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ORIGINAL PAPER

Proteins involved in nodulation competitiveness of two *Bradyrhizobium diazoefficiens* strains induced by soybean root exudates

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Abstract Competitiveness for nodulation is one of the major restrictive factors in symbiotic nitrogen fixation between rhizobia and their host legumes. Soybean root exudates that include a variety of compounds are thought to act as signals to trigger the early symbiotic events between *Bradyrhizobium diazoefficiens* and soybeans, and thus they act as a key determinant of the competitiveness for nodulation. To gain a better understanding of the molecular mechanism of competitiveness at the level of protein expression, we compared the proteomic responses of two *B. diazoefficiens* strains that demonstrated completely different nodulation abilities, strain 4534 being the most competitive and strain 4222 being the least competitive in nodulation. In the proteomic analysis, 40 of the 65 and 22 of the 29 differential proteins were identified in response to soybean root exudates in strain 4534 and strain 4222, respectively. Compared to strain 4222, a higher amount and a number of differential proteins were detected in strain 4534, including *S*-adenosylmethionine synthetase (SAMS), PhyR-σ^{EcfG} regulon, ABC-type transporters, flagellar proteins, molecular chaperones, and proteins involved in redox state maintenance as well as several unknown proteins. Noteworthy was the induction of the PhyR-σ^{EcfG} regulon and flagellar proteins, recently demonstrated to be involved in the competitiveness for nodulation in *Bradyrhizobium japonicum*. Our results indicate that the role of root exudates can go far beyond inducing the expression of nodulation genes in *B. diazoefficiens*. Many other proteins/enzymes involved in the metabolism and environmental fitness were also upregulated when exposed to root exudates. More proteins were upregulated by the high nodulation competitive strain than that by the low, and the reasons for this need further investigation. The outcome of such study may contribute to our understanding of molecular mechanisms of different competitiveness in *B. diazoefficiens* as well as specific adaptation in the legume host.

Keywords *Bradyrhizobium diazoefficiens* · Soybean root exudates · Nodulation competitiveness · Proteomic

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Introduction

Biological nitrogen fixation is a fundamental process in the global N cycles and agricultural production (Xiao et al. 2010). Legume–*rhizobium* symbiosis accounts for about 60 % of total inputs of biologically fixed N in world agriculture (David et al. 2008). To increase crop production and reduce N fertilizer input, farmers usually treat leguminous seeds with highly efficient rhizobial inoculants (McInnes et al. 2004; Argaw 2012; Juge et al. 2012). Among rhizobia, some strains of *Bradyrhizobium japonicum*, now renamed *Bradyrhizobium diazoefficiens*, draw attention because they fix N with soybean, the most important oil and protein crop in the world (Hungria et al. 1998; Delamuta et al. 2013). This symbiosis involves a molecular cross talk of multistep dialogue based on the exchange of diffusible complex signals between the symbionts (Oldroyd et al. 2011; Cesco et al. 2012; Palacios et al. 2014).

Secondary metabolites in the rhizosphere released by plants, serving as nutritional and signaling substances, are capable of positively affecting the microbial colonization of

the root and microbial survival in the rhizosphere (Kennedy and Powell 1985; Mark et al. 2005; Palacios et al. 2014). The ability to percept and interpret these signals allows bacteria to sense their environment, leading to the expression of genes, which are important for establishing symbiosis between rhizobia and their host plants, especially for a successful competition for nodule occupancy (Cesco et al. 2012). Despite the advance in the knowledge of molecular cross talks, researchers have long been puzzled as to why the so-called superior strains failed to occupy a significant portion of nodules compared with indigenous rhizobia (McInnes et al. 2004). For this reason, studies on mechanism of competitiveness are critical for the development of commercial inoculants with high-efficient N fixation and to be used to improve crop productivity. Successful nodule formation is determined by factors such as the rhizobial strain's growth rate in the rhizosphere, communication via signal recognition, and physical attachment (Yates et al. 2011). Specific microbial communities can be selected in the rhizosphere by different plants (Cooper 2004; Mark et al. 2005; Jones et al. 2007; Kidaj et al. 2012).

In response to legume-derived flavonoids, rhizobia synthesize host-specific lipooligosaccharides (LCOs), known as nodulation (Nod) factors, which allow rhizobia to enter the host plant via root hairs (D'Haeze and Holsters 2002; Cesco et al. 2012). The backbone of the Nod factors is produced by enzymes encoded by *nodABC*, the common *nod* genes found in most rhizobia studied so far. The specificity of each Nod factor is achieved by the addition of different groups to the backbone, a process facilitated via species-specific *nod* gene-encoded proteins (Takakazu et al. 2002; Takeshima et al. 2013). In *B. japonicum*, NodD (a *Lys*R family of prokaryotic transcriptional regulators), NodVW (the two-component regulators), and FrrA (a *Tet*R family transcriptional regulators) positively activate the expression of the nodulation genes in response to host-specific flavonoids (Göttfert et al. 1990, 1992; Loh et al. 1997; Wenzel et al. 2012). So far, more than 4000 types of flavonoids have been found in legumes (Ferrer et al. 2008), and many of them have been studied for their effects on *nod* gene-inducing activity in different legume–rhizobia interactions (Barbour et al. 1991; Kape et al. 1991, 1992; Lang et al. 2008; Cesco et al. 2012). Daidzein and genistein are the two major isoflavones presented in soybean exudates that induce *nod* gene expression of *B. japonicum* (Lang et al. 2008). Recently, it was found that many non-flavonoid components also participate in the induction of nodulation genes in rhizobia such as *B. japonicum* (Mabood et al. 2006, 2008; Lee et al. 2012). In addition, it has also been reported that flavonoids released as root exudates by lupine roots can inhibit microbial proliferation and mineralization of root exudates (Tomasi et al. 2008). Therefore, the regulation of nod gene is complex. Despite the widely accepted fact that extracellular signals produced by plants can influence the performance of bacteria in the rhizosphere, the effects of naturally occurred soybean root exudates on the interaction of legumes with rhizobia, especially regarding the physiology and nodulation competitiveness of rhizobia, are poorly known.

The fine-tuning of transcriptomics and mass spectrometry technology has greatly facilitated the investigation of the transcriptome-level changes of *B. diazoefficiens* global gene expression in response to a given compound (Donati et al. 2013). However, due to the presence of posttranslational regulation, quantitative mRNA data are insufficient to predict the function of proteins in the cells (Kawamura and Uemura 2003). Proteomics is the study of the protein expression profiles of organisms and can be used for monitoring the microbial responses to external stimuli. Proteomic profiles of rhizobia during the establishment of the symbiotic relation with leguminous plants have been reported (Nomura et al. 2010; Da Silva Batista and Hungria 2012). However, the proteomic responses of rhizobial strains with different nodulation efficiencies to root exudates as well as the role of specific compounds of root exudates in the initiation of the symbiotic relation between rhizobia and leguminous plants are still poorly understood (Giagnoni et al. 2011).

In this study, we investigated and compared the effects of soybean root exudates on the protein expression of two *B. diazoefficiens* strains, which are characterized by different nodulation competitiveness: one occupies 90 % of nodules and the other only 10 % when an equal amount of cells was applied to soybean roots (Xiao et al. 2010). Thus it is interesting to know the differences in the proteomic level of these two strains when they are treated by root exudates. The optimal conditions for nod genes induction by soybean root exudates were determined by quantitative reverse transcription PCR (qRT-PCR). Then we analyzed the total proteins produced by the two strains after exposure to soybean root exudates. The results of the study can contribute to a better understanding of molecular mechanisms of competitiveness in *B. diazoefficiens* as well as the specific adaptation to legume host.

Materials and methods

Bacterial strains, medium, and culture conditions

The bacterial strains used in this work were *B. diazoefficiens* 4534 and *B. diazoefficiens* 4222. The soybean cultivar was Zhonghuang 13, which is widely cultivated in Huang-Huai-Hai region on the North China Plain. All rhizobial strains were cultured in TY broth (Beringer 1974) shaking at 180 rpm and 30 °C. Bacterial growth was evaluated by measuring its optical absorbance at 600 nm.

Preparation of root exudates

Root exudates were collected from the soybean variety Zhonghuang 13. Soybean seeds were surface disinfected and transferred into a nitrogen-free medium (Albareda et al. 2006). Twenty-five pre-germination seeds were aseptically transferred into a polypropylene lattice placed in a glass cylinder containing 300 mL of sterilized modified N-free solution as reported by Rigaud and Puppo (1975). No seeds were sown in the control. Microcosms were arranged in a replicate-randomized block design and incubated at 30 °C for 16 h and 15 °C for 8 h. The root exudates and control eluant (liquid from blank control) were tested on TY medium to monitor the microbial contamination, no growth indicating no microbial contamination present. Soybean root exudates (SREs) were sterilized by filtration through a 0.22-μm filter (Millipore Company, USA) and stored at −20 °C until use (Mark et al. 2005). Each treatment was replicated three times.

Assays for induction of *nod* genes

Before proteomic analysis, the influence of root exudates on nodulation of *B. diazoefficiens* was evaluated. Expression levels of *nod* genes were chosen as criteria on judging the effective induction of root exudates. Though it is generally accepted that root exudates have profound influence on bacterial physiology, the gene expression pattern in response to root exudates is seldom studied. The optimal conditions of incubation should be established for the highest upregulation of *nod* genes. At the start of this study, three parameters were compared: (i) concentrations of nutrient solution, i.e., soybean plants were grown in different concentrations of nutrient solution; (ii) time intervals of root exudate collection, i.e., root exudates were collected from 0, 6, 12, and 16-day-old soybean plants; and (iii) optimal period exposure of rhizobia to root exudates for maximal expression of *nod* genes, i.e., the bacterial cells were harvested after exposure to root exudates for 0.25, 1, 3, and 6 h. We measured the expression of *nodD1*, *nodD2*, and *nodC* genes. Both *B. diazoefficiens* strains 4534 and 4222 were grown up to the exponential phase and then incubated with 200 mL root exudates.

RT-PCR and quantitative real-time RT-PCR

Total RNA was extracted using the SV Total RNA Isolation System (Promega, Madison, WI, USA) purified with RNase-free DNase I (Promega). The first strand of cDNA was synthesized with 1.0 μg of RNA using the ProtoScript First-Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA, USA).

Specific primers for *nodC*, *nodD1*, and *nodD2* genes to be used in the quantitative real-time PCR (Q-PCR) were designed using Primer 5 Server based on the published genomic sequence of *B. diazoefficiens* USDA110 targeting an amplicon size of 150–200 bp. The reaction specificity was assayed by agarose gel electrophoresis and product dissociation curves. The primers used are listed in Table 1. Quantitative RT-PCR experiments following the manufacturer's instruction were performed as described by Yan et al. (2008). The equipment used included the 7500 Sequence Detection system (Applied Biosystems) and the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA).

Whole-cell protein extraction and two-dimensional electrophoresis

Bacterial cells were harvested by centrifugation at 8000×*g* for 5 min at 4 °C and resuspended in the mixture of 1.5 mL W1 buffer, 50 μL DTT, and 1.5 mL W2 buffer (Wang et al. 2008). Bacterial cells were lysed by 3 cycles of sonication at 35 kHz for 30 s on ice and incubated on ice for 30 s. Clear lysates obtained after centrifugation at 12,000×*g* for 10 min were subjected to protein precipitation by adding 1.5 mL 50 % TCA solution to 6 mL of clear lysates; then the mixture was incubated at 4 °C and then centrifuged at 12,000×*g* for 10 min at 4 °C; the precipitate was washed with W3 buffer for three times, dried in a vacuum dryer, and stored in an ultrafreezer (−80 °C) until analysis, and total protein concentration was determined as reported by Bradford (1976).

Immobilized pH gradient (IPG) strips (11 cm, pH 4–7; GE Healthcare Bio-Sciences) were used for isoelectric focusing (IEF). The IPG gels were rehydrated with the protein solution (about 600 μg) and covered with cover fluid (GE Healthcare) for 12 h. Sodium dodecyl sulfate (SDS)-PAGE was carried out with the following gradient: 4 and 15 % polyacrylamide gel; it was run with 0.3 % Tris, 1.44 % glycine, and 0.1 % SDS buffer. The electrophoresis was performed as described previously with minor modifications (Liu et al. 2013). The preparative gels were stained with Coomassie brilliant blue (CBB) R-250. At least three independent replicates were performed for each sample.

Table 1 Sequences of the primers

Gene	Primer sequence	Amplicon size (bp)
16S	CCTACGGGAGGCAGCAG CCTACGGGAGGCAGCAG	170
nodC	AGGACCGCCACCTAACG AGCGGAGTTGCTGTCGTAG	126
nodD1	CTGAGCCAGCCTGCTATGAGC CGTCCCGCGATATGATTGAGA	174
nodD2	CATAGTTGGGACGAACCGCATAG CGGGTCGCTGTTGTGAAGTG	151

Gel image analysis

The two-dimensional electrophoresis (2-DE) profiles of different samples were acquired by scanning the 2-DE gels with Image Scanner III (GE Healthcare Bio-Sciences) and analyzed by ImageMatser 2D-platinum v5.0 software (GE Healthcare Bio-Sciences). The spot volumes were normalized as the proportion of the sum of total spots per gel. All selected spots were automatically detected and matched before the manual confirmation. Three well-separated gels of biological replicates were used to create "replicate groups." After image analysis, normalized spot volumes were obtained for each gel and statistical differences were calculated between the control group and each treated group by the Student's t test considering a significance level of 95 %. The spot were selected as differential proteins if their volume showed more than 1.5-fold of differences and a statistically significant difference ($p<0.05$) between exudate-induced and non-induced treatments.

MALDI-TOF/TOF-TOF analysis and protein identification

MS and MS/MS spectra were acquired using the ultrafleXtreme™ MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) and ABI 4800 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems, Foster City, CA, USA) and analyzed by the software GPS Explorer, 3.6 software (Applied Biosystems) and MASCOT 2.1 software with the parameter settings as described for ultrafleXtreme™. The instrument was operated in the positive reflection mode and externally calibrated using the peptide calibration kit (Bruker Daltonics). The mass accuracy and mass resolution were set as the default. The samples (1 μL) were spotted onto the AnchorChip™ MALDI target plate (Bruker Daltonics, Billerica, MA, USA) and allowed to dry at room temperature. One milliliter of matrix solution (1 mg/mL, a-cyano-4-hydroxycinnamic acid in 70 % acetonitrile containing 0.1 % trifluoroacetic acid) was then manually applied onto samples to allow cocrystallization.

MS and MS/MS data analysis was performed using the BioTools software (V3.2, Bruker Daltonics) and GPS Explorer Software (Applied Biosystems), which uploads the peptide mass fingerprinting and MS/MS ion to Mascot for database searching on the Matrix Science (London, UK) public web site (http://www.matrixscience.com) and searched against NCBInr protein databases. The parameters for searching were set as follows: an enzyme of trypsin; one missed cleavage; a fragment tolerance of ±0.5 Da; a peptide mass tolerance of 100 ppm; carbamidomethylation (Cys) set as fixed modification; and oxidation of methionines (Met) set as variable modification. Only significant hits, as defined by the MASCOT probability analysis, were accepted (Liu et al. 2013).

Results

Transcriptional analysis of two strains after exposure to root exudates

Transcriptional analysis indicated that the *nod* genes of both strains were upregulated to varying degrees in response to root exudates, and the patterns of both strains were quite similar. However, under the same conditions, the upregulation of *nod* genes was greater in *B. diazoefficiens* 4534 than in *B. diazoefficiens* 4222. By considering these results, the optimal conditions for *nod* gene expression were determined: the root exudates were collected from soybean grown in one-third strength of modified N-free Rigaud–Puppo solution for 7 days and a 1-h incubation period for gene induction; this gave the highest upregulation of *nod*C, *nodD1*, and *nodD2* genes.

Proteomic analysis of two strains treated with soybean root exudates

Sixty-five differential proteins were detected in *B. diazoefficiens* 4534 (Fig. 1(b)) in response to root exudate induction. Only 40 of the 65 differentially expressed proteins were excised for tryptic digestion and identified by MALDI-TOF (Table 2), because the remaining ones showed a low abundance. Although 29 differential protein spots were detected, only 22 differential protein spots were selected in *B. diazoefficiens* 4222 in response to the root exudates induction, as shown by comparing Fig. 1(c) with Fig. 1(d) (Table 3).

Proteins responsible for changes in the proteome profile of the two *B. diazoefficiens* strains, identified in this study, fell in the following six functional categories with numbers of the different proteins associated with different functions in strain 4534 and strain 4222: (i) signal transduction (2, 0); (ii) transport and substrate binding (4, 1); (iii) chemotaxis and motility (1, 0); (iv) metabolic fitness and energy metabolism (12, 9); (v) transcription, translation, and protein folding (5, 2); and (vi) unclassified conserved and hypothetical proteins (16, 11).

Discussion

Proteins related to signal transduction

We observed that the *S*-adenosylmethionine (SAM) synthetase was increased in *B. diazoefficiens* 4534 by incubation with root exudates, which is consistent with what was previously reported for the proteome of *B. japonicum* induced by genistein (Da Silva Batista and Hungria 2012). SAM is the methyl donor for the synthesis of *N*-acyl-homoserine lactones (acyl-HSLs), which positively activates the expression of quorum sensing (QS)-dependent genes. Previous studies

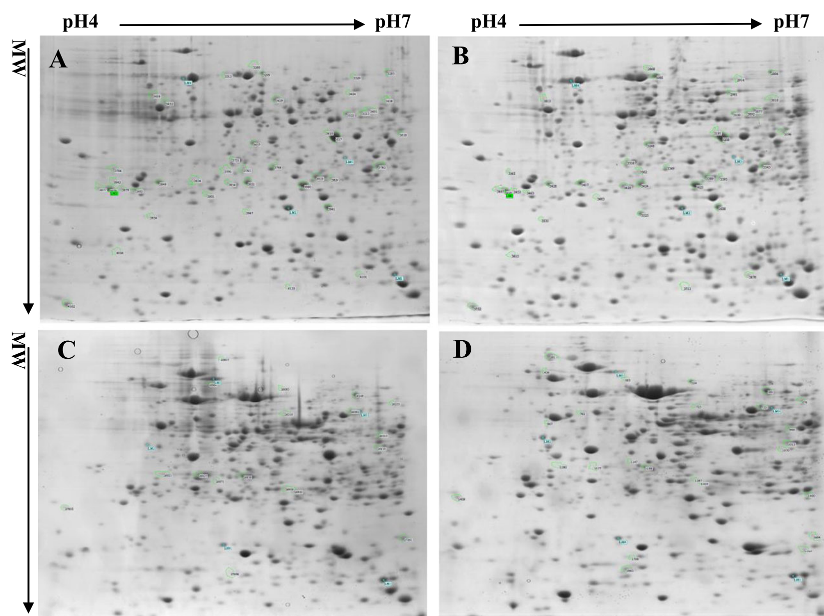

Fig. 1 Two-DE protein images of protein extracted from *B. diazoefficiens* 4534 and *B. diazoefficiens* 4222 either with the treatment control (*a*, *c*) or with the root exudate treatment; 2-DE protein images of *B. diazoefficiens* 4534 and *B. diazoefficiens* 4222 with the root exudates treatment (*b*, *d*)

confirmed that QS was involved in several processes in rhizobia, including biofilm formation, exopolysaccharide synthesis, swimming motility, nodulation efficiencies, and nitrogen fixation efficiencies (Sanchez-Contreras et al. 2007; Mueller and González 2011). Biofilm formation of rhizobia is important for nodule infection (Williams et al. 2008) and is influenced by environmental conditions as well as legume-produced compounds such as lectins (Perez-Giménez et al. 2009) and flavonoids (Fujishige et al. 2008). In addition, EPS biosynthesis is also regulated by QS and is important for rhizobium infection, attachment, and competitiveness (Davies and Walker 2007; Edwards et al. 2009).

Another important protein for the establishment of the symbiosis identified in this study was the two-component regulator PhyR, a special type of response regulator consisting of a receiver domain and an extracytoplasmic function sigma factor-like domain; the latter linked in the signaling cascade following the interaction with the anti-sigma factor PhyR-σ^{EcfG} regulon, was involved in the *Bradyrhizobium*–legume interaction, and was a general stress response whose mechanism is still unknown (Gourion et al. 2008). The PhyR-σ^{EcfG}-lacking mutants of *B. japonicum* were deficient in nodulation (Cytryn et al. 2007; Gourion et al. 2009).

We observed a clear difference in the upregulation of the signal transduction proteins between the high and the low competitiveness strains: two differential proteins (e.g., SAM synthetase and PhyR) associated with signal transduction were only detected in *B. diazoefficiens* 4534, whereas none was detected in *B. diazoefficiens* 4222.

Our results suggest that the root exudate-induced signal transduction plays an important role in nodulation competitiveness of *B. diazoefficiens*, which is important for the nodulation efficiency (Gourion et al. 2009).

Proteins related to transport and substrate binding

ABC-type transporter substrate-binding protein (ATP-binding cassette), one of the largest superfamilies of membrane transport proteins, was differentially expressed in the presence of soybean root exudates. ABC-type transporters can participate in nutrient uptake, polysaccharide secretion, signal transduction, and drug resistance through energy produced by ATP hydrolysis (Nicolás et al. 2007). It is clear that efficient transport systems are an essential requisite for nutrient competition, which leads to the difference in competitiveness (Sarma and Emerich 2006).

An abundance of ABC-type transporters was confirmed in *B. japonicum*, and the relative gene expression of proteins was induced by genistein (Da Silva Batista and Hungria 2012). Here, four different proteins related to transport and substrate-binding were upregulated in *B. diazoefficiens* 4534, but only one transporter protein was upregulated in *B. diazoefficiens* 4222. Therefore, root exudates led to the increased expression of ABC-type transporters and that expression differed in the two strains.

Proteins related to chemotaxis and motility

Here, flagellar proteins were significantly induced by root exudates. Recent evidence suggests that flagellum biosynthesis is critical for bacterial adaptation to numerous environmental conditions and enhanced competitiveness for nodule occupancy under laboratory conditions (Mongiardini et al. 2009; Covelli et al. 2013). Microarray experiments indicated that all genes within the flagellar cluster are slightly upregulated in response to genistein (Lang et al. 2008), and highly abundant flagellar proteins were detected in the secretome analysis of

Table 2 Proteins expressed in *B. diazoefficiens* 4534

Spot number	Score	Protein source	Protein name	Mr (Da)	pI	Pept match	Sequence coverage (%)	Accession number
1	459	*B. diazoefficiens* USDA110	Phosphoribosylformylglycinamidine synthase I	25,266	5.64	5/3	37	NP_772363
2	264	*B. diazoefficiens* USDA110	Phosphoglycolate phosphatase	22,785	5.37	5/2	36	NP_767602
3	576	*B. japonicum*	Flagellin	74,988	5.12	6/5	14	WP_018642833
4	264	*B. japonicum*	ABC transporter substrate-binding protein	40,095	8.5	5/1	22	WP_018645478
5	132	*B. diazoefficiens* USDA110	Two-component response regulator	29,606	5.04	2/1	19	NP_774435
6	665	*B. diazoefficiens* USDA110	*S*-Adenosylmethionine synthetase	43,617	5.88	11/4	32	NP_772585
7	84	*Rhodopseudomonas palustris BisB*18	Cold-shock DNA-binding domain-containing protein	7485	8.15	1/1	32	YP_533559
8	89	*B. diazoefficiens* USDA110	ABC transporter substrate-binding protein	47,375	9.41	2/1	7	NP_773837
9	484	*B. diazoefficiens* USDA110	Trigger factor	50,061	4.87	8/3	20	NP_771585
10	699	*B. diazoefficiens* USDA6	Acetyl-CoA acetyltransferase	41,797	6.96	8/5	29	YP_005606777
11	199	*B. diazoefficiens* USDA110	Methyltransferase	31,305	5.89	3/2	15	NP_771579
12	127	*B. diazoefficiens* USDA110	Hypothetical protein bll5588	30,897	6.54	2/1	10	NP_772228
13	326	*B. diazoefficiens* USDA110	Molecular chaperone GroEL	57,213	5.39	4/3	12	NP_774173
14	406	*B. diazoefficiens* USDA110	ABC transporters substrate-binding protein	44,856	7.67	5/4	20	NP_767527
15	399	*B. diazoefficiens* USDA110	Gluconolactonase	33,159	5.54	8/4	26	NP_773337
16	445	*B. diazoefficiens* USDA110	Enoyl CoA hydratase	27,834	5.44	5/4	28	NP_769676
17	791	*B. diazoefficiens* USDA110	Molybdopterin biosynthesis protein B	25,999	5.97	8/8	41	NP_769151
18	72	*B. diazoefficiens* USDA110	Outer membrane immunogenic protein bll7469	22,055	5.25	2/1	15	NP_774109
19	68	*B. diazoefficiens* USDA110	Phosphopyruvate hydratase	45,318	5.08	1/1	14	NP_771434
20	231	*B. diazoefficiens* USDA110	Hypothetical protein blr2921	29,597	5.57	3/2	19	NP_769561
21	77	*B. diazoefficiens* USDA110	Hypothetical protein blr7761	14,125	5.39	1/1	8	NP_774401
22	290	*B. diazoefficiens* USDA110	Hypothetical protein bll0800	20,502	4.46	5/3	29	NP_767440
23	414	*B. diazoefficiens* USDA110	Hypothetical protein bll5807	34,776	8.92	4/4	21	NP_772447
24	493	*B. japonicum*	D-2-Hydroxyacid dehydrogenase	50,839	5.63	5/4	16	WP_018648751
25	757	*B. diazoefficiens* USDA110	Methylmalonate-semial ehydehydrogenase	53,785	6.24	11/7	30	NP_770594
26	282	*B. japonicum*	virR	18,564	5.40	2/1	20	WP_018646933
27	158	*B. diazoefficiens* USDA 110	Preprotein translocase subunit SecB	17,503	4.71	3/2	20	NP_767281
28	292	*B. diazoefficiens* USDA110	Oxidoreductase	29,784	6.21	4/2	21	NP_772616
29	99	*Bradyrhizobium* sp. S23321	Outer membrane immunogenic protein	21,819	5.23	2/1	15	YP_005453400
30	385	*B. japonicum*	Membrane protein	21,562	5.54	4/3	28	WP_018317790
31	546	*Bradyrhizobium* sp. WM9	Heat shock protein	65,115	5.27	6/5	15	AF222752_1
32	122	*B. diazoefficiens* USDA110	ABC transporter ATP-binding protein	25,365	5.79	2/1	11	NP_767100
33	611	*B. diazoefficiens* USDA110	Hypothetical protein (no related data)	34,275	5.88	6/3	23	1TAB_E
34	333	*B. diazoefficiens* USDA110	Hypothetical protein blr2921	29,597	5.57	4/3	22	NP_769561
35	188	*B. diazoefficiens* USDA110	Hypothetical protein blr4003	24,105	5.24	2/2	13	NP_770643
36	269	*B. diazoefficiens* USDA110	Hypothetical protein blr2425	26,396	9.05	5/2	22	NP_769065
37	333	*B. diazoefficiens* USDA110	Hypothetical protein bll7551	27,566	6.9	3/3	19	NP_774191
38	446	*B. japonicum* USDA110	Hypothetical protein bll0805	49,152	8.68	6/4	22	NP_767445
39	513	*B. japonicum*	Beta-lactamase	32,136	6.33	6/4	29	WP_018642650
40	128	*B. japonicum* USDA6	*O*-Acetylhomoserine aminocarboxypropyltransferase	45,015	6.10	3/1	10	NP_771607

B. japonicum (Süß et al. 2006; Hempel et al. 2009). The mutants of *B. japonicum* with increased motility had the advantage when competing with indigenous strains for nodulation (López-García et al. 2002, 2009; Bogino et al. 2008).

Here, the significantly upregulated proteins related to chemotaxis and motility only occurred in *B. diazoefficiens* 4534 (Table 2). Since *B. diazoefficiens* 4534 is highly efficient in nodulation, the upregulation of chemotaxis and motility-

Table 3 Proteins expressed in *B. diazoefficiens* 4222

Spot number	Score	Protein source	Protein name	Mr (Da)	pI	Pept match	Sequence coverage (%)	Accession number
1	414	*B. japonicum*	5-Carboxymethyl-2-hydroxymuconate isomerase	28,328	8.69	6/3	23	WP_018641157
2	367	*Ainsliaea acerifolia*	Ribulose-1,5-bisphosphate carboxylase	53,010	6.00	7/2	15	AAW78406
3	521	*B. diazoefficiens* USDA110	Phosphoglycolate phosphatase	22,756	5.58	7/4	38	YP_005454139
4	272	*B. diazoefficiens* USDA110	Short-chain dehydrogenase	29,015	5.68	6/1	26	NP_772637
5	117	*B. diazoefficiens* USDA110	Carbon monoxide hydrogenase	29,795	5.94	3/1	14	YP_005447835
6	249	*B. japonicum*	(2Fe-2*S*)-binding protein	17,029	5.09	2/1	20	WP_018646166
7	1350	*B. japonicum*	2-Isopropylmalate synthase	57,040	5.61	14/14	33	WP_018321622
8	329	*B. japonicum*	Isopropylmalate isomerase	51,118	6.23	6/3	18	WP_018643931
9	1105	*B. diazoefficiens* USDA110	Transcription termination factor Rho	47,122	6.08	12/8	36	NP_767275
10	191	*B. japonicum* USDA6	ABC transporter substrate-binding protein	39,699	6.62	3/1	12	YP_005605964
11	73	*B. diazoefficiens* USDA110	1-Deoxy-D-xylulose-5-phosphate synthase	71,393	6.25	1/1	12	NP_769291
12	142	*B. japonicum* USDA6	Glutamine synthetase	34,989	6.44	3/1	15	AF169576_1
13	687	*B. diazoefficiens* USDA110	Hypothetical protein blr2865	28,094	6.25	9/6	40	NP_769505
14	258	*B. diazoefficiens* USDA110	Hypothetical protein bll3051	34,076	9.08	4/3	10	NP_769691
15	757	*B. diazoefficiens* USDA110	RNA polymerase sigma-E factor (Sigma-24) protein	19,979	5.30	7/7	41	NP_774437
16	267	*B. diazoefficiens* USDA110	Acetolactate synthase regulatory subunit	20,119	6.22	3/2	23	NP_773141
17	626	*B. diazoefficiens* USDA110	Hydrolase	24,577	5.29	6/6	33	NP_767540
18	284	*B. japonicum*	NAD-dependent deacetylase	29,132	5.29	4/3	18	YP_005611292
19	432	*B. diazoefficiens* USDA110	Hydroxylase	25,646	5.37	6/5	31	NP_770545
20	317	*Bradyrhizobium* sp. CCGE-LA001	Molybdopterin biosynthesis protein	28,443	5.33	4/4	17	WP_008540093
21	160	*B. diazoefficiens* USDA6	Glutamine synthetase	34,989	6.44	2/1	20	AF169576_1
22	222	*B. diazoefficiens* USDA110	Hypothetical protein bll5581	18,749	6.29	2/2	24	NP_772221

related proteins by root exudates indicates that chemotaxis and motility are probably important for nodulation competitiveness. In addition, compared with the results by Lang et al. (2008), root exudates are more efficient in inducing the upregulation of chemotaxis and motility-related proteins than genistein (Lang et al. 2008).

Proteins related to metabolic fitness and energy metabolism

In rhizosphere, many substrates coexist at relatively low concentrations; therefore, the ability to efficiently utilize different nutrients by a strain should be an ecological advantage (Prell and Poole 2006). In this experiment, an abundance of proteins related to metabolic fitness was induced in both strains when incubated with root exudates. Proteins involved in purine metabolism were only found upregulated in *B. diazoefficiens* 4534, which confirmed previous studies. The purine pathway in *Rhizobium* is important during the nodulation processes, since it is significantly and positively correlated with competitive nodulation abilities of the bacteria (Xie et al. 2009).

Proteins associated with C and N cycling were generally more upregulated in *B. diazoefficiens* 4534 than in *B. diazoefficiens* 4222 by root exudates. Phosphopyruvate hydratase is one enzyme of the glycolysis pathway, which is a catabolic pathway. Acetyl-CoA acetyltransferase, an enzyme involved in generating poly-β-hydroxybutyrate (PHB), was also found to be upregulated. The ability to build up PHB is a key factor in the survival of bacteria, which can use the accumulated PHB under nutrient-limited conditions (Aneja et al. 2005; Marroquí et al. 2001).

Furthermore, intermediate metabolites and energy are indispensable for the proliferation of rhizobia during rhizobium–legume symbiotic nodulation (Li et al. 2011). Our proteome analysis showed that under the induction by soybean root exudates, more proteins related to metabolic fitness were found upregulated in *B. diazoefficiens* 4534 than in *B. diazoefficiens* 4222, suggesting that these metabolic properties may also be essential traits in determining the competitiveness of rhizobia.

Proteins related to transcription, translation, and protein folding

The molecular chaperone GroEL in *B. diazoefficiens* 4534 was significantly induced by root exudates. Previous studies indicated that GroEL chaperones are essential for the

formation of a functional nitrogenase during the initial bacteroid developmental stage, and the expression of the relative gene seems to be regulated during the symbiotic development through a stringent regulatory circuit (Sarma and Emerich 2005). Molecular chaperones are a ubiquitous family of abundant proteins in cellular regulation (Wang et al. 2004) and play a pivotal role in preventing both new synthesis of polypeptide chains and assembly of subunits, preventing newly synthesized polypeptide chains from folding into nonfunctional protein; in addition, they facilitate protein refolding (Yan et al. 2006).

Root exudates also upregulated the trigger factor (TF) that is the only ribosome-associated chaperone known in bacteria. This protein ensures efficient protein folding and translocation of newly synthesized polypeptide chains (Hoffmann et al. 2010). This observation is in agreement with the downregulation of chaperone SecB in *B. diazoefficiens* 4534 because its access to nascent chains is normally restrained by TF (Ullers et al. 2004). Our data support the conclusion that protein folding is secured by the action of several chaperones which, despite their different mechanisms of action, are able to substitute each other, thus endowing *B. diazoefficiens* with maximal flexibility in response to varying environmental conditions (Hoffmann et al. 2010; Nomura et al. 2010).

Five proteins associated with transcription and translation were detected in *B. japonicum* 4534, but only two were found in *B. diazoefficiens* 4222. These data might reflect the activity of cellular metabolism during critical stages of symbiotic infection with alterations in the proteome pattern.

Conclusions

A comparison of proteomic analyses of the two strains induced by soybean root exudates reveals that proteins related to transport and substrate binding, metabolic fitness and energy metabolism, transcription, translation, and protein folding in *B. diazoefficiens* 4534 were more abundant than those in *B. diazoefficiens* 4222. The expression of proteins involved in signal transduction, chemotaxis, and motility was only detected in strain 4534, not in strain 4222. This may be due to variations in the response of individual *B. diazoefficiens* genotypes to soybean root exudates. Although the different regulation mechanisms of different metabolic processes are not fully characterized, it may act in the early establishment of rhizobium–legume symbiosis, demonstrating greater complexity of responses to soybean root exudates. The striking difference between high nodulation competitive strain 4534 and low competitive strain 4222 is that more proteins were upregulated when exposed to soybean root exudates. A possible explanation for this phenomenon is that high competitive strain might be more sensitive to the induction of special compounds in the root exudates.

We are unaware that other plant signals may change the protein expression of *B. diazoefficiens* during competition for nodulation. Flavonoids (genistein, coumestrol, daidzein, etc.) and non-flavonoids (jasmonates) can only partially substitute soybean root exudates (Mabood et al. 2006; Lee et al. 2012). Our data not only improve our understanding of the genetic and functional responses of *B. diazoefficiens* in competitive nodulation but also support further studies on functions of the 20 hypothetical proteins only induced by root exudates, not by pure flavonoids.

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