Curr Microbiol (2012) 65:465–474
DOI 10.1007/s00284-012-0180-x

Soybean Seed Lectin Prevents the Accumulation of S-Adenosyl Methionine Synthetase And the S1 30S Ribosomal Protein in *Bradyrhizobium japonicum* Under C and N Starvation

Julieta Pérez-Giménez · Julieta M. Covelli · M. Florencia López · M. Julia Althabegoiti · Mario Ferrer-Navarro · Elías J. Mongiardini · Aníbal R. Lodeiro

Received: 4 April 2012 / Accepted: 19 June 2012 / Published online: 11 July 2012

Abstract Soybean lectin (SBL) participates in the recognition between *Bradyrhizobium japonicum* and soybean although its role remains unknown. To search for changes in the proteome in response to SBL, *B. japonicum* USDA 110 was incubated for 12 h in a C- and N-free medium with or without SBL (10 μg ml $^{-1}$), and the soluble protein profiles were compared. Two polypeptides, S-adenosylmethionine synthetase (MetK) and the 30S ribosomal protein S1 (RpsA), were found only in the fractions from rhizobia incubated without SBL. Transcript levels of *metK* and *rpsA* were not correlated with polypeptide levels, indicating that there was regulation at translation. In support of this proposal, the 5′ translation initiation-region of *rpsA* mRNA contained folding elements as those involved in regulation of its translation in other species. Disappearance of MetK and RpsA from the soluble protein fractions of SBL-treated rhizobia suggests that SBL might have attenuated the nutritional stress response of *B. japonicum*.

J. Pérez-Giménez · J. M. Covelli · M. F. López · M. J. Althabegoiti · E. J. Mongiardini · A. R. Lodeiro (✉)
Instituto de Biotecnología y Biología Molecular (IBBM), Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata y CCT La Plata-CONICET, Calles 47 y 115 (1900), La Plata, Argentina
e-mail: lodeiro@biol.unlp.edu.ar

M. Ferrer-Navarro
Institut de Biotecnologia i de Biomedicina (IBB), Universitat Autònoma de Barcelona (UAB) Laboratorio de Biologia Computacional i Proteòmica, 08193 Bellaterra, Barcelona, Spain

Introduction

Legume lectins are (glyco)proteins involved in cell–cell recognition, which is performed by specific binding of the lectins to cell surface polysaccharides. The participation of these proteins in the symbiosis between nitrogen-fixing rhizobia and legume plants was recognized in 1974 by Bohlool and Schmidt [2] in the *Bradyrhizobium japonicum* soybean symbiosis, where the bacterial exopolysaccharide (EPS) specifically binds the soybean lectin (SBL). Later, Dazzo and Truchet [9] proposed that rhizobia-legume recognition might occur by specific bridges through the lectin's multivalent sugar binding sites between the plant and rhizobial surface polysaccharides. However, further experiments shed doubts about this hypothesis, and observations in transgenic plants expressing heterologous lectins suggested another role related with stabilization of infection thread structures into the root hairs [12, 34, 35]. Infection threads are the channels through which rhizobia enter the plant root cells, and plant defense reactions that could be triggered against this invasion are attenuated during the passage of symbiotic rhizobia toward the developing root nodule. Bacterial surface polysaccharides are among the rhizobial molecules involved in suppressing plant defense reactions, and the experimental data obtained to date indicate the relationship among some activity of these polysaccharides, plant defense suppression, and root hairs infection [14]. In *B. japonicum*, mutants producing an EPS devoid of galactose, the specific SBL-binding sugar moiety, triggered a rapid plant defense response in contrast to mutants producing EPS devoid of galacturonic acid, which is not a SBL hapten [27, 29]. However, a link between this polysaccharide activity and plant lectin activity has not been yet reported.

Halverson and Stacey [15] observed that low concentrations of SBL increased the infectivity of *B. japonicum*. This

stimulation required prolonged incubation times of the rhizobia with the lectin before inoculation to the roots, and was inhibited by the addition of rifampin and chloramphenicol. Thus, this study indicated that at least this infection-simulating role of SBL, at low concentrations, is not restricted to a passive bridge between both cell surfaces, suggesting an active role which involves protein synthesis in the rhizobia. Further studies confirmed this activity and extended it to rhizobial adhesion to root surfaces and competition for nodulation [23]. In these experiments, the rhizobia were incubated for different times in C- and N-free plant nutrient solution, where the bacterial growth was transiently arrested for the first 12 h after which cell duplication was resumed. Interestingly, the adhesion to plant roots was maximal during this lag phase, and decreased at the onset of cell division. In parallel, the adhesion stimulated by SBL also peaked at the end of the lag phase. In other studies [24], incubation of *Rhizobium etli* with the common bean lectin phytohemagglutinin increased the bacterial respiration rate, another indication that plant lectins have an active role in modifying the metabolic status of the bacterial cell. However, in none of these works the possible differences that could arise in rhizobia protein profiles in response to SBL were studied.

Proteomics allows the simultaneous screening of multiple polypeptides in cell extracts. By means of this technique, the polypeptide profiles from different cell types or from the same cell in different physiological situations may be accurately compared. Therefore, proteomes have been studied in different bacterial species to better understand a variety of phenomena such as quorum-sensing [11], biofilm formation [31], two-components signaling [7], virulence [1], etc. These studies are facilitated by the availability of complete bacterial genomic sequences and the coupling of polypeptide separation to mass spectrometric analyses, which allow rapid identification of differentially expressed polypeptides. In *B. japonicum,* the complete genomic sequence of the type strain USDA 110 is available [19], and therefore proteomics was already applied in this species to compare the symbiotic and free-living states [30] and to characterize the secreted proteins from free-living bacteria [17]. In this study, we employed proteomics to analyze the changes in protein profiles of *B. japonicum* USDA 110 produced in response to low concentrations of SBL.

Materials and Methods

SBL Preparation

SBL was purified from soybean seeds by affinity chromatography with ε-aminocaproyl-N-acetyl-β-D-galactosamine agarose and analyzed as described [23].

Bacteria Incubation and Proteins Extraction

Bradyrhizobium japonicum USDA 110 was grown in yeast extract-mannitol (YM) medium [36] at 28 °C and 180 rpm until the logarithmic growth phase attained at an optical density at 500 nm (OD_{500}) of around 0.6. Then, rhizobia were centrifuged at 10,000×*g* and resuspended in the same volume of C- and N-free plant nutrient Fåhraeus solution [13]. This suspension was divided in two halves. To one half, SBL was added to a final concentration of 10 μg ml^{-1}, while the other half was kept without SBL. Both suspensions were incubated without agitation for 12 h at 28 °C [15, 23] and were then centrifuged at 10,000×*g* and washed three times with 1 M D-galactose, for the first two washes in PBS and for the last in 3 mM KCl, 1.5 mM KH_2PO_4, 68 mM NaCl, and 9 mM NaH_2PO_4. The pellet was resuspended in 10 mM Tris–HCl pH 8.5 up to an $OD_{500} = 10$, and 1 μl of phenylmethylsulfonyl fluoride (PMSF) in Tris–HCl per ml was added. Cells were then broken using a Sonifier 150 (Branson) sonicator with six pulses of three min at 38 % power in an ice bath. The lysate was centrifuged at 30,000×*g* for 20 min to remove unbroken cells and cell debris. The supernatant was centrifuged again at 70,000×*g* for 1 h to discard the microsomal fraction. The supernatant was precipitated with four volumes of acetone for 12 h at −20 °C, centrifuged at 10,000×*g* for 20 min, and resuspended in approx. 400 μl of rehydration solution containing 8 M urea and 2 % 3-[(3-cholamidopropyl)dimethylammonium]-1-propanesulfonate (CHAPS). Proteins were quantified according to the method of Bradford [4] and the quality of the preparation was assessed in 1D sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [20].

2D Gel Electrophoresis and Polypeptides Identification

Proteins in the rehydration buffer were mixed with 0.28 % dithiotreitol (DTT) in 0.5 % commercial buffer for isoelectrofocusing (IPGbuffer) supplemented with bromophenol blue to a final volume of 340 μl. This mixture was agitated for 20 min and centrifuged in a microcentrifuge at a maximal speed for 3 min. The supernatant was loaded onto an Immobiline DryStrip (pH 3–10 or pH 4–7, 18 cm, GE Healthcare) and subjected to isoelectrofocusing. The second dimension was performed in SDS-PAGE and proteins were stained with Coomassie Brilliant Blue.

In all experiments, three independent cultures (launched in different days) were processed as described above, and from each protein extract, three independent pairs of gels were run and compared. Only the spots that were consistently repeated in each member of the nine pairs of gels

were analyzed. For this analysis, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) was performed as described [39] in a mass spectrometer (Ultraflex II TOF, Bruker) in a reflectron-positive mode, and peak intensities between 800 and 3,000 Da were registered. The chromatograms were analyzed by means of the software Flex Analysis 3.0 with the algorithm SNAP. The peptide sequences from the *B. japonicum* USDA 110 genome, which were able to give rise to these same peaks, were identified by means of the software Bio-Tools and the Mascot program with $p < 0.05$ for type I error. Spots were identified and analyzed by Image Master 2D Platinum v 5.0 software (GEHealthcare).

Retrotranscribed-PCR

Rhizobial suspensions cultured in the presence or absence of SBL as above were centrifuged at 11,000×*g* at 4 °C for 40 min and washed twice with 1 M NaCl. Then, the cells were disrupted with lysozyme in buffer TE, pH 8.0. Total RNA was obtained with Trizol (Invitrogen) following manufacturer's instructions and then treated with DNase I at 37 °C 15 min. cDNA was synthesized using random primers with M-MLV reverse transcriptase (Invitrogen) following the manufacturer's instructions. After retrotranscription, PCR was performed as described [29] using the following primers: for *metK*, SadenFw: 5′-CGGATTCCAAGAGCCAGGT-3′ and SadenRv: 5′-CGCCGATGTAGAACTTGCC-3′; for *rpsA*, S1Fw: 5′-AACCTCGAAGAGGGTCAGGT-3′ and S1Rv: 5′-CTCGAGACCCAGGAACAGAC-3′; and for *sigA* (internal control, [16]): sigAFw: 5′-CTGATCCAGGAAGGCAACATC-3′ and sigARv: 5′-TGGCGTAGGTCGAGAACTTGT-3′. The following reaction controls were employed: (1) reaction without template to test for the absence of contamination in the reagents, (2) reaction with a product obtained omitting the retrotranscription step to test for the absence of DNA remaining after DNAse treatment, and (3) reaction with genomic DNA as template to test primers' accuracy and amplicon size. Results from retrotranscribed PCR reactions were analyzed only when controls (1) and (2) gave negative results and control (3) gave a positive result.

RNA Folding

Possible folding of the translation-initiation region (TIR) of *metK* and *rpsA* mRNAs were estimated by the mfold Web Server [http://mfold.rna.albany.edu/?q=mfold]. A possible riboswitch element in *metK* was searched by means of Riboswitch Explorer [http://132.248.32.45/cgi-bin/ribex.cgi] and Rfam [http://rfam.sanger.ac.uk]. Models for folding of *rpsA* RNA TIR were obtained as described previously [3, 33] with the −80 to +18 sequence regions of *rpsA* from *B. japonicum* USDA 110, *Bradyrhizobium* sp. ORS278, and *Bradyrhizobium* sp. BTAi obtained from Rhizobase [http://genome.kazusa.or.jp/rhizobase]. Multiple sequence alignments were performed with Vector-NTI 10.1.1 [http://www.invitrogen.com/site/us/en/home/LINNEA-Online-Guides/LINNEA-Communities/Vector-NTI-Community/vector-nti-software.html].

Results and Discussion

Differences in Polypeptides of *B. japonicum* Incubated with or Without SBL

When *B. japonicum* USDA 110 cells grown in rich YM medium [36] are transferred to C- and N-free Fåhraeus plant nutrient solution [13], the nutritional shift leads to growth arrest [23]. It was previously observed that in this starving condition, SBL induces an increase in *B. japonicum* adhesiveness and infectiveness on soybean roots through a process that seems to require protein synthesis [15]. To observe the possible effects of SBL on protein profiles of these nutrient-deprived *B. japonicum* cells, we compared the proteomes of soluble protein fractions from rhizobia incubated in Fåhraeus solution with or without SBL at a final concentration of 10 μg ml^{-1}. Since SBL is considered to play a passive role in symbiosis by establishing a molecular bridge between the surface polysaccharides of plants and rhizobia, we did not expect to find extensive differences in the proteomes; therefore, we evaluated three independent protein extracts per condition, each one in triplicate gels. Only those differential spots that were consistently observed in the nine gels were analyzed. In addition, we only focused on spots that were either present or absent in each different condition. Representative gels are shown in Fig. 1.

We started with a pH range of 3–10 in the first dimension, and clearly distinguished 340–350 spots in 20 × 20 cm gels (Fig. 1a, b). However, most of the spots were grouped in the central region of the gels, and therefore it was difficult to isolate them for identification. The spot number 10 in Fig. 1a was clearly observed in the incubations without SBL, but was undetectable in the incubations with SBL. This spot could be identified and matched with the 30S ribosomal protein S1 (RpsA).

To better separate the polypeptides in the central region of the gels, we employed a pH gradient of 4–7 in the first dimension (Fig. 1c, d). Since the aim of this study was not to establish the complete proteome of *B. japonicum* in this culture condition but only to detect possible differences due to the presence of SBL, we extracted a set of 29 spots for their identification and mapping in the gels to unequivocally determine the position of the observed differential spots in

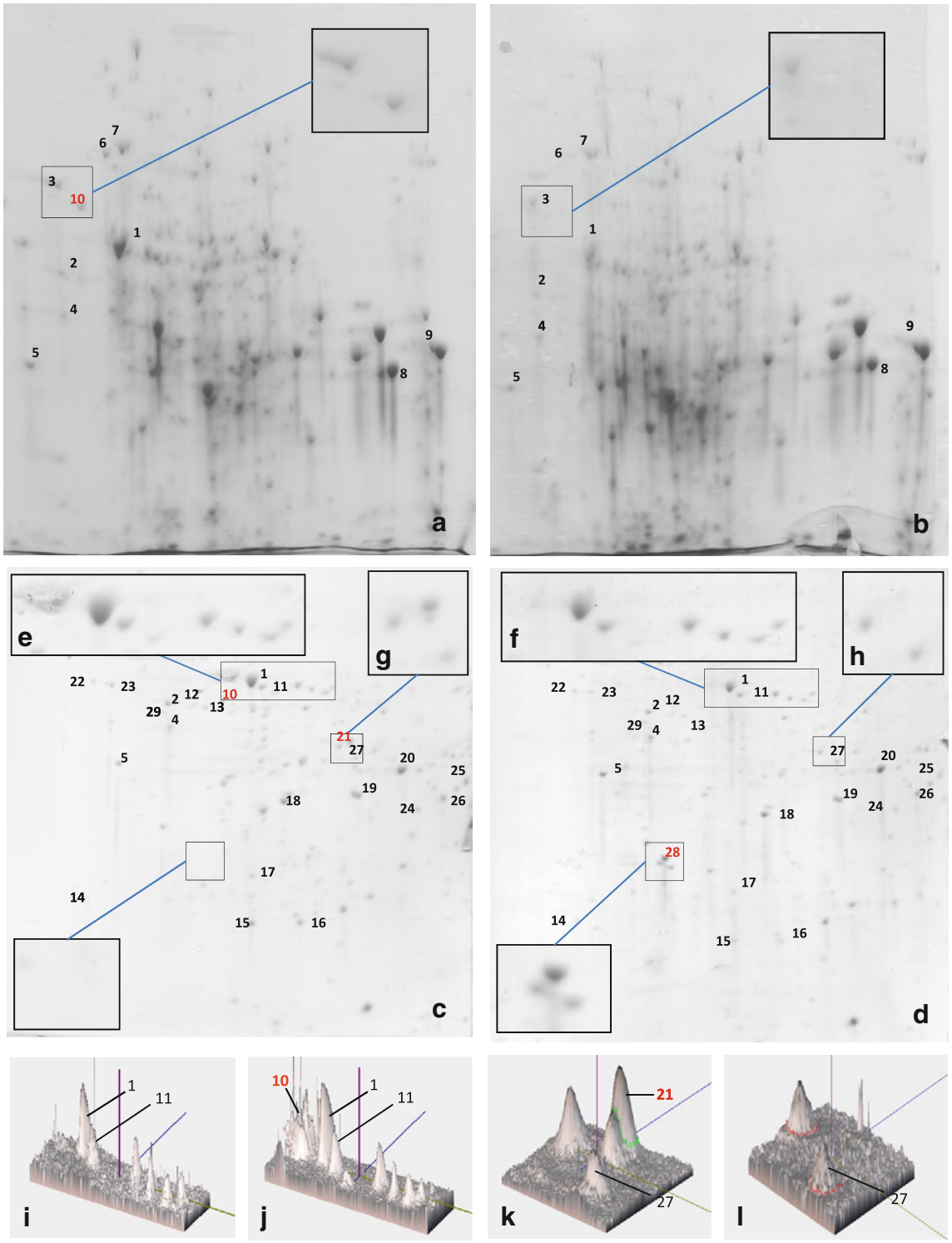

Fig. 1 Electrophoretic analysis of soluble protein fractions from *B. japonicum* USDA 110. The rhizobia were incubated for 12 h in Fåhraeus solution in the absence (**a**, **c**) or presence (**b**, **d**) of SBL 10 μg ml^{-1}. 2D-PAGE separations were done by pI in the pH range from 3 to 10 (**a**, **b**) or 4 to 7 (**c**, **d**) in the horizontal dimension and by size in the vertical dimension. *Numbers* indicate the proteins identified by tryptic digestion and MALDI-TOF that are described in Table 1. Insets: amplification of the images where relevant differences were reproducibly encountered; *e*, *f* differential expression of S1 (spot 10), *g*, *h* differential expression of SAM-synthetase (spot 21). Densitometric quantification of the spots are shown in panels **i–j** (from *insets e–f*) and **k–l** (from *insets g–h*). In all cases numbers refer to the same identified proteins. Spots without numbers were not identified

Table 1 Proteins identified in this study using the program MASCOT with a mass tolerance of 50 ppm and the NCBInr database

	Protein	Spot	Differential	Score Mascot	GI	Theoretical PI	Theoretical MW	No. of mass values matched	No. of mass values searched
Transport proteins									
1	ABC transporter substrate-binding protein	9	–	85	27380786	8.95	40,020	10	47
2[a]	ABC transporter substrate-binding protein	11	–	91	27380707	5.72	55,372	9	35
3	ABC transporter amino acid-binding protein	18	–	92	27379557	5.61	34,659	6	7
4[a]	Probable amino acid-binding protein	26	–	89	27378020	6.22	33,880	7	13
5[a]	ABC transporter substrate-binding protein	27	–	76	27378936	6.32	40,722	6	19
Proteins related to energy metabolism.									
6	F0F1 ATP synthase subunit β	2	–	234	27375551	5.13	50,987	11	48
7	Enolase	4	–	103	27379905	5.08	45,314	8	35
8	Glyceraldehyde-3-phosphate dehydrogenase	8	–	95	27376634	7.77	35,826	8	36
9	Probable succinate-semialdehyde dehydrogenase [NADP+]	13	–	206	27379109	5.30	50,087	16	31
10	NADH dehydrogenase subunit C	14	–	78	27380028	4.80	23,201	6	23
11	Enoyl CoA hidratase	17	–	117	27378147	5.44	27,829	7	19
12	Inorganic pyrophosphatase	16	–	78	27375659	5.61	20,096	6	27
13	Malate dehydrogenase	19	–	82	27375567	5.88	34,275	9	35
14	**S-adenosylmethionine synthetase**	**21**	**without SBL**	**145**	**27381056**	**5.88**	**43,613**	**11**	**30**
15	Pyruvate dehydrogenase subunit β	22	–	105	27379893	4.81	48,906	10	35
16	Pyruvate dehydrogenase subunit β	23	–	116	27379893	4.81	48,906	11	34
17	Glutamine synthetase II	20	–	197	27379280	6.02	38,570	13	18
Proteins related to transcription, translation and protein folding									
18	Chaperonin GroEL	1	–	99	27380737	5.45	57,716	9	18
19	Molecular chaperone DnaK	3	–	81	27375790	5.12	68,364	8	23
20	DNA-directed RNA polymerase subunit α	5	–	117	27380487	4.90	38,035	9	16
21	Elongation factor G	6	–	159	27380514	5.32	76,067	22	77
22	Polynucleotide phosphorylase/ polyadenylase	7	–	214	27375890	5.47	78,307	22	37
23	**30S ribosomal protein S1**	**10**	**without SBL**	**75**	**27375851**	**5.27**	**64,213**	**10**	**42**
24	Aspartyl/glutamyl-tRNA amidotransferase subunit B	12	–	87	27380198	5.22	54,388	9	19
25	HspC2 heat shock protein	15	–	139	27379748	5.47	19,422	9	27
26	Elongation factor Ts	24	–	75	27379971	6.17	32,175	5	14
Proteins unclassified and hypothetical									
27[a]	Periplasmic mannitol-binding protein	25	–	85	27378854	7.02	37,422	8	40

Table 1 continued

	Protein	Spot	Differential	Score Mascot	GI	Theoretical PI	Theoretical MW	No. of mass values matched	No. of mass values searched
28	**Chain A, Soybean Agglutinin Complexed With 2,6-Pentasaccharide**	**28**	**with SBL**	**74**	**6729836**	**5.15**	**27,555**	**6**	**39**
29	Hypothetical protein blr4341	29	–	122	27379452	5.11	45,880	11	28

[a] MW and pI are recalculated (http://www.expasy.ch/tools/pi_tool.html) without considering the sequence of signal peptide (http://www.cbs.dtu.dk/services/SignalP)

Proteins in bold are those identified as differential

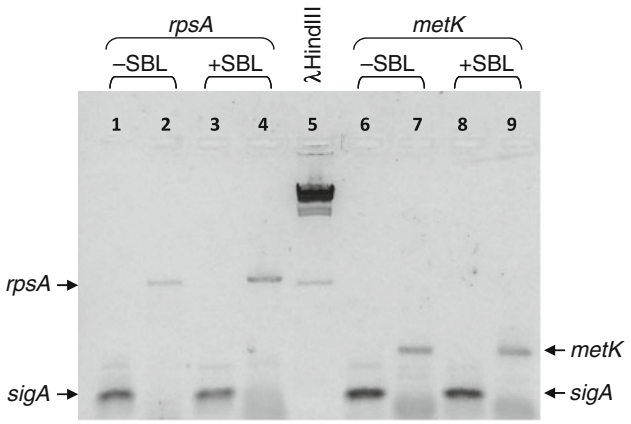

Fig. 2 Transcription of *metK* and *rpsA* in *B. japonicum* USDA 110. RNA was obtained from rhizobia incubated for 12 h in Fåhraeus solution in the absence (*lanes 1*, *2*, *6*, *7*) or presence (*lanes 3*, *4*, *8* and *9*) of SBL 10 μg ml^{-1} and the RT-PCR was performed with specific primers for *rpsA* (*lanes 2* and *4*), *met K* (*lanes 7* and *9*), or *sigA* (constitutive control, *lanes 1*, *3*, *6* and *8*). *Lane 5* λ HindIII molecular weight marker

the nine gels analyzed for each condition. The spots, which included polypeptides related to transport, energy metabolism, transcription, translation, and protein folding, and without assigned function, are numbered in Fig. 1 and their identification is shown in Table 1.

Despite the better separation, only two differences in addition to S1 (spot 10) could be detected: spots 21 and 28 (Fig. 1c–d). One of them (spot 21) was again detected only in the condition without SBL (Fig. 1c) and was identified as S-adenosylmethionine (SAM) synthetase (MetK). By densitometric quantification (Fig. 1i–l), we observed that the quantities of S1 and SAM-synthetase varied significantly between both treatments, by comparing to the other proteins, which were at similar amounts, e.g., chaperonin GroEL, and ABC transporter substrate-binding proteins GI27380707 and GI27378936 (spots 1, 11, and 27, Fig. 1i–l). Meanwhile, another spot (No. 28) was detected only in the condition with SBL (Fig. 1d), but it did not match with any sequence in the *B. japonicum* genome. After searching other databases, it turned out that this spot was SBL itself, thus confirming its strong binding to the bacterial cell surface [28]. Since SBL was used at low concentration and cells were washed with a method aimed to remove most of the SBL, this result indicates the sensitivity and specificity of the detection of differential polypeptides.

In our experimental design, rhizobia grown in a rich culture medium were suspended in C- and N-free plant nutrient solution, a similar nutritional shift to what occurs when rhizobia grown in rich broths (inoculants) are inoculated to the soybean seeds or the soil. Therefore, during the initial period in this poor medium, rhizobial growth is arrested [23]. Accumulation of SAM-synthetase and S1 was already observed in other bacterial species under nutritional stress and during growth arrest periods [10, 26]. In particular, S1 was increased in the viable but non culturable state of *Vibrio parahaemolyticus* [21]; conversely, artificially induced increase of S1 in vivo leads to lower growth rate [6, 32]. Therefore, the high levels of SAM-synthetase and S1 in the soluble protein fractions of *B. japonicum* incubated without SBL with respect to similar fractions from cells incubated with SBL might indicate more nutritional stress without SBL at the end of the 12-h incubation period in the C- and N-free plant nutrient solution. For the case of S1, its free presence in the cytoplasm might indicate an impairment in ribosomes assembly due to more nutritional stress in the absence of SBL.

The simplest explanation for these observations is that rhizobia might have degraded SBL and used its aminoacids and carbohydrates as nutrients. However, the lectin concentration in these experiments was too low to be consumed as nutrient and, even so, significant amounts of intact SBL were recovered after incubation of rhizobia in the presence of this lectin even after extensive washing of the cells (Fig. 1d). Moreover, the use of SBL at 30-fold higher concentration did not increase the total biomass of *B. japonicum* in mineral medium [28], thus indicating that this protein cannot be used as nutrient by this bacterial species.

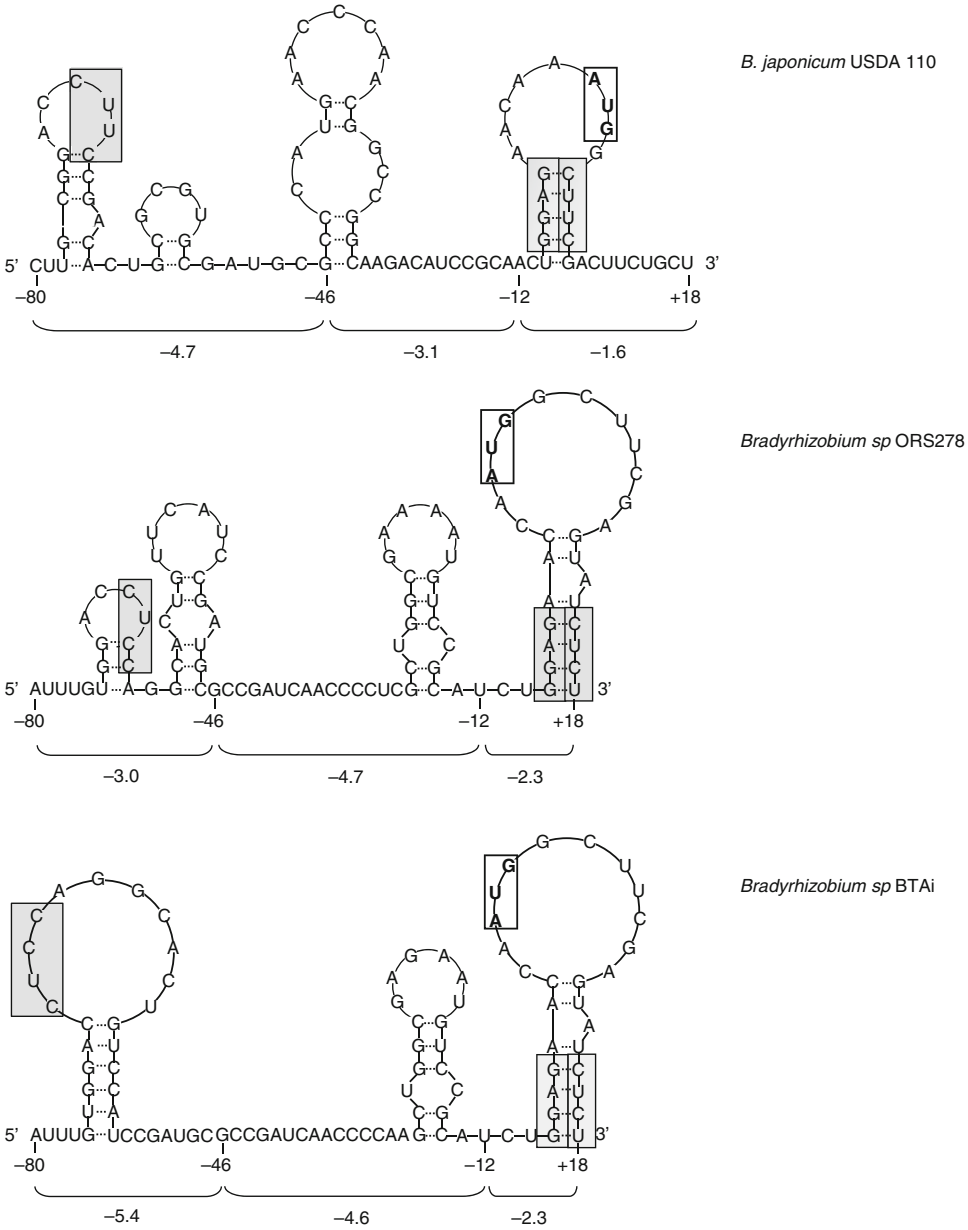

Fig. 3 Proposed secondary structures for the 5′ TIR of the 30S ribosomal protein S1. Nucleotide sequences are from −80 to +18 with respect to the translation start nucleotide. *Top panel B. japonicum* USDA 110, *middle panel Bradyrhizobium* sp. ORS278, *bottom panel Bradyrhizobium* sp. BTAi1. The sequences were divided in three parts (indicated by *brackets*) with the estimated $\Delta G^{\circ\prime}$ values for folding indicated below. *White rectangles* translation start codons, *gray rectangles* SD and anti-SD elements

mRNA Levels Evaluated by Retrotranscribed-PCR

To evaluate if transcription of *metK* and *rpsA* also responds to SBL, we performed semi-quantitative retrotranscribed PCRs with *metK*- and *rpsA*-specific primers using total *B. japonicum* cDNA as template. As internal control, we included primers for *sigA*, the expression of which was previously reported as constitutive [16]. We could not observe differences in *metK* mRNA abundance; however, *rpsA* mRNA was more abundant in the bacterial cells incubated with SBL (Fig. 2). This last trend is opposite to that observed above for polypeptide accumulation of S1. However, stability of S1 protein is higher than *rpsA* mRNA, especially when translation is arrested [6]. Therefore, the relative abundance of polypeptides and transcripts may be influenced by different degradation rates.

In *E. coli*, S1 is a very stable protein, the biosynthesis of which is repressed in starved cells at the transcription level by the ppGpp alarmone [22], and is further autoinhibited at the translation level by binding of S1 in excess (due to impairments in ribosomal assembly) to a translation-initiation region (TIR) in its own mRNA [3, 6, 32, 33]. If our system behaves similarly, starvation might have led to an increase of soluble S1 in the cytoplasm, together with a decrease in *rpsA* transcription, as observed. In turn, the addition of SBL might have prevented this part of the nutritional stress response.

To further investigate whether regulation might be at the level of translation, we searched if the TIR of *metK* and *rpsA* possess identifiable regulatory structures.

Analysis of TIR in *metK* and *rpsA* mRNAs

In other bacteria, the TIR of *metK* mRNA forms a riboswitch [38] that, upon binding of SAM, hides the Shine-Dalgarno (SD) element, thus arresting translation when SAM is abundant. A SAM element was detected in other riboswitches that respond to SAM, such as *metX* and *metZ* in *B. japonicum* USDA 110 [8], but in *metK* we could only find the four conserved base-pairing sequences although separated by much longer distance than in those SAM elements. We also could not detect riboswitch structures in *metK* of *B. japonicum* USDA 110 by Riboswitch Explorer [http://132.248.32.45/cgi-bin/ribex.cgi] or Rfam [http://rfam.sanger.ac.uk].

Regarding S1, the current hypothesis postulates that the ability of this protein to bind to the 16S rRNA as well as to the TIR of *rpsA* mRNA prevents S1 accumulation by autoinhibition of translation when the soluble form of this protein is in excess over the fraction bound to ribosomes [3, 32]. The TIR of *E. coli rpsA* mRNA has distinct features that distinguish it from the other mRNAs. The most prominent are the absence of a canonical SD element and the formation of secondary structures where the AUG start codon lies in a hairpin with low free energy of folding. However, in *Pseudomonas putida* and *P. aeruginosa rpsA,* there is a SD element although the secondary structures are similar to that of *E. coli*. Thus, we investigated whether these features are also present in the *rpsA* TIR sequences of *B. japonicum* USDA 110*, Bradyrhizobium* sp. BTAi1, and *Bradyrhizobium* sp. ORS278. We observed that all rhizobial *rpsA* TIR possess the SD sequence element. In particular, our mutliple sequence alignment revealed that the *B. japonicum* USDA 110 DNA *rpsA* sequence annotated as *blr0740* in the Rhizobase [http://genome.kazusa.or.jp/rhizobase] might contain 14 additional codons at the 5' end, and therefore we propose that the coding sequence should start at nucleotide No. 788135 instead of 788093.

To see if secondary structures of the *B. japonicum rpsA* mRNA TIR are similar to that of *E. coli* or *Pseudomonas*, we submitted the –80 to +18 sequence regions of *rpsA* from *B. japonicum* USDA 110, *Bradyrhizobium* sp. BTAi, and *Bradyrhizobium* sp. ORS278 to the mfold Web Server [http://mfold.rna.albany.edu/?q=mfold]. We retrieved predicted secondary structures without a clear trend, except that in all cases the AUG start codon was in a loop (not shown). Therefore, we divided this region in three parts: −80 to −47, −46 to −13, and −12 to +18, and analyzed the possible secondary structures of each part, according to previous works on *rpsA* TIR regions of other bacterial species [3, 33]. This approach rendered secondary structure proposals with a more defined trend, and quite similar to that reported in other species (Fig. 3). Although the exact folding remains to be experimentally confirmed, the salient features in these proposals are that the AUG start codon is at a loop in a hairpin with the lowest $\Delta G^{o\prime}$ of folding of all the RNA segment and that the SD element becomes entrapped in a double helix at the 3′ end. Interestingly, the same SD complementary sequence can be found near the 5′ end in a hairpin that was suggested to play a role in retroinhibition of translation by an excess of S1 in other bacterial species [3, 33].

Concluding Remarks

The activity of SBL seems to be exerted during root hairs' penetration by the rhizobia through infection threads [12, 34, 35]. The interior of infection threads is subjected to acidification, nutrients extraction, and desiccation [5, 18, 25, 37], and therefore the rhizobia inside infection threads are under stressing conditions, which might induce them to release plant defense-inducing factors. Our results suggest that this rhizobial stress might be attenuated by SBL. Consistent with this proposal, mutant rhizobia that produce EPS without galactose, the receptor moiety of SBL in *B. japonicum*, induce strong plant defense reactions and aberrant nodules devoid of bacteria, while mutants that produce EPS without galacturonic acid do not [27, 29]. In addition, wild type *B. japonicum* may induce the formation of nodules in transgenic *Lotus corniculatus* that produces SBL, but this ability is lost in a mutant strain that produces an EPS devoid of galactose [34]. Therefore, SBL might be the plant partner of EPS for the suppression of plant defense during *B. japonicum* infection of soybean roots. This may also explain why the addition of SBL to *B. japonicum* liquid cultures increases their competitiveness for nodulation [23]. Thus, this activity might be exploited by adding SBL to commercial inoculants.

Acknowledgments The authors are grateful to Paula Giménez, Abel Bortolameotti and Bernabé Castillo for excellent technical assistance. Supported by the National Agency for Promotion of Science and Technology (ANPCyT, Argentina). JPG, JMC, MFL, and MJA are fellows of the National Council of Scientific and Technological Research (CONICET, Argentina). EJM and ARL are members of the Scientific Career of CONICET (Argentina).

References

1. Andrade AE, Silva LP, Pereira JL, Noronha EF, Reis FB Jr., Bloch C Jr., dos Santos MF, Domont GB, Franco OL, Mehta A (2008) In vivo proteome analysis of *Xanthomonas campestris* pv.

campestris in the interaction with the host plant *Brassica oleracea*. FEMS Microbiol Lett 281:167–174
2. Bohlool BB, Schmidt EL (1974) Lectins: a possible basis for specificity in the *Rhizobium*-legume root nodule symbiosis. Science 185:269–271
3. Boni IV, Artamonova VS, Tzareva NV, Dreyfus M (2001) Non-canonical mechanism for translational control in bacteria: synthesis of ribosomal protein S1. EMBO J 20:4222–4232
4. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
5. Brechenmacher L, Lei Z, Libault M, Findley S, Sugawara M, Sadowsky MJ, Sumner LW, Stacey G (2010) Soybean metabolites regulated in root hairs in response to the symbiotic bacterium *Bradyrhizobium japonicum*. Plant Physiol 153:1808–1822
6. Briani F, Curti S, Rossi F, Carzaniga T, Mauri P, Dehò G (2008) Polynucleotide phosphorylase hinders mRNA degradation upon ribosomal protein S1 overexpression in *Escherichia coli*. RNA 14:2417–2429
7. Charles RC, Harris JB, Chase MR, Lebrun LM, Sheikh A, LaRocque RC, Logvinenko T, Rollins SM, Tarique A, Hohmann EL, Rosenberg I, Krastins B, Sarracino DA, Qadri F, Calderwood SB, Ryan ET (2009) Comparative proteomic analysis of the PhoP regulon in *Salmonella enterica* serovar *Typhi* versus *Typhimurium*. PLoS One 4:e6994
8. Corbino KA, Barrick JE, Lim J, Welz R, Tucker BJ, Puskarz I, Mandal M, Rudnick ND, Breaker RR (2005) Evidence for a second class of S-adenosylmethionine riboswitches and other regulatory RNA motifs in alpha-proteobacteria. Genome Biol 6:R70. doi:10.1186/gb-2005-6-8-r70
9. Dazzo FB, Truchet GL (1983) Interactions of lectins and their saccharide receptors in the *Rhizobium*-legume symbiosis. J Membr Biol 73:1–16
10. Di Cagno R, De Angelis M, Coda R, Minervini F, Gobbetti M (2009) Molecular adaptation of sourdough *Lactobacillus plantarum* DC400 under co-cultivation with other lactobacilli. Res Microbiol 160:358–366
11. Di Cagno R, De Angelis M, Calasso M, Gobbetti M (2011) Proteomics of the bacterial cross-talk by quorum sensing. J Proteomics 74:19–34
12. Díaz CL, Melchers LS, Hooykaas PJJ, Lugtenberg BJJ, Kijne JW (1989) Root lectin as a determinant of host plant specificity in the *Rhizobium*-legume symbiosis. Nature 338:579–581
13. Fåhraeus G (1957) The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J Gen Microbiol 16:374–381
14. Fraysse N, Couderc F, Poinsot V (2003) Surface polysaccharide involvement in establishing the rhizobium-legume symbiosis. Eur J Biochem 270:1365–1380
15. Halverson LJ, Stacey G (1986) Effect of lectin on nodulation by wild-type *Bradyrhizobium japonicum* and a nodulation-defective mutant. Appl Environ Microbiol 51:753–760
16. Hauser F, Lindemann A, Vuilleumier S, Patrignani A, Schlapbach R, Fischer HM, Hennecke H (2006) Design and validation of a partial-genome microarray for transcriptional profiling of the *Bradyrhizobium japonicum* symbiotic gene region. Mol Genet Genomics 275:55–67
17. Hempel J, Zehner S, Göttfert M, Patschkowski T (2009) Analysis of the secretome of the soybean symbiont *Bradyrhizobium japonicum*. J Biotechnol 140:51–58
18. Jones KM, Kobayashi H, Davies BW, Taga ME, Walker GC (2007) How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. Nat Rev Microbiol 5:619–633
19. Kaneko T, Nakamura T, Sato S, Minamisawa K, Uchiumi T, Sasamoto S, Watanabe A, Idesawa K, Iriguchi M, Kawashima K, Kohara M, Matsumoto M, Shimpo S, Tsuruoka H, Wada T, Yamada M, Tabata S (2002) Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA 110. DNA Res 9:189–197
20. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
21. Lai C-J, Chen S-Y, Lin I-H, Chang C-H, Wong H-C (2009) Change of protein profiles in the induction of the viable but nonculturable state of *Vibrio parahaemolyticus*. Int J Food Microbiol 135:118–124
22. Lemke JJ, Sánchez-Vázquez P, Burgos HL, Hedberg G, Ross W, Gourse RL (2011) Direct regulation of *Escherichia coli* ribosomal protein promoters by the transcription factors ppGpp and DksA. Proc Natl Acad Sci USA 108:5712–5717
23. Lodeiro AR, López-García SL, Vázquez TEE, Favelukes G (2000) Stimulation of adhesivenes, infectivity, and competitivenesss for nodulation of *Bradyrhizobium japonicum* by its pretreatment with soybean seed lectin. FEMS Microbiol Lett 188:177–184
24. Martínez CR, Albertini AVP, Figueiredo MVB, Silva VL, Sampaio AH, Cavada BS, Lima-Filho JL (2004) Respiratory stimulus in *Rhizobium* sp. by legume lectins. World J Microbiol Biotechnol 20:77–83
25. Miyahara A, Richens J, Starker C, Morieri G, Smith L, Long S, Downie JA, Oldroyd GE (2010) Conservation in function of a SCAR/WAVE component during infection thread and root hair growth in *Medicago truncatula*. Mol Plant Microbe Interact 23:1553–1562
26. Mohamed Fahmy Gad El-Rab S, Abdel-Fattah Shoreit A, Fukumori Y (2006) Effects of cadmium stress on growth, morphology, and protein expression in *Rhodobacter capsulatus* B10. Biosci Biotechnol Biochem 70:2394–2402
27. Parniske M, Schmidt PE, Kosch K, Müller P (1994) Plant defense response of host plants with determinate nodules induced by EPS defective *exoB* mutants of *Bradyrhizobium japonicum*. Mol Plant-Microbe Interact 7:631–638
28. Pérez-Giménez J, Mongiardini EJ, Althabegoiti MJ, Covelli J, Quelas JI, López-García SL, Lodeiro AR (2009) Soybean lectin enhances biofilm formation by *Bradyrhizobium japonicum* in the absence of plants. Int J Microbiol 1. doi:10.1155/2009/719367.
29. Quelas JI, Mongiardini EJ, Casabuono A, López-García SL, Althabegoiti MJ, Covelli JM, Pérez-Giménez J, Couto A, Lodeiro AR (2010) Lack of galactose or galacturonic acid in *Bradyrhizobium japonicum* USDA 110 exopolysaccharide leads to different symbiotic responses in soybean. Mol Plant-Microbe Interact 23:1592–1604
30. Sarma AD, Emerich DW (2006) A comparative proteomic evaluation of culture grown vs nodule isolated *Bradyrhizobium japonicum*. Proteomics 6:3008–3028
31. Sauer K (2003) The genomics and proteomics of biofilm formation. Genome Biol 4:219
32. Skouv J, Schnier J, Rasmussen MD, Subramanian AR, Pedersen S (1990) Ribosomal protein S1 of *Escherichia coli* is the effector for the regulation of its own synthesis. J Biol Chem 265:17044–17049
33. Tchufistova LS, Komarova AV, Boni IV (2003) A key role for the mRNA leader structure in translational control of ribosomal protein S1 synthesis in α-proteobacteria. Nucleic Acids Res 31:6996–7002
34. van Rhijn P, Goldberg RB, Hirsch AM (1998) *Lotus corniculatus* nodulation specificity is changed by the presence of a soybean lectin gene. Plant Cell 10:1233–1249
35. van Rhijn P, Fujishige NA, Lim PO, Hirsch AM (2001) Sugar-binding activity of pea lectin enhances heterologous infection of transgenic alfalfa plants by *Rhizobium leguminosarum* biovar *viceae*. Plant Physiol 126:133–144
36. Vincent JM (1970) A manual for the practical study of the root nodule bacteria. IBP handbook No. 15. Blackwell Scientific Publications, Oxford.

37. Vinuesa P, Neumann-Silkow F, Pacios-Bras C, Spaink HP, Martínez-Romero E, Werner D (2003) Genetic analysis of a pH-regulated operon from *Rhizobium tropici* CIAT899 involved in acid tolerance and nodulation competitiveness. Mol Plant-Microbe Interact 16:159–168
38. Wakeman CA, Winkler WC, Dann CE III (2007) Structural features of metabolite-sensing riboswitches. Trends Biochem Sci 32:415–424
39. Watt SA, Patschkowski T, Kalinowski J, Niehaus K (2003) Qualitative and quantitative proteomics by two-dimensional gel electrophoresis, peptide mass fingerprint and a chemically-coded affinity tag (CCAT). J Biotechnol 106:287–300