactéria e a simbiose, limitando a sobrevivência, a persistência e a multiplicação das bactérias diazotróficas (BALA et al., 2003).

Entre os fatores que limitam a FBN já citados está a acidez dos solos, uma vez que o pH ótimo para o crescimento das bactérias pertencentes à família *Rhizobiaceae* está na faixa de 6.0 a 7.0 (JORDAN, 1984). Poucas espécies apresentam representantes capazes de crescer em faixas de pH abaixo de 5.0, dentre essas destacam-se *Rhizobium tropici* (GRAHAM et al., 1994), *Rizobium freirei* (DALL'AGNOL et al., 2013) e *Bradyrizobium japonicum* (BATISTA et al., 2012). A tolerância ao pH ácido apresentada por algumas espécies de bactérias diazotróficas parece depender da capacidade de manterem o pH intracelular (pHi) entre 7.2 e 7.5 (O'HARA et al., 1989). Por exibirem essa característica, estirpes pertencentes à espécie *R. tropici* apresentam elevada ocorrência em solos ácidos da América Central, Brasil e África (HUNGRIA et al., 2000).

Tão limitante quanto a acidez, as altas temperaturas dos solos prejudicam fortemente a simbiose entre bactérias diazotróficas e plantas leguminosas. O processo de nodulação das raízes de leguminosas, entre elas o feijoeiro e a soja, ocorre entre as temperaturas de 25 a 30 °C, de modo que a eficiência deste processo é reduzida em temperaturas acima ou abaixo dessa faixa (HERNANDEZ-ARMENTA et al., 1989). Contudo, é comum que os solos localizados entre os trópicos atinjam 40°C (MARTÍNEZ-ROMERO, 1991; MICHIELS et al., 1994). Neste cenário, é fundamental que as bactérias fixadoras de nitrogênio mantenham a eficiência do processo de fixação biológica do nitrogênio, uma vez que alterações ou perdas das propriedades simbióticas comprometem a utilização destas em inoculantes agrícolas (MARTÍNEZ-ROMERO, 1991; MICHIELS et al., 1994).

A salinidade do solo também representa uma condição prejudicial à FBN. Quando estão em solos salinos, as bactérias perdem água para o ambiente, resultando em distúrbios osmóticos nocivos às atividades metabólicas (CHALLOUGUI et al., 2010). Além disso, muitos efeitos negativos na interação simbiótica entre plantas leguminosas e seus microssimbiontes têm sido atribuídos à salinidade. Problemas no crescimento e sobrevivência das bactérias no solo, na colonização das raízes e no desenvolvimento de nódulos

são ocasionados por essa condição de estresse, limitando a FBN (KULKARNI et al., 2000).

1.4.2 Tratamento das sementes

Muitas doenças atingem as plantas e geram prejuízos aos agricultores. Na soja, por exemplo, mais de 40 doenças causadas por vírus, fungos e nematoides ocasionam perdas anuais de aproximadamente 20% (Embrapa 2006). Como a maior parte dos agentes responsáveis por essas doenças encontram-se nas sementes, atualmente é comum tratá-las com fungicidas antes do plantio. Na tentativa de eliminar um problema surge outro; a incompatibilidade desses fungicidas com as bactérias fixadoras de nitrogênio presentes nos inoculantes. Apenas duas horas na presença de fungicidas utilizados nas sementes, mais de 62% das bactérias morrem. Vinte e quatro horas depois, somente 5% das bactérias sobrevivem. Além de afetarem a sobrevivência dos simbiontes, os fungicidas também reduzem o número de nódulos e o nitrogênio total nas sementes (CAMPO et al., 2009).

Outra prática comum é a adição de micronutrientes nas sementes, entre eles o molibdênio. Assim como o que ocorre com a aplicação dos fungicidas, a toxicidade das fontes deste composto afeta negativamente as bactérias presentes nos inoculantes, resultando na redução do número de nódulos e, no caso da soja, no rendimento de grãos (CAMPO et al., 2009).

1.5 CARACTERIZAÇÃO FUNCIONAL

Durante anos foi investido muito tempo e esforço no sequenciamento do genoma de diversos organismos, com o objetivo de elucidar completamente o mecanismo de funcionamento celular a nível molecular (VILLAS-BÔAS e GOMBERT, 2006). É verdade que esses estudos geraram muitas informações. Mesmo assim, o conhecimento acumulado com os sequenciamentos de genomas trouxe adiante um novo desafio, entender como e porque os genes são expressos e qual a implicação funcional da expressão gênica.

Na era denominada pós-genômica, diferentes abordagens têm possibilitado o estudo funcional dos organismos, agregando novos conhecimentos à biologia desses. Neste contexto, a transcriptômica e a proteômica têm tido destaque, pois possibilitam detectar adaptações

metabólicas dos organismos em diferentes condições (SUBRAMANIAN e SMITH, 2013). Nesse sentido, por apresentarem grande importância ambiental e econômica, bactérias fixadoras de nitrogênio passaram a ser caracterizadas e selecionadas com o auxílio dessas metodologias.

1.5.1 Transcriptoma

Os estudos transcriptômicos fornecem informações qualitativas e quantitativas do conteúdo de RNA de uma célula, tecido ou organismo. Em alguns casos, todos os transcritos são objeto de estudo, independentemente de sua estrutura ou função. Outros estudos focam em subconjuntos do transcriptoma, como os RNAs mensageiros (mRNA) ou os micro RNAs (miRNA).

Atualmente, três técnicas são empregadas para o estudo do transcriptoma; O PCR quantitativo em tempo real (RT-qPCR), os microarranjos e o RNA-seq. Por meio do RT-qPCR é possível quantificar precisamente o transcrito de interesse em diferentes amostras (GINZINGER, 2002). A limitação dessa abordagem se dá pela dificuldade em trabalhar com muitos alvos simultaneamente. Os microarranjos e o RNA-seq permitem estudar vários transcritos de uma só vez, entretanto, no primeiro apenas os transcritos presentes no arranjo serão analisados. No RNA-seq é possível realizar análises ditas “imparciais”, sendo ideal para novas descobertas. O custo elevado da tecnologia e a dificuldade em analisar os resultados são os pontos negativos dessa abordagem.

Entre as diferentes tecnologias para o estudo de transcritos, o RT-qPCR é bastante acessível, tanto pela praticidade de preparação da amostra, como na análise dos resultados. Através da PCR em tempo real é possível monitorar a amplificação do alvo a cada ciclo devido à emissão de uma determinada quantidade de fluorescência. A intensidade da fluorescência emitida é proporcional à quantidade do fragmento amplificado, aumentando exponencialmente em cada ciclo da reação. Desse modo, é possível quantificar o produto gerado a cada ciclo, sendo que na fase exponencial da reação a quantidade de fluorescência gerada é proporcional à quantidade inicial do fragmento alvo. Em outras palavras, durante a fase exponencial de amplificação é possível determinar um valor de intensidade de fluorescência,

na qual todas as amostras podem ser comparadas. Este valor é denominado limiar ou *threshold* e é calculado em função da quantidade de fluorescência basal. Neste ponto, o sinal de fluorescência gerado pela amostra é significativamente maior que a fluorescência basal. A quantidade de ciclos de PCR requerida para que cada amostra emita fluorescência suficiente para alcançar este ponto é definida como *cycle threshold* ou Ct. O Ct é específico para cada amostra e é inversamente proporcional à quantidade inicial do alvo presente na reação. Este valor é a base para a quantificação baseada em PCR quantitativo (MARCELINO, 2006).

1.5.2 Proteoma

Em 1975 foram realizados os primeiros estudos caracterizados como proteômicos nos quais foi empregada a técnica de eletroforese bidimensional, desenvolvida, independentemente, por O'Farrel (1975), Klose (1975) e Scheele e Palade (1975). Entretanto, o termo proteoma foi sugerido pela primeira vez apenas em 1993 por Mark Wilkins e Keith Williams, que utilizaram como definição para este "identificação sistemática das proteínas totais expressas por um genoma" (LOPES, 1999). Com um melhor entendimento das possibilidades oferecidas por essa abordagem, o termo proteoma foi redefinido como; "a caracterização em larga escala do conjunto de proteínas expressas por um organismo, célula ou tecido em um determinado momento" (WILKINS et al., 1997).

Na atualidade, o principal objetivo dos estudos proteômicos consiste em obter uma visão global e integrada, examinando simultaneamente as proteínas expressas em uma determinada condição (GRAVES e HAYSTEAD, 2002). Entretanto, é importante salientar que a proteômica também permite analisar as propriedades das proteínas, seus níveis de expressão, suas funções e as modificações pós-traducionais (BLACKSTOCK e WEIR, 1999). Para explorar todas essas possibilidades é necessário o envolvimento de diferentes áreas do conhecimento, tais como a biologia molecular, a bioquímica e a bioinformática.

Uma questão importante na caracterização do proteoma de um organismo é a dinâmica que este apresenta, variando de acordo com o estado fisiológico das células e com os estímulos internos e externos. Deste modo, as proteínas podem sofrer modificações pós-traducionais, translocação ou, ainda,

terem suas concentrações alteradas, devido á síntese ou degradação e revelando, assim, os processos em que estas estão envolvidas. Apesar das proteínas serem produtos da expressão de um gene, as modificações pós-traducionais permitem que elas ocorram em múltiplas formas nas células, de modo que estas modificações não podem ser observadas, nem mesmo previstas pelo estudo do genoma, que é sempre o mesmo em todas as células de um organismo (GRAVES e HAYSTEAD, 2002).

Com o decorrer do tempo, novas tecnologias aplicadas aos estudos proteômicos foram desenvolvidas. Alguns desses métodos apresentam grande poder em separar e identificar proteínas de amostras complexas, resultando em estudos valiosos. Entre esses, vários foram conduzidos para a caracterização de bactérias fixadoras de nitrogênio, tanto em vida livre como em simbiose (DELMOTE et al., 2010; MENESES et al., 2010). Mesmo diante dessas novas abordagens proteômicas, estudos que empregam a eletroforese em gel bidimensional foram aprimorados, gerando dados importantes, incluindo a caracterização funcional de diversos aspectos relacionados às bactérias diazotróficas (HEMPEL et al., 2009; BATISTA et al., 2010; BATISTA e HUNGRIA, 2012).

2 OBJETIVOS

2.1 OBJETIVO GERAL

Caracterizar o perfil de proteínas expressas por *Rhizobium freirei* PRF 81 e *Bradyrhizobium diazoefficiens* CPAC 7 através da análise proteômica bidimensional e espectrometria de massas MALDI-TOF-MS/MS.

2.2 OBJETIVOS ESPECÍFICOS

- Estabelecer os mapas proteomicos de referência para as estirpes PRF 81 e CPAC 7;
- Identificar proteínas relacionadas com etapas importantes para o estabelecimento da simbiose;
- Identificar fatores importantes para a adaptação e tolerância a condições de estresse ambiental, características comuns entre as estirpes estudadas;
- Realizar a inferência funcional de proteínas hipotéticas identificadas no perfil proteômico da estirpe CPAC 7;
- Determinar o padrão de expressão dos genes para proteínas hipotéticas de *B. diazoefficiens* CPAC 7 na presença do flavonoide genisteína.

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4 CAPÍTULO 1

DATASET BRIEF

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Two-dimensional proteome reference map of *Rhizobium tropici* PRF 81 reveals several symbiotic determinants and strong resemblance with agrobacteria

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List of Abbreviations: HCCA: α-cyano- 4-hydroxy-cinnamic acid, COG: Clusters of orthologous groups, TBDR: TonB-dependent receptor, EPS: exopolysaccharide, EPSI: succinoglycan exopolysaccharide, Tat: twin-arginine translocation system, Omp: Outer membrane lipoprotein, T4SS: Type IV secretion system.

Key words: Nitrogenase, proteomics reference map, symbiosis.

Abstract

Rhizobium tropici strain PRF 81 is used in commercial inoculants for common-bean crops in Brazil due its high efficiency in nitrogen fixation and, as in other strains belonging to this species, its tolerance of environmental stresses, representing a useful biological alternative to chemical nitrogen fertilizers. In this study, a proteomic reference map of PRF 81 was obtained by 2-DE and MALDI-TOF/TOF-TOF mass spectrometry. In total, 115 spots representing 109 different proteins were successfully identified, contributing to a better understanding of the rhizobia-legume symbiosis and supporting, at proteomics level, a strong resemblance with agrobacteria.

Article

Alternative technologies to improve agricultural productivity are necessary; however, many of them are harmful to the environment. In contrast, biological nitrogen fixation by rhizobia-legume symbiosis represents an environmentally friendly and economically sound strategy to achieve high yields [1]. *Rhizobium tropici* strain PRF 81 (=SEMIA 4080) is recognized by both its high efficiency in fixing nitrogen in association with common bean (*Phaseolus vulgaris* L.), and its high tolerance to environmental stresses [2]. These features, in addition to the higher genetic stability in comparison with other common bean rhizobia [2], resulted in this strain being broadly used in inoculants in Brazil [1]. The genome of PRF 81 [3] is now in progress (http://www.bnf.lncc.br), but proteomic studies are fundamental to understand gene expression. In this study, we present a two-dimensional (2-DE) proteomic reference map of *R. tropici* PRF 81, which allowed the identification of several molecular determinants related to the symbiosis and highlighted a close phylogenetic relationship with strains of *Agrobacterium* (=*Rhizobium*).

R. tropici strain PRF 81, isolated from common bean nodules in Brazil, is deposited at the *"*Diazotrophic and Plant Growth Promoting Bacteria Culture Collection*"* of Embrapa Soja. More details about the strain are given elsewhere [1, 3]. To achieve the whole-cell protein extract, the bacterium was grown in TY medium and proteins were extracted as described by Batista et al. [4], except for that forty cycles of freezing and thawing were used.

The whole protein extract was purified with phenol and dissolved with DeStreak buffer (GE Healthcare) to a final concentration of 300 µg. For 2-DE, performed in triplicate, the procedure was as described before [4], modified only by the IPG-strips pH range (pH 4–7). After being stained with Coomassie Blue PhastGel^{TM} R-350 (GE Healthcare), the gels were analyzed by Image Master 2D Platinum v 5.0 (GE Healthcare). Well-defined spots, present in all three gels were selected, excised and processed as described before [5]. Digestion was achieved with trypsin (Gold Mass Spectrometry Grade, Promega) at 37ºC overnight.

Mass spectra were acquired in a MALDI-TOF-TOF Autoflex Spectrometer (Bruker Daltonics), which 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 Flex Control software v. 2.2 and processed using Flex Analysis v. 3.0 (Bruker Daltonics, Billerica, MA). The PMFs and MS/MS ion spectra generated were searched against the public database NCBInr using Mascot software search engine v. 2.3 (http://www.matrixscience.com/) as previously described [4]. Identifications, available at PRIDE (http://ebi.ac.uk/pride/) with the experiment accession number 14817, were validated only when the Mowse (molecular weight search) score was significant, and both decoy score and false discovery rates were considered.

To improve the separation of *R. tropici* PRF 81 proteins, we employed IPG strips with a narrow pH range, 4.0 to 7.0. This procedure achieved better protein resolution than with a broader range, of pH 3.0 to 10.0 (data not shown). Among 880 spots detected on gels, 150 were randomly selected and 115 spots, representing 109 different proteins, were successfully identified (Figure 1 - Supporting Information Table S1).

Although most proteins extracted by this methodology are located in cell cytoplasm and periplasmatic space, some membrane and extra cellular proteins can also be obtained. In addition, the presence of distinct spots for the same protein may be the result of post-translational modifications, e.g., glutamine synthetase I (spots 12, 13 and 14), which in *Escherichia coli* is an example of post-translational regulated enzyme [6].

According to the functional classification in COG, proteins were distributed in 17 categories, belonging to four functional groups: metabolic functions (C, E,

F, G, H, I, P); cellular processes and signaling (D, T, O, M, N); information storage and processing (J, K, L); and poorly characterized proteins (R, S) (Figure 2). Six proteins were classified as hypothetical/conserved hypothetical and only four did not fit in any of the categories, being assigned as “not in COG”.

Besides its ability to establish a symbiotic partnership with common bean, *R. tropici* PRF 81 is also characterized by high competitiveness against indigenous rhizobial populations [1, 7, 8]. This feature is directly related to metabolic plasticity shown *in vitro* by this species [9], which can assimilate a wide range of carbon and nitrogen sources by the Entner-Doudoroff pathway, glycolysis and the tricarboxylic acid cycle [10]. Our proteomic study confirmed metabolic diversity, since more than 51% of identified proteins were associated with metabolic functions. The most representative, grouping 26% of the metabolic proteins, was that of amino acid metabolism, that can directly influence nitrogen fixation in the legume-rhizobia symbiosis [9].

Nitrogen-fixing bacteria also have high requirements for iron, essential for the activity of many enzymes, including those involved in nitrogen fixation—such as nitrogenase, cytochromes and ferredoxin—which contain Fe as their prosthetic groups [11]. Like other microbes, those belonging to rhizobial genera use various strategies to acquire iron, and one of the most well studied is the TonB-dependent receptor (TBDR), identified in our study [12].

Biological nitrogen fixation process involves several intricate steps, initiated by the exchange of molecular signals between the symbionts. Several proteins related to these steps encoded by PRF 81 were evidenced among the 19 proteins distributed in the different categories of the cellular process and signaling functional group, e.g., ChvI. This protein is the response-regulator of a two-component regulatory system that, in response to specifics plant signals, regulates positively the transcription of *exo* genes, encoding enzymes for succinoglycan exopolysaccharide (EPSI), critical for the establishment of the symbiosis [13].

Present in many bacteria, and now reported in PRF 81, the twin-arginine translocation (Tat) system transports folded proteins across the cytoplasmic membrane, including symbiosis and pathogenicity determinants [14]. In

addition, the essential role of the Tat system for the rhizobia-legume symbiosis has been highlighted [15].

Outer membrane lipoprotein (Omp), a peptidoglycan-associated protein, was also identified in our study. Well characterized in *E. coli*, it has a multifunctional role, affecting cell morphology, virulence, and host-pathogen interaction [16]. Omp also acts as a host-cell adhesion factor, and differential expression in *Gluconacetobacter diazotrophicus* co-cultivated with sugarcane plantlets has been reported [17], suggesting that the protein acts in the plant-bacterium interaction.

Also noteworthy are two proteins expressed in PRF 81, VirD4 and ATP-dependent Clp protease (ClpA). VirD4 is a member of the type IV secretion system (T4SS), which in *Agrobacterium tumefacies* participates in DNA processing and transfer reactions [18]. The second, ClpA, is part of the ClpAP protease system, whose importance has been reported in the regulation of bacterial polysaccharide production [19], stress tolerance, cell division and motility [20].

The transcription factors have fundamental roles in controlling metabolic pathways, because they regulate gene-expression levels [17]. In our study, nine were assigned this functional category. Among these, we report the expression of LysR family of transcriptional regulatory proteins, known to act in the establishment of the symbiosis *S. meliloti*-alfalfa (*Medicago sativa* L.), probably through the regulation of *nod*-gene expression [21]; a similar role might be found in *R. tropici*.

Several antioxidant factors were identified in our study, among them alkyl hydroperoxide reductase that plays an important role in cell defense against oxidative stress, since it uses H_2O_2 as substrate [22]. Reactive oxygen species (ROS) are by-products of normal metabolic processes and at high levels were first thought to be lethal for cells. However, in both symbiotic and pathogenic relations, transient production of ROS, detected in early events of plant-microorganism interactions, may be considered as specific signals during the interaction process [23]; therefore, it should be overcome by the microsymbionts. Other two proteins encoded by PRF 81 that show antioxidant activity were the bacterioferritin (Bfr) and bacterioferritin comigratory (Bcp) proteins.

Species of rhizobia and agrobacteria are closely related, and the inclusion of *Agrobacterium* species in the genus *Rhizobium* has been proposed [24]. Consistent with this, *Rhizobium tropici* strain PRF 81 has demonstrated high resemblance in many genes [3], and now also of many proteins to those of strains belonging to the genus *Agrobacterium*.

Among the proteins resembling agrobacteria, it is worth mentioning the LysR family transcriptional regulator. In agrobacteria they are related to *chvE* gene expression, the product of which induces a set of bacterial virulence (*vir*) genes that are necessary for plant infection [25]. Another protein now identified at proteomic level, and previously identified in the draft genome of PRF 81 [3] was the VirD4, playing a role in the DNA transfer from agrobacteria to the host plant [26].

Noteworthy is also that most of the PRF 81 proteins matched—with high scores—*Agrobacterium* proteins (Figure 3), even though the number of peptide sequences of *Rhizobium* in the NCBI database is about three times higher than that of *Agrobacterium*. Therefore, our data strengthen previous observations that *Rhizobium tropici* and *Agrobacterium* share a high level of resemblance.

In this paper, we present a two-dimensional reference map of *R. tropici* strain PRF 81, an agronomically important nitrogen-fixing microsymbiont that is poorly characterized at the molecular level. Various molecular determinants of the establishment of symbiosis between *R. tropici* and common bean are highlighted, contributing to a better understanding of the symbiosis. We have also reported important proteins related to cell protection against several stresses. Finally, we have shown that *R. tropici* presents high resemblance with agrobacteria, as previously observed at the genomic level [3], and now for the first time confirmed in a proteomic study.

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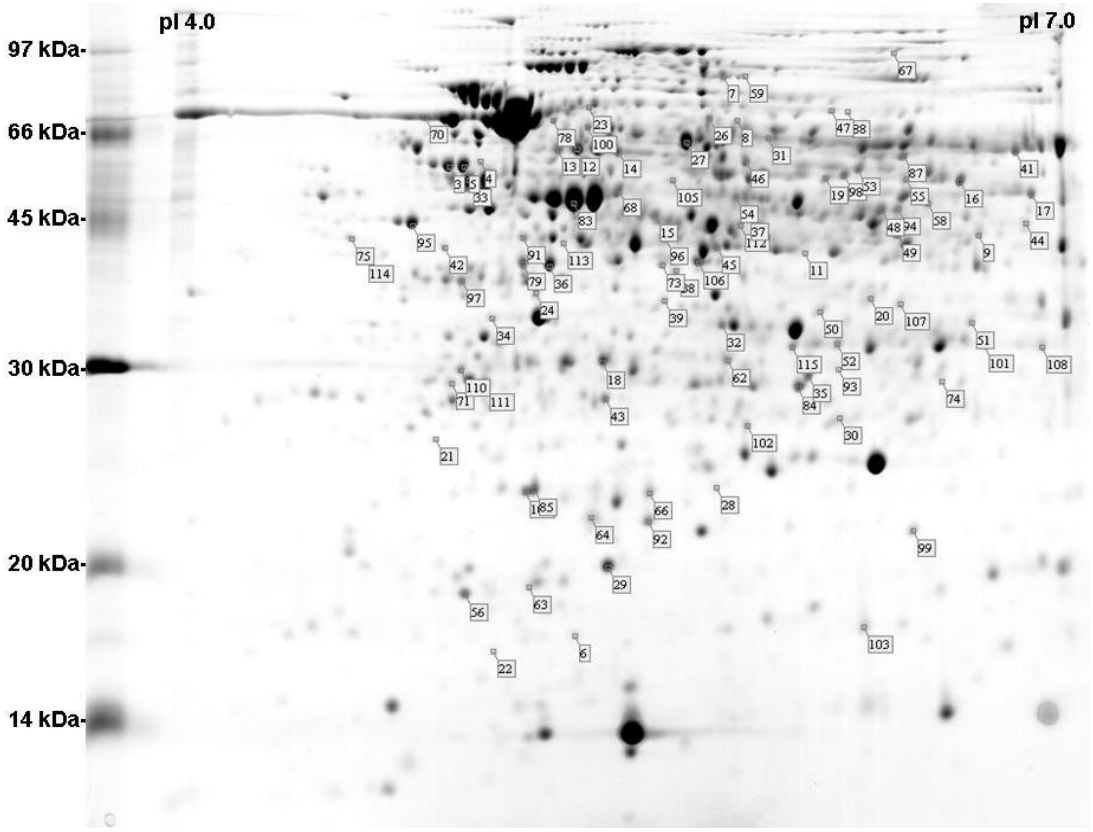

Figure 1: 2-DE protein profile of *Rhizobium tropici* strain PRF 81 whole cell extract. More information about identified proteins assigned is available in supporting information Table S1.

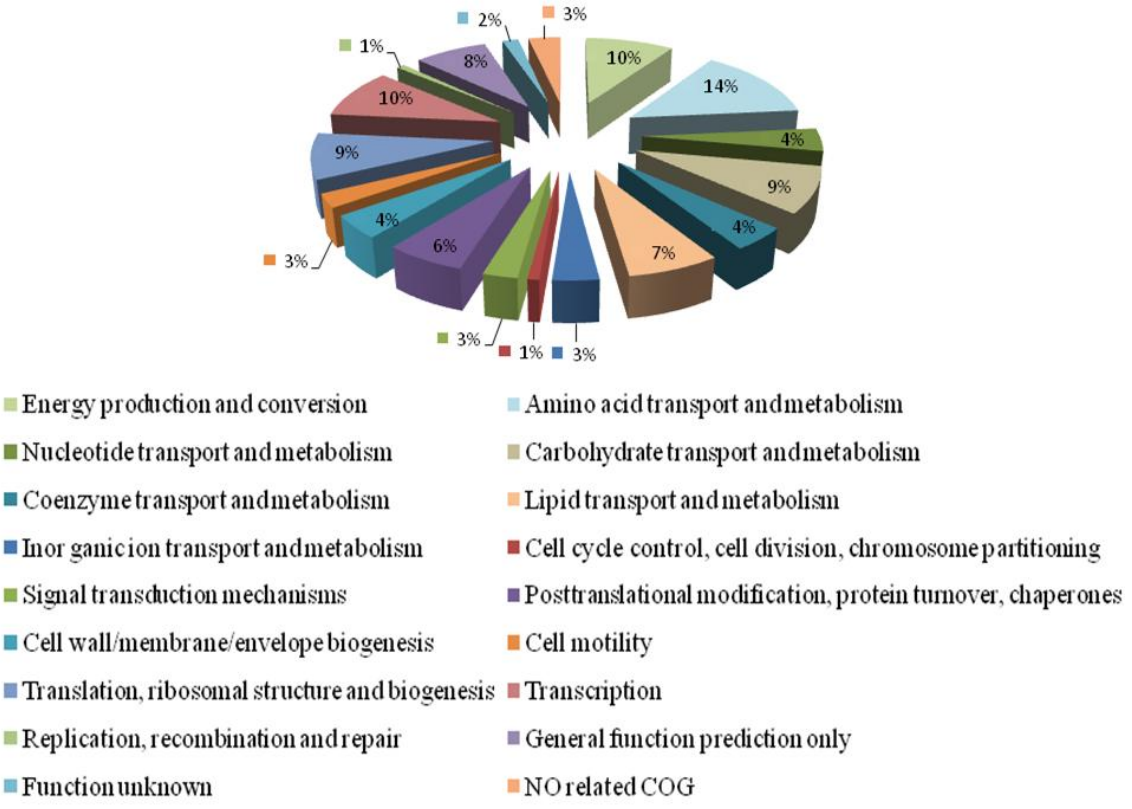

Figure 2: Distribution of *R. tropici* PRF 81 proteins identified by MS into COG categories.

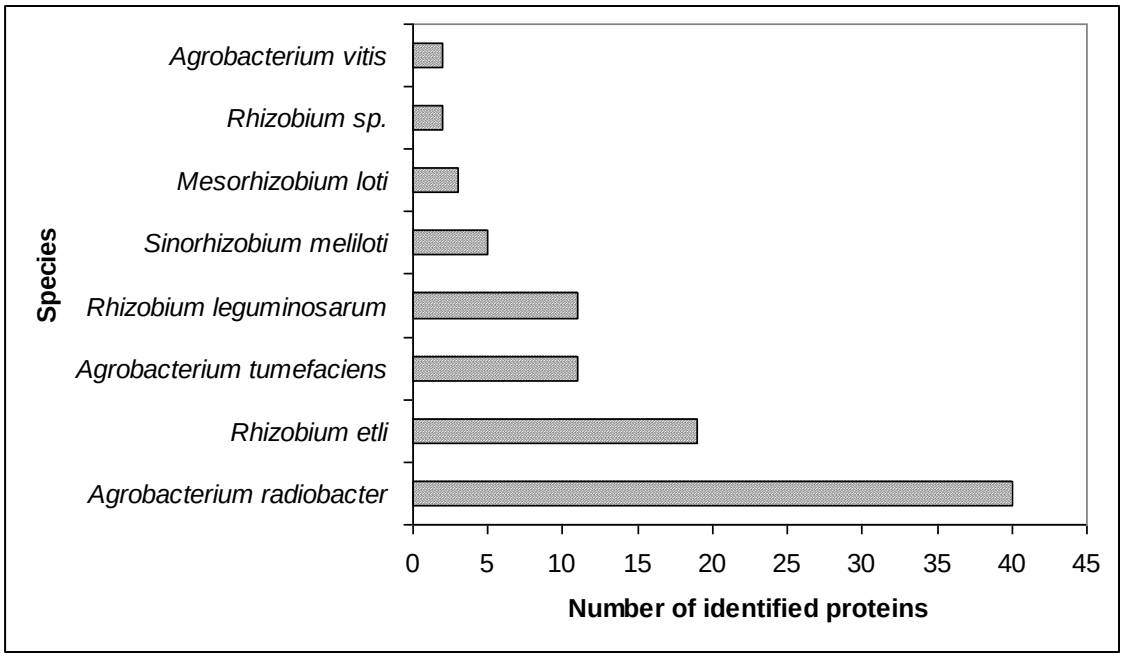

Figure 3: Amount of proteins identified with higher score for each different species. Species with only one protein identified are not shown.

Supplementary file

Table S1: Identified proteins of *Rhizobium tropici* PRF 81 whole cell extracts. Matched peptides masses and MS/MS combined results are available in PRIDE (http://ebi.ac.uk/pride/) under the experiment accession number 14817.

Spot ID	NCBI ID	Gene	Protein description	Organism	*T. pI	**E. pI	T. mass	E. mass	Cellular location
Metabolism									
C - Energy production and conversion									
1	gi|222087461	*sucC*	Succinyl-CoA synthetase beta subunit protein	*Agrobacterium radiobacter*	4,98	4,96	42028	46000	Cytoplasmic
2	gi|86359524	*acnA*	Aconitate hydratase	*Rhizobium etli*	5,48	5,69	97180	98000	Cytoplasmic
3	gi|116254139	*atpD*	F0F1 ATP synthase subunit beta	*Rhizobium leguminosarum*	5,03	4,88	50885	56000	Cytoplasmic
4	gi|15966787	*atpD*	F0F1 ATP synthase subunit beta	*Sinorhizobium meliloti*	5,35	4,99	53250	57000	Cytoplasmic
5	gi|227823626	*atpD*	ATP synthase F1, beta subunit	*Rhizobium sp.*	5,27	4,94	53408	56000	Cytoplasmic
6	gi|86359450	*atpC*	F0F1 ATP synthase subunit epsilon	*Rhizobium etli*	5,21	5,32	15097	17000	Cytoplasmic
7	gi|222085583		NADH-quinone oxidoreductase, chain G	*Agrobacterium radiobacter*	5,44	5,83	75528	85000	Cytoplasmic
8	gi|222086219	*fumB*	Fumarate hydratase class I protein	*Agrobacterium radiobacter*	6,01	5,88	59014	69000	Cytoplasmic
9	gi|13471013		Glutathione dependent formaldehyde dehydrogenase	*Mesorhizobium loti*	12	6,71	40481	43000	Cytoplasmic
10	gi|222087510	*acnA*	Aconitate hydratase 1	*Agrobacterium radiobacter*	5,23	5,76	98443	97000	Cytoplasmic
11	gi|222086991		Oxidoreductase protein	*Agrobacterium radiobacter*	5,66	6,11	36032	41000	Cytoplasmic
E- Amino acid transport and metabolism									
12	gi|1245379	*glnA*	Glutamine synthetase I	*Sinorhizobium meliloti*	5,2	5,33	52287	61000	Cytoplasmic
13	gi|153009417	*glnA*	Glutamine synthetase I	*Ochrobactrum anthropi*	5,38	5,26	52469	61000	Cytoplasmic
14	gi|6601379	*glnA*	Glutamine synthetase	*Rhizobium leguminosarum*	6,07	5,47	34686	60000	Cytoplasmic
15	gi|222087908	*Asd*	Aspartate-B-semialdehyde dehydrogenase protein	*Agrobacterium radiobacter*	5,46	5,59	37925	45000	Cytoplasmic
16	gi|209551290		Argininosuccinate synthase	*Rhizobium leguminosarum*	6,00	6,64	45203	53000	Cytoplasmic
17	gi|86357146	*glyA*	Serine hydroxymethyltransferase	*Rhizobium etli*	6,29	6,89	46740	50000	Cytoplasmic
18	gi|15887731	*argB*	Acetylglutamate kinase	*Agrobacterium tumefaciens*	5,16	5,41	31083	30000	Cytoplasmic
19	gi|241204468		Homoserine dehydrogenase	*Rhizobium leguminosarum*	5,39	6,18	47035	53000	Cytoplasmic
20	gi|15964271	*argF1*	Ornithine carbamoyltransferase	*Sinorhizobium meliloti*	6,09	6,34	33643	36000	Cytoplasmic
21	gi|86359669	*leuD*	Isopropylmalate isomerase small subunit	*Rhizobium etli*	5,02	4,83	22397	26000	Cytoplasmic
22	gi|22208582	*gloA*	Lactoylglutathione lyase	*Agrobacterium radiobacter*	5,03	5,03	16541	16000	Cytoplasmic

23	gi|116253255	*leuA*	2-Isopropylmalate synthase	*Rhizobium leguminosarum*	5,15	5,35	62565	73000	Cytoplasmic
24	gi|222087414	*rocF*	Arginase	*Agrobacterium radiobacter*	5,3	5,17	33045	36000	Cytoplasmic
25	gi|16264577	*hutU*	Urocanate hydratase	*Sinorhizobium meliloti*	5,94	5,87	61859	86000	Cytoplasmic
26	gi|190893841		Dipeptide ABC transporter	*Rhizobium etli*	5,23	5,78	60139	70000	Periplasmic
27	gi|222084939		Dipeptide ABC transporter	*Agrobacterium radiobacter*	5,21	5,7	59112	62000	Periplasmic
F- Nucleotide transport and metabolism									
28	gi|159184890	*Gtp*	Xanthine-guanine phosphoribosyltransferase	*Agrobacterium tumefaciens*	5,35	5,8	18487	23000	Cytoplasmic
29	gi|116251346	*Ndk*	Nucleoside diphosphate kinase	*Rhizobium leguminosarum*	5,65	5,43	15314	20000	Cytoplasmic
30	gi|190889872	*upp*	Uracil phosphoribosyltransferase protein	*Rhizobium etli*	5,98	6,23	23305	27000	Cytoplasmic
31	gi|209551875	*guaA*	GMP synthase	*Rhizobium leguminosarum*	5,56	5,98	57742	64000	Cytoplasmic
32	gi|86146888	*pyrH*	Uridylate kinase	*Vibrio sp.*	5,08	5,82	26284	33000	Cytoplasmic
G - Carbohydrate transport and metabolism									
33	gi|222085874	*eno*	Phosphopyruvate hydratase	*Agrobacterium radiobacter*	4,84	4,95	45120	53000	Cytoplasmic
34	gi|282887091		Alpha amylase catalytic region	*Burkholderia sp.*	6,26	5,03	64245	34000	Cytoplasmic
35	gi|241206422	*talB*	Transaldolase	*Rhizobium leguminosarum*	5,32	6,12	35091	29000	Cytoplasmic
36	gi|91975363		Polysaccharide deacetylase	*Rhodopseudomonas palustris*	8,31	5,23	39900	39000	Cytoplasmic
37	gi|222086498		Sugar ABC transporter	*Agrobacterium radiobacter*	5,39	5,91	46099	45000	Periplasmic
38	gi|86356131	*Frk*	Fructokinase protein	*Rhizobium etli*	5,22	5,66	32940	39000	Cytoplasmic
39	gi|222084754	*frcB*	Fructose ABC transporter	*Agrobacterium radiobacter*	5,64	5,62	35424	36000	Periplasmic
40	gi|86359086	*Tkt*	Transketolase	*Rhizobium etli*	6,07	6,49	70055	85000	Cytoplasmic
41	gi|222084912	*Zwf*	Glucose-6-phosphate 1-dehydrogenase	*Agrobacterium radiobacter*	6,13	6,84	55064	60000	Cytoplasmic
42	gi|222084346		Sugar kinase protein	*Agrobacterium radiobacter*	4,7	4,87	36089	41000	Cytoplasmic
H - Coenzyme transport and metabolism									
43	gi|296105270		Biotin--protein ligase	*Enterobacter cloacae*	5,23	5,42	35255	28000	Cytoplasmic
44	gi|159184833		Coproporphyrinogen III oxidase	*Agrobacterium tumefaciens*	6,88	6,67	50241	44000	Cytoplasmic
45	gi|148265868		Phosphomethylpyrimidine kinase	*Geobacter uraniireducens*	5,8	5,81	52613	41000	Cytoplasmic
46	gi|209551535		S-Adenosyl-L-homocysteine hydrolase	*Rhizobium leguminosarum*	5,49	5,9	51007	57000	Cytoplasmic
47	gi|240850082	*Dxs*	1-Deoxy-D-xylulose-5-phosphate synthase Dxs	*Bartonella grahamii*	6,44	6,2	69040	73000	Cytoplasmic
I – Lipid transport and metabolism									
48	gi|159186213	*pcaF*	Beta-ketoadipyl CoA thiolase	*Agrobacterium tumefaciens*	5,51	6,37	41850	46000	Cytoplasmic
49	gi|261823376	*pldB*	Lysophospholipase	*Pectobacterium wasabiae*	6,43	8,22	39779	43000	Inner membrane

50	gi|197122598		Acyl-CoA dehydrogenase domain protein	*Anaeromyxobacter sp.*	5,7	6,17	40084	35000	Cytoplasmic
51	gi|15966435		Acetyl-CoA carboxylase carboxyltransferase subunit alpha	*Sinorhizobium meliloti*	6,04	6,68	34716	34000	Cytoplasmic
52	gi|222085109	*fabI2*	Enoyl-(acyl-carrier-protein) reductase (NADH) protein	*Agrobacterium radiobacter*	5,7	6,22	29153	32000	Cytoplasmic
53	gi|222085639		Acetyl-CoA carboxylase, biotin carboxylase	*Agrobacterium radiobacter*	5,64	6,29	49554	55000	Cytoplasmic
54	gi|13474638		3-Oxoacyl-(acyl carrier protein) synthase I	*Mesorhizobium loti*	5,5	5,87	43201	49000	Cytoplasmic
55	gi|222085444	*fabF1*	3-Oxoacyl-(acyl-carrier protein) synthase II	*Agrobacterium radiobacter*	5,73	6,46	44384	53000	Cytoplasmic
P - Inor ganic ion transport and metabolism									
56	gi|222087891	*Bfr*	Bacterioferritin	*Agrobacterium radiobacter*	4,81	4,94	16860	19000	Cytoplasmic
57	gi|87199081		TonB-dependent receptor	*Novosphingobium aromaticivorans*	5,82	5,01	87810	75000	Extra Cellular
58	gi|238795940		Glucans biosynthesis protein	*Yersinia mollaretii*	6,41	6,53	58198	48000	Periplasmic
59	gi|46580895		ABC transporter, ATP-binding protein	*Desulfovibrio vulgaris*	6,04	5,9	67370	86000	Cytoplasmic
Cellular processes and signaling									
D - Cell cycle control, cell division, chromosome partitioning									
60	gi|222086436	*ftsZ2*	Cell division protein FtsZ	*Agrobacterium radiobacter*	5,21	5,39	63014	81000	Cytoplasmic
T - Signal transduction mechanisms									
61	gi|222087232	*prkA*	Serine protein kinase protein	*Agrobacterium radiobacter*	5,42	5,69	74417	84000	Cytoplasmic
62	gi|159184131	*chvI*	Two component response regulator	*Agrobacterium tumefaciens*	5,56	5,85	27253	30000	Cytoplasmic
63	gi|209549558		Transcriptional regulator, TraR/DksA family	*Rhizobium leguminosarum*	5,15	5,16	16338	19000	Cytoplasmic
O - Posttranslational modification, protein turnover, chaperones									
64	gi|118590060	*Bcp*	Bacterioferritin comigratory protein	*Stappia aggregata*	5,63	5,37	16749	22000	Cytoplasmic
65	gi|222085003	*groEL*	Chaperonin GroEL	*Agrobacterium radiobacter*	5,03	5,11	57836	69000	Cytoplasmic
66	gi|53716307	*ahpC*	Alkyl hydroperoxide reductase, subunit c	*Burkholderia mallei*	5,17	5,57	20749	23000	Cytoplasmic
67	gi|222085851	*clpA*	ATP-dependent Clp protease	*Agrobacterium radiobacter*	5,93	6,41	92748	97000	Cytoplasmic
68	gi|15888589	*clpX*	ATP-dependent protease ATP-binding subunit	*Agrobacterium tumefaciens*	5,3	5,47	47266	49000	Cytoplasmic
69	gi|15888590	*Lon*	ATP-dependent protease LA	*Agrobacterium tumefaciens*	5,63	6,36	88751	100000	Cytoplasmic
70	gi|222085803	*Tig*	Trigger factor protein	*Agrobacterium radiobacter*	4,85	4,78	54566	72000	Cytoplasmic
M - Cell wall/membrane/envelope biogenesis									
71	gi|86359655		Putative metalloendopeptidase protein	*Rhizobium etli*	5,36	4,89	49514	29000	Periplasmic
72	gi|222085864	*omp1*	Outer membrane lipoprotein	*Agrobacterium radiobacter*	5,26	5,76	84589	79000	Outer Membrane

73	gi|222087790	*galE*	UDP-glucose 4-epimerase	*Agrobacterium radiobacter*	5,39	5,62	35969	39000	Cytoplasmic
74	gi|222084436		Hypothetical protein Arad_0321	*Agrobacterium radiobacter*	6,34	6,58	31726	29000	Cytoplasmic
75	gi|103487746		Twin-arginine translocation pathway signal	*Agrobacterium radiobacter*	8,63	4,54	45113	42000	Periplasmic
N - Cell motility									
76	gi|222087318	*secA*	Protein-export translocase protein	*Agrobacterium radiobacter*	5,13	5,37	102186	104000	Cytoplasmic
77	gi|13473495		GTP-binding protein TypA	*Mesorhizobium loti*	5,57	5,47	67143	85000	Cytoplasmic
78	gi|18033179	*virD4*	VirD4	*Agrobacterium tumefaciens*	6,82	5,24	73380	69000	Cytoplasmic
Information storage and processing									
J - Translation, ribosomal structure and biogenesis									
79	gi|222085858	*Tsf*	Translation elongation factor Ts	*Agrobacterium radiobacter*	5,15	5,14	32268	40000	Cytoplasmic
80	gi|227821753	*fusA*	Elongation factor G	*Rhizobium sp.*	5,17	5,3	77966	89000	Cytoplasmic
81	gi|86355771	*pnp*	Polynucleotide phosphorylase/polyadenylase	*Rhizobium etli*	5,2	5,19	77491	89000	Cytoplasmic
82	gi|294624706	*infB*	Translation initiation factor IF-2	*Xanthomonas fuscans*	5,89	5,76	83626	75000	Cytoplasmic
83	gi|218672404		Elongation factor EF-Tu protein	*Rhizobium etli*	4,87	5,31	31884	48000	Cytoplasmic
84	gi|222086595	*rplY*	Ribosomal protein L25, Ctc-form	*Agrobacterium radiobacter*	5,6	6,09	21953	29000	Cytoplasmic
85	gi|86357075	*rplI*	50S ribosomal protein L9	*Rhizobium etli*	4,82	5,18	21057	23000	Cytoplasmic
86	gi|15887437	*pnpA*	Polynucleotide phosphorylase/polyadenylase	*Agrobacterium tumefaciens*	5,21	5,23	77366	89000	Cytoplasmic
87	gi|222085595	*proS*	Prolyl-tRNA synthetase protein	*Agrobacterium radiobacter Sphingopyxis alaskensis*	5,65	6,45	53483	59000	Cytoplasmic
88	gi|222086050	*thrS*	Threonyl-tRNA synthetase	*Agrobacterium radiobacter*	5,59	6,25	75209	72000	Cytoplasmic
K – Transcription									
89	gi|222081230		Transcriptional regulator protein	*Agrobacterium radiobacter*	6,38	5,6	98220	98000	Cytoplasmic
90	gi|190895600		Transcriptional regulator AraC family	*Rhizobium etli*	6,91	5,42	42937	85000	Cytoplasmic
91	gi|222106466		Transcriptional regulator ROK family	*Agrobacterium vitis*	7,03	5,14	41156	42000	Cytoplasmic
92	gi|222082875		Transcriptional regulator MarR family	*Agrobacterium radiobacter*	5,46	5,57	18141	22000	Cytoplasmic
93	gi|222109081		LysR family transcriptional regulator	*Agrobacterium radiobacter*	6,75	5,23	32302	30000	Cytoplasmic
94	gi|218662071	*hrcA*	Heat-inducible transcription repressor	*Rhizobium etli*	5,05	6,43	36513	46000	Cytoplasmic
95	gi|86357329	*rpoA*	DNA-directed RNA polymerase subunit alpha	*Rhizobium etli*	4,75	4,76	37330	44000	Cytoplasmic
96	gi|167836822	*nusA*	Transcription elongation factor NusA	*Burkholderia thailandensis*	4,59	5,63	54873	42000	Cytoplasmic
97	gi|15890078	*Rho*	Transcription termination factor Rho	*Agrobacterium tumefaciens*	5,87	4,93	47017	38000	Cytoplasmic
98	gi|86359702	*Rho*	Transcription termination factor Rho	*Rhizobium etli*	5,86	6,25	47058	54000	Cytoplasmic
99	gi|86357292	*nusG*	Transcription antitermination protein NusG	*Rhizobium etli*	6,01	6,48	20024	21000	Cytoplasmic

L - Replication, recombination and repair									
100	gi|222084927		ATP-dependent RNA helicase protein	*Agrobacterium radiobacter*	9,17	5,36	69955	67000	Cytoplasmic
Poorly characterized									
R - General function prediction only									
101	gi|222082138	*Cpo*	Chloride peroxidase protein	*Agrobacterium radiobacter*	7,88	6,37	34965	32000	Periplasmic
102	gi|186472508	*wrbA*	Flavoprotein WrbA	*Burkholderia phymatum*	6,19	5,91	20930	26000	Cytoplasmic
103	gi|170699364		NADPH-dependent FMN reductase	*Burkholderia ambifaria*	6,71	6,31	8539	17000	Periplasmic
104	gi|194431754	*dkgA*	2,5-Diketo-d-gluconic acid reductase A	*Shigella dysenteriae*	6,22	5,15	19399	23000	Cytoplasmic
105	gi|222085370		Ferredoxin reductase protein	*Agrobacterium radiobacter*	5,88	5,65	43777	53000	Cytoplasmic
106	gi|222087374	*cobS*	Cobalamin synthase protein	*Agrobacterium radiobacter*	5,62	5,74	36882	40000	Cytoplasmic
107	gi|159184816		Hypothetical protein Atu1564	*Agrobacterium tumefaciens*	5,95	5,68	32803	35000	Cytoplasmic
108	gi|222087548		Oxidoreductase protein	*Agrobacterium radiobacter*	6,72	6,93	30503	31000	Cytoplasmic
109	gi|190892610		Probable ABC transporter, ATP-binding Protein	*Rhizobium etli*	5,34	5,62	61173	74000	Cytoplasmic
S – Function unknown									
110	gi|86359066		Hypothetical protein RHE_CH03475	*Rhizobium etli*	4,84	4,92	26773	30000	Cytoplasmic
111	gi|222149801		Hypothetical protein Avi_3814	*Agrobacterium vitis*	5,03	5,01	24632	29000	Periplasmic
NO related COG									
112	gi|209547526		Hypothetical protein Rleg2_5527	*Rhizobium leguminosarum*	6,02	5,89	33584	44000	Cytoplasmic
113	gi|254511108		TPR domain protein	*Rhodobacteraceae bacterium*	5,03	5,28	62677	42000	Cytoplasmic
114	gi|262377567		Conserved hypothetical protein	*Acinetobacter lwoffii*	4,95	4,59	37453	40000	Cytoplasmic
115	gi|222087920		Hypothetical protein Arad_4933	*Agrobacterium radiobacter*	5,89	6,07	26112	31000	Cytoplasmic

* Theoretical and ** Experimental.

5 CAPÍTULO 2

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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 (N_2) (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 N_2 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 N_2-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 N_2 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 transcriptomic 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 pI 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 the result of posttranslational modifications.

Figure 1 Two-dimensional electrophoresis protein profile of *Bradyrhizobium diazoefficiens* CPAC 7 whole cell extract at free-living state. More information about expressed proteins assigned is available in Tables 1 and 2.

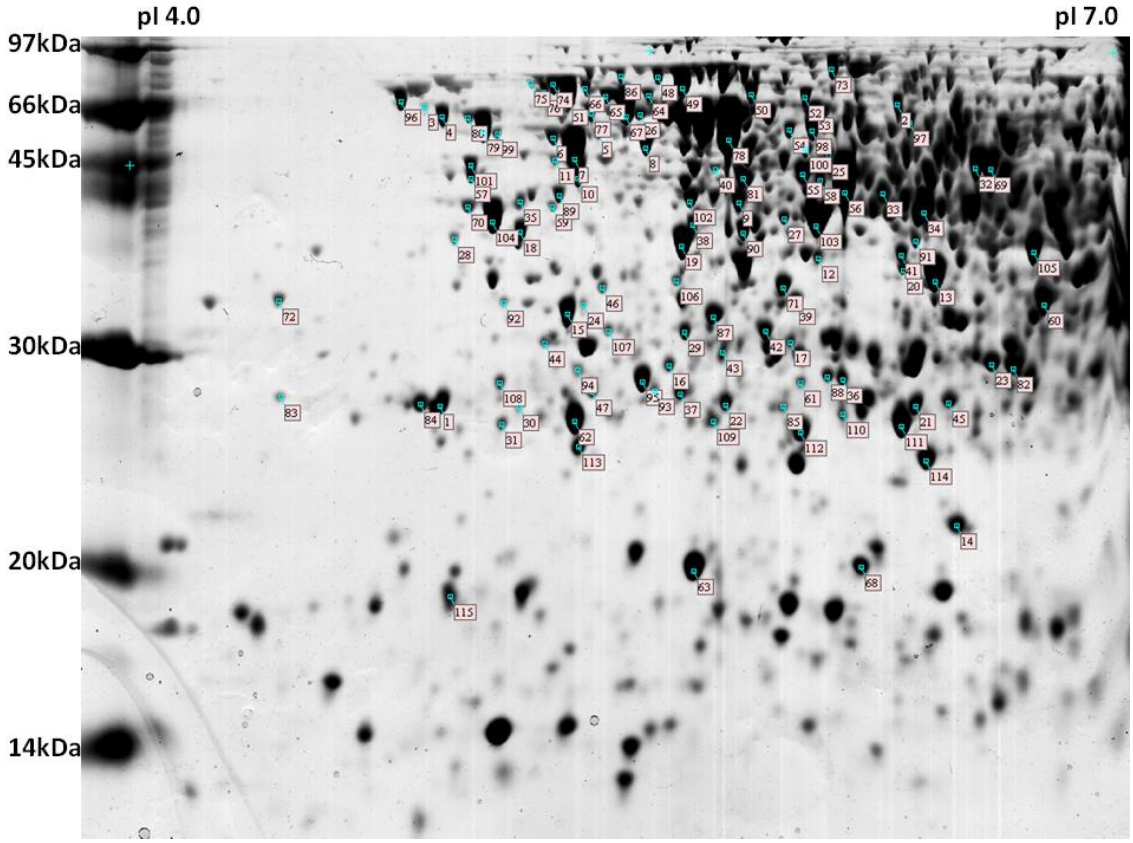


Table 1 Identified proteins of *Bradyrhizobium diazoefficiens* CPAC 7 whole cell extract and classification according to COG

Spot ID	Gene	Product	NCBI ID	Cellular location	*T/**E pI	*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 F0F1 subunit beta	gi|27375551	Cytoplasmic	5.13/5.42	50987/62000	*B. diazoefficiens* USDA 110
6	*atpD*	ATP synthase F0F1 subunit beta	gi|27375552	Cytoplasmic	5.13/5.28	50987/62000	*B. diazoefficiens* USDA 110
7	*atpD*	ATP synthase F0F1 subunit beta	gi|27375551	Cytoplasmic	5.13/5.35	50987/57000	*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|384214148	Cytoplasmic	5.88/6.09	34259/41000	*B. japonicum* USDA 6
13	*Mdh*	Malate dehydrogenase	gi|27375567	Cytoplasmic	5.88/6.09	34275/41000	*B. diazoefficiens* USDA 110
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 NADP + 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
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	*fadB*	Enoyl CoA hydratase	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	*nifH*	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							
Translation, ribosomal structure and biogenesis							
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
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	*rpoA*	DNA-directed RNA polymerase alpha subunit	gi|354957001	Cytoplasmic	4.91/4.97	38082/41000	*B. diazoefficiens* USDA 110
71	*rpoA*	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	5.27/5.21	65113/72000	*Bradyrhizobium* sp. WM9
75	*htpG*	Heat shock protein 90	gi|27382900	Cytoplasmic	5.08/5.16	69004/72000	*B. diazoefficiens* USDA 110
76	*htpG*	Heat shock protein 90	gi|27382900	Cytoplasmic	5.08/5.19	69004/710000	*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	*tig*	Trigger factor	gi|27380056	Cytoplasmic	4.87/5.06	50061/57000	*B. diazoefficiens* USDA 110
80	*tig*	Trigger factor	gi|27380056	Cytoplasmic	4.87/4.97	50061/64000	*B. diazoefficiens* USDA 110
81	*clpX*	ATP-dependent protease ATP-binding subunit ClpX	gi|27380054	Cytoplasmic	5.575.80	46932/44000	*B. diazoefficiens* USDA 110
82		Anti-oxidant protein	gi|27380419	Cytoplasmic	6.1/6.66	24420/29000	*B. diazoefficiens* USDA 110
84	*grpE*	Heat shock protein GrpE	gi|27375787	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
Poorly characterized							
General function prediction only							
89	*ychF*	GTP-dependent nucleic acid-binding protein EngD	gi|27382550	Cytoplasmic	5.13/5.22	39493/42000	*B. diazoefficiens* USDA 110
90	*cobS*	Cobalt insertion protein	gi|383768898	Cytoplasmic	5.62/5.80	37304/39000	*Bradyrhizobium* sp. S23321
91		Dehydrogenase	gi|27378316	Cytoplasmic	5.92/6.32	33290/38000	*B. diazoefficiens* USDA 110
93	*cinA*	Competence-damage associated protein	gi|27380722	Cytoplasmic	5.29/5.51	26451/28000	*B. diazoefficiens* USDA 110
NO related COG							
96	*nusA*	Transcription elongation factor NusA	gi|27375896	Cytoplasmic	4.70/4.77	59333/67000	*B. diazoefficiens* USDA 110
97		ATP-dependent phosphoenolpyruvate carboxykinase	gi|398824719	Cytoplasmic	6.01/6.34	59235/62000	*Bradyrhizobium* sp. YR681
98		ABC transporter substrate-binding protein	gi|27380707	Periplasmic	6.93/6.00	59019/58000	*B. diazoefficiens* USDA 110
100	*tldD*	TldD protein	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*	Fructose 1,6-bisphosphatase II	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*	Ketol-acid reductoisomerase	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.

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

Spot ID	Hypothetical protein	NCBI ID	Cellular location	*T/**E pI	*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 (Pfan); 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 (Ypuh-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).
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

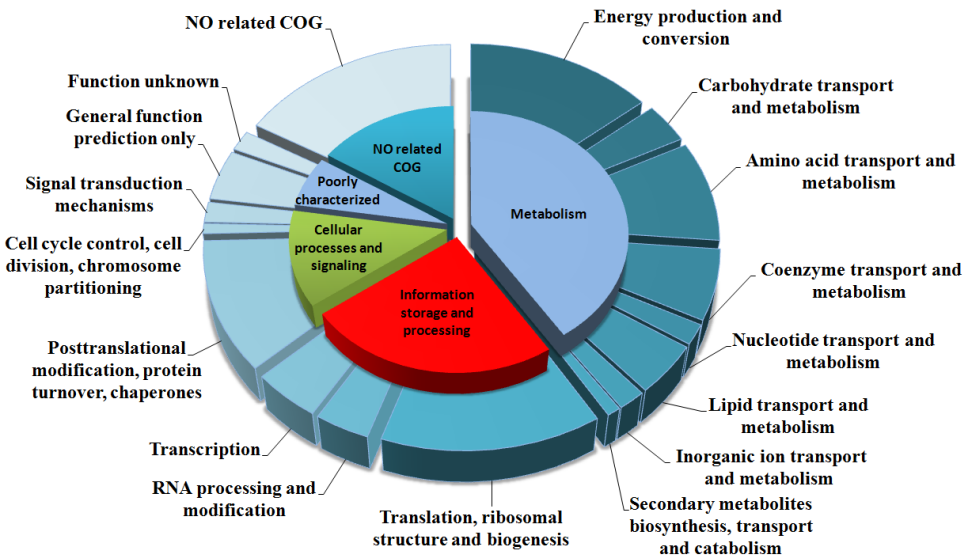
107	Bll5131	gi|27380242	Extracellular	7.68/5.80	34214/32000	Protein of unknown function DUF (Pfam/InterPro).
108	Blr2961	gi|27378072	Cytoplasmic	4.96/5.06	25510/29000	GO: catalytic activity/Metabolic process (InterPro); Protein of unknown function (Pfam).
109	Bll5307	gi|27380418	Periplasmic	5.55/5.70	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.

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).

Figure 2 Functional distribution of identified proteins of *Bradyrhizobium diazoefficiens* CPAC 7 into COG categories.



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 N_2-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 N_2-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*-genes, 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.

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 translation, ribosomal structure and biogenesis functional category (Table 1). Similarly to NifA, this protein

participates in controlling the expression of sigma-54 (RpoN, NtrA), which, in turn, helps initiate the transcription of genes encoding proteins for diverse cell functions [53,54]. Among several roles, RpoN is directly involved in the N_2-fixation process, being required for control of major N_2-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 N_2-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 N_2-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 N_2 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. diazoeficiens* 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 (O_2^-) and hydrogen peroxide (H_2O_2). 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 hyperosmosis 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 ArgJ-like domain.

Classified according to the COGnitor in the coenzyme transport and metabolism functional category, proteins Blr3798 and Bll4565, 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 Bll4752, 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.

BJ6T_08050, classified in the posttranslational modification, protein turnover, chaperones functional category, shows 97% of similarity with Bll0800 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—Bll5663 and Blr5067—remained annotated as hypothetical in the NCBInr database (Table 2). Bll5663 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

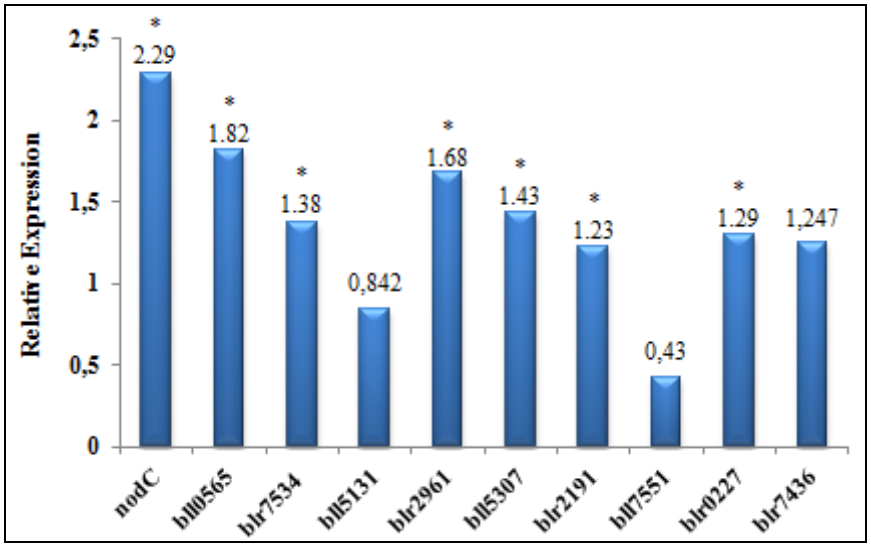
expressed in bacteroids of *B. diazoefficiens* USDA 110, and is still poorly characterized [14]; it could be a LabA-like protein with a putative metal-binding site. This family of proteins has been studied in cyanobacteria and reported to affect both gene expression and cellular metabolic state [94,95]. 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 3: Figure S1. Identified as one of the main components present in soybean root extract [96], genistein induces the expression of *nod* genes in *Bradyrhizobium* [16,97]. In addition, it has been shown that flavonoids, such as genistein, can induce the expression of other genes besides *nod* genes [15,47].

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, Additional file 2: Table S2). 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.

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.



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: Table 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* 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 N_2-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 N_2-fixation process. We also report the constitutive expression of proteins such as DnaK, ferredoxin-$NADP^+$ reductase (FRN) and trigger factor (*tig*) 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-gluconate (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 KH_2PO_4; 68 mM NaCl; and 9 mM NaH_2PO_4. Washed cells were resuspended in 600 µL of a buffer containing 10 mM Tris–HCl pH 8.0; 1.5 mM $MgCl_2$; 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 N_2 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 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. 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 iodoacetamine, 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^{TM} 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 NCBInr (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: Figure 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.nih.gov/COG) [117]. Software packages PSORT-B [118] and PSLpred [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 (Quiagen) and quantified by NanoDrop ND-1000 (NanoDropTechnologies, 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: Table 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.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the experiments: M.H., L.V.G.T. Performed the experiments: D.F.G., J.S.S.B., A.A.P.R., L.P.S., C.B. Analyzed the data: D.F.G., J.S.S.B., A.A.P.R., L.P.S., C.B., L.V.G.T, M.H. Contributed reagents/materials/analysis tools: M.H., L.P.S., C.B. Wrote the paper: D.F.G., J.S.S.B., L.P.S., M.H. All authors read and approved the final manuscript.

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Additional files

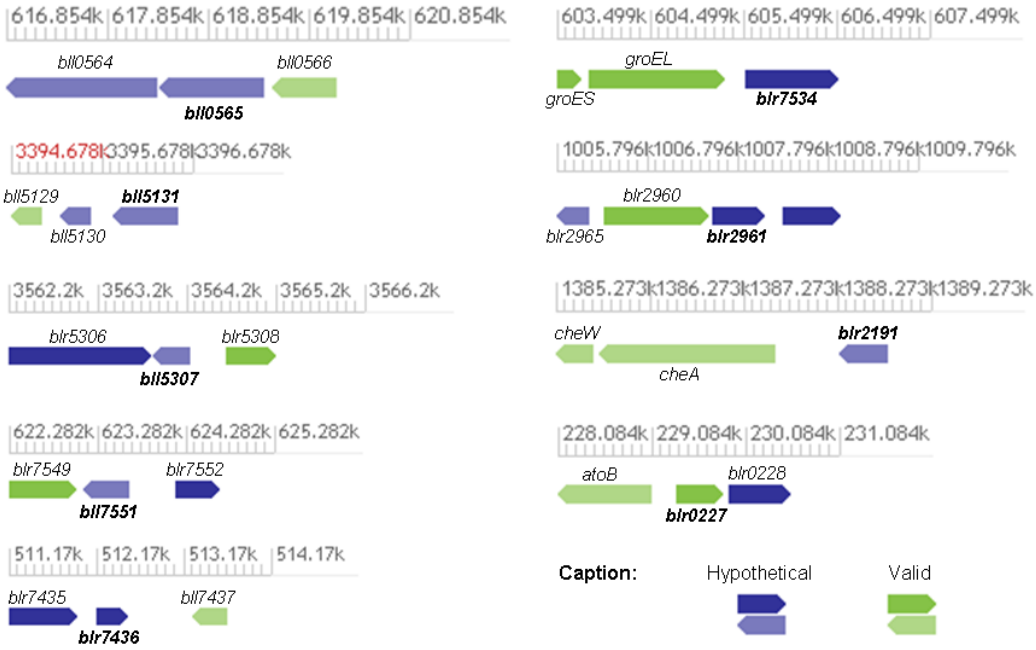
Additional file 1: Table S1: Complementary information about protein identifications. All searches were performed with Mascot software v. 2.3 (http://www.matrixscience.com/) against the public database NCBInr (National Center for Biotechnology Information non-redundant). *Identified by MS; **Identified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

Disponível em: http://www.biomedcentral.com/imedia/1990429630138373/supp2.doc

Additional file 2: Table S2: Statistical results of RT-qPCR data provided by Rest2009 software package.

Hypotetical protein-coding genes	Expression	Std. Error	95% C.I.	P(H1)	qPCR Result
nodC	2,291	1,490 - 3,407	1,158 - 4,834	0	Up regulated
bll0565	1,82	1,440 - 2,214	1,248 - 2,570	0	Up regulated
blr7534	1,379	1,076 - 1,722	0,907 - 1,933	0,002	Up regulated
bll5131	0,842	0,477 - 1,427	0,309 - 1,932	0,381	
blr2961	1,681	1,280 - 2,151	1,042 - 2,684	0	Up regulated
bll5307	1,433	1,117 - 1,835	1,003 - 2,210	0,001	Up regulated
blr2191	1,228	0,993 - 1,484	0,850 - 1,706	0,011	Up regulated
bll7551	0,43	0,168 - 2,316	0,140 - 2,542	0,07	
blr0227	1,294	1,068 - 1,599	0,870 - 1,879	0,003	Up regulated
blr7436	1,247	0,772 - 2,149	0,519 - 3,159	0,204	

Additional file 3: Figure S1: Localization in the genome of *B. diazoefficiens* CPAC 15 of the genes coding hypothetical proteins used in our study.



6 CONCLUSÕES GERAIS

- Mesmo sendo cultivadas na ausência de flavonoides, diversas proteínas importantes para o estabelecimento da simbiose foram identificadas em *R. freirei* PRF 81 e *B. diazoefficiens* CPAC 7;
- Foram identificadas proteínas que participam das defesas celulares em resposta a condições de estresse. A expressão dessas proteínas está diretamente relacionada à capacidade de suportar diferentes condições de estresses ambientais, característica importante para o sucesso da interação simbiótica;
- O estabelecimento dos mapas proteômicos para as estirpes PRF 81 e CPAC 7 proporcionou a identificação de proteínas pertencentes a diferentes vias metabólicas, potencialmente envolvidas com a elevada competitividade apresentada por essas estirpes.
- Através da eletroforese bidimensional e espectrometria de massas aplicadas para o estudo da estirpe CPAC 7 detectou-se a expressão de 20 proteínas até então denominadas hipotéticas. Entre essas proteínas, dez tiveram suas prováveis funções determinadas com o auxílio de ferramentas da bioinformática, agregando novas informações à biologia desse microssimbionte;
- Mediante PCR quantitativo, foi constatada, na estirpe CPAC 7, a indução de seis genes codificantes para proteínas hipotéticas em resposta ao flavonoide genisteína. Novos estudos devem ser conduzidos para determinar qual o papel desses genes no processo de estabelecimento da simbiose.

7 PERSPECTIVAS DE NOVAS PESQUISAS

Os estudos proteômicos permitem obter uma visão geral das respostas dos organismos às condições em que se encontram. Esse tipo de caracterização enquadra-se entre as abordagens da genomica funcional, ou seja, através dessa metodologia é possível compreender quais as “estratégias” empregadas pelos organismos para adaptarem-se às diferentes condições a que são submetidos. Nesse sentido, os mapas proteômicos obtidos para

Rhizobium tropici PRF 81 e *Bradyrhizobium diazoefficiens* CPAC 7 possibilitaram acessar diversas informações sobre a expressão constitutiva de fatores relacionados ao estabelecimento da simbiose e outros relacionados à tolerância a condições de estresse. Para complementar a caracterização, obtida na perspectiva proteômica, os genes que codificam esses fatores poderão ter sua expressão relativa quantificada por PCR quantitativo em resposta às condições onde exercem suas funções adaptativas. Além disso, o sequenciamento em larga escala dos transcritos (RNA-seq) dessas estirpes bacterianas, através da plataforma Illumina-Miseq, poderá revelar novas informações que, somadas ao alcançado com a análise proteômica, ajudarão a compreender quais fatores são efetivos na torerância aos estresses ambientais, na elevada competitividade e eficiência simbiótica apresentada por elas.

Outra abordagem para o desenvolvimento de novas pesquisas é a mutação dirigida dos genes que codificam proteínas relacionadas com o estabelecimento da simbiose. Diversos genes codificando para proteínas identificadas nos mapas proteômicos, bem como para genes relacionados a proteínas hipotéticas que foram induzidos em *B. diazoefficiens* CPAC 7 na presença do flavonoide genisteína podem ser utilizados como objeto dessa modalidade de estudo. A obtenção desses microrganismos mutantes pode ajudar a definir como esses genes afetam as propriedades simbióticas das bactérias frente a seus hospedeiros.