Arch Microbiol (2017) 199:737–755
DOI 10.1007/s00203-017-1351-8

ORIGINAL PAPER

The naringenin-induced exoproteome of *Rhizobium etli* CE3

Niurka Meneses[1,2,3] · Hermenegildo Taboada[1] · Michael F. Dunn[1] · María del Carmen Vargas[1] · Natasha Buchs[2] · Manfred Heller[2] · Sergio Encarnación[1]

Received: 16 August 2016 / Revised: 25 January 2017 / Accepted: 1 February 2017 / Published online: 2 March 2017

Abstract Flavonoids excreted by legume roots induce the expression of symbiotically essential nodulation (*nod*) genes in rhizobia, as well as that of specific protein export systems. In the bean microsymbiont *Rhizobium etli* CE3, *nod* genes are induced by the flavonoid naringenin. In this study, we identified 693 proteins in the exoproteome of strain CE3 grown in minimal medium with or without naringenin, with 101 and 100 exoproteins being exclusive to these conditions, respectively. Four hundred ninety-two (71%) of the extracellular proteins were found in both cultures. Of the total exoproteins identified, nearly 35% were also present in the intracellular proteome of *R. etli* bacteroids, 27% had N-terminal signal sequences and a significant number had previously demonstrated or possible novel roles in symbiosis, including bacterial cell surface modification, adhesins, proteins classified as MAMPs (microbe-associated molecular patterns), such as flagellin and EF-Tu, and several normally cytoplasmic proteins as Ndk and glycolytic enzymes, which are known to have extracellular "moonlighting" roles in bacteria that interact with eukaryotic cells. It is noteworthy that the transmembrane ß (1,2) glucan biosynthesis protein NdvB, an essential symbiotic protein in rhizobia, was found in the *R. etli* naringenin-induced exoproteome. In addition, potential binding sites for two *nod*-gene transcriptional regulators (NodD) occurred somewhat more frequently in the promoters of genes encoding naringenin-induced exoproteins in comparison to those ofexoproteins found in the control condition.

Keywords Rhizobium · Nitrogen Fixation · Proteomics · Naringenin

Communicated by Jorge Membrillo-Hernández.

Niurka Meneses and Hermenegildo Taboada contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00203-017-1351-8) contains supplementary material, which is available to authorized users.

✉ Sergio Encarnación
encarnac@ccg.unam.mx

[1] Programa de Genómica Funcional de Procariotes, Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, 62210 Cuernavaca, Morelos, Mexico

[2] Mass Spectrometry and Proteomics Laboratory, Department of Clinical Research, University of Bern, 3010 Bern, Switzerland

[3] Faculty of Science, Department of Chemistry and Biochemistry, University of Bern, 3010 Bern, Switzerland

Introduction

Protein excretion by bacteria is a vital component of their interaction with the environment and with eucaryotic cells reviewed in (Dou and Zhou 2012; Sánchez et al. 2010; Zanotti and Cendron 2014; von Tils et al. 2012). During interactions of the latter kind, proteins excreted by bacteria (exoproteins) include virulence factors and effectors that elicit host responses required for the development of the interaction, including cytoplasmic or cell surface proteins having secondary or "moonlighting" roles when exported to the environment (Henderson and Martin 2011) (http://www.moonlightingproteins.org/moonlighting.php).

Several species of Gram-negative alpha- and beta-proteobacteria, collectively called rhizobia, establish nitrogen-fixing symbioses with leguminous plants. Rhizobial

proteins localized on the cell surface or exoproteins excreted by specific export systems have been shown to play important roles in the symbiosis reviewed in (Downie 2010; Fauvart and Michiels 2008).

The symbiotic signalling cascade begins when flavonoids like naringenin diffuse across the rhizobial membranes and activate NodD (Deakin and Broughton 2009; Downie 2010), a LysR type regulator that binds to "*nod*-box" sequences in the promoters of the *nod* genes (Feng et al. 2003; Poupot et al. 1995). The rhizobial nod factors, synthesized by the products of the *nod* genes, are perceived by specific legume receptors whose activation brings about plant responses required for rhizobial infection and nodule formation (Downie 2010). In addition to controlling Nod-factor production, NodD also activates genes for producing symbiotically important protein secretion systems and lipopolysaccharides (Deakin and Broughton 2009), while other genes are induced by flavonoids in a NodD-independent manner (Pérez-Montaño et al. 2016a). Furthermore, in different rhizobial species other regulatory proteins different to NodD controlling nodulation, such as SyrM, and TtsI (López-Baena et al. 2016), suggesting an alternative way based on the secretion of effectors through the T3SS; however, homologous to *syrM* and *ttsI* genes is absent in the *R. etli* genome.

Once inside the nodules rhizobia are enveloped in a plant-derived symbiosome membrane where, in a metabolically differentiated form called bacteroids, they reduce atmospheric nitrogen to ammonium (Udvardi and Poole 2013).

A study investigating the intracellular proteome induced by naringenin in Rhizobium leguminosarum bv. viciae has been done (Arrigoni et al. 2013). In addition, many proteins in the exoproteomes of *R leguminosarum* bv. *viciae* (Krehenbrink and Downie 2008) and *Rhizobium etli* CE3 (Meneses et al. 2010) grown in minimal medium cultures have been identified, but little is known about the effect of *nod*-gene inducing flavonoids on the export of proteins by free-living rhizobia (Afroz et al. 2013). Exoproteins known to be involved in symbiosis include adhesins that function in bacterial attachment to roots, enzymes required for the modification of surface polysaccharides, and effectors that suppress plant defense responses or induce specific signalling pathways (Downie 2010). The symbiosome space, located between the symbiosome membrane and the bacteroid outer membrane, contains rhizobial enzymes for glycolysis and the tricarboxylic acid (TCA) cycle (Emerich and Krishnan 2014). The symbiotic requirement for protein secretion by rhizobia is also shown by the finding that *Sinorhizobium meliloti* mutants lacking TolC, an exporter protein, are unable to fix nitrogen (Cosme et al. 2008). Mutants of *S. meliloti* in genes encoding proteins predicted to be exported to the outer membrane or periplasmic space by the twin arginine transport (TAT) system had modest symbiotic defects on alfalfa and/or sweet clover (Pickering et al. 2012). However, deletion of the TAT system in *R. leguminosarum* bv. *viciae* resulted in nodules that were unable to fix nitrogen (Krehenbrink and Downie 2008). Mutants of *R. leguminosarum* bv. *viciae* lacking the PrsDE general secretion system had a reduced ability to fix nitrogen (Finnie et al. 1997).

R. etli CE3 establishes a nitrogen-fixing symbiosis with bean plants (*Phaseolus vulgaris*). During growth in minimal medium cultures, strain CE3 develops a fermentative-like metabolism that in several aspects resembles its metabolism during symbiosis, including poly-ß-hydroxybutyrate (PHB) accumulation and a decrease or disappearance of the activities of several central metabolic enzymes (Encarnación et al. 1995, 2003; Dunn et al. 2002). Here, we report the first extracellular proteome in *R. etli* CE3 induced by naringenin in minimal medium. The aim of this work was to obtain a global view of whether proteins excreted during the early phase of the fermentative-like response in *R. etli* would contain a significant portion of symbiotically relevant proteins, and how this response might be altered by the presence of the *nod*-gene inducing flavonoid naringenin.

Methods

Cell culture and preparation of culture supernatants

Rhizobium etli strain CE3 was grown in minimal medium as described previously (Encarnación et al. 1995). At mid-log phase (12 h, OD_{540} of 0.5–0.6), naringenin was added to a final concentration of 1.5 µM from a 50 mg/ml stock solution in ethanol and growth was continued for 12 h at 30 °C to late-log phase. Control cultures lacking naringenin contained ethanol only. Cells were pelleted by centrifugation at 7500×*g* for 30 min at 4 °C, after which the supernatant was recentrifuged at 6500×*g* for 45 min at 4 °C. The cell-free supernatant was passed through a 22 µm filter, lyophilized to dryness and the samples rehydrated with phosphate buffer (7 mM K_2HPO_4; 3 mM KH_2PO_4; 0.15 mM NaCl; pH 7.2). Seven hundred fifty µl of extraction buffer (0.7 M sucrose; 0.5 M Tris–HCl; 30 mM HCl; 50 mM EDTA; 0.1 M KCl and 40 mM DTT; pH 8.5) was then added, followed by incubation for 15 min at 25 °C. The samples were then vortexed with an equal volume of phenol for 15 min, the emulsion was centrifuged at 14,000×*g* for 3 min at 4 °C and the phenolic phase recovered. The extraction was repeated two more times and proteins in the combined phenolic phases were precipitated by adding 5 volumes of 0.1 M ammonium acetate in methanol. The precipitate was washed with 1 ml of 80% acetone and solubilized with 50 mM Tris–HCl, pH 8.

SDS–PAGE and protein digestion

Two technical replicates were done, each using 40 ug of total protein mixed with Laemmli buffer (Laemmli UK 1970), boiled at 95 °C for 5 min and resolved on a 1 mm thick 12.5% polyacrylamide gel. Gels were stained with Coomassie Brilliant Blue G-250 (Gunasekera et al. 2012) and stored for up to 2 days in 20% (v/v) ethanol at 4 °C. MS analysis was done in duplicate on two SDS–PAGE lanes for each sample by cutting each gel lane into ten segments. Each segment was transferred to a low-binding reagent tube (Sarstedt, Nümbrecht, Germany) (Gunasekera et al. 2012) and washed with a 1:1 mixture of 100 mM Tris–HCl (pH 8.0)/acetonitrile, followed by reduction with 50 mM DTT for 30 min at 37 °C. Samples were alkylated with 50 mM iodoacetamide for 30 min at 37 °C. Sequencing-grade trypsin was added to a final concentration of 10 ng/µl and samples were incubated for 5 h at 37 °C. Tryptic fragments were extracted with 20 µl of 20% (v/v) formic acid.

Peptide separation by nano-LC-MS/MS

Peptide sequencing was performed on an LTQ XL-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a Rheos Allegro nano flow system with AFM flow splitting (Flux Instruments, Reinach, Switzerland) and a nano electrospray ion source operated at a voltage of 1.6 kV. Peptide separation was performed on a Magic C18 nano column (5 µm, 100 Å, 0.075 × 70 mm) using a flow rate of ~400 nl/min and a 60 min linear gradient of 5 to 40% acetonitrile in H_2O containing 0.1% formic acid. Data acquisition was in data dependant mode on the top five peaks with an exclusion for 15 s. Survey full scan MS spectra were from 300 to 1800 m/z, with resolution $R = 60{,}000$ at 400 m/z, and fragmentation was achieved by collision-induced dissociation with helium gas.

Protein identification and sequence analysis

Mascot generic files (mgf) were created using a pearl script with Hardklor software, v1.25 (Michael Hoopmann and Michael MacCoss, University of Washington). MS/MS data (mgf files) were submitted to PHENYX (Geneva Bioinformatics) and searched against the Uniprot-SwissProt protein database [Rhizobium_Homo_Tryp_For-Rev (20100415)]. The process was performed in two rounds First search parameters were: parent error tolerance 20 ppm; normal cleavage mode with 1 missed cleavage, allowed; amino-acid modifications (fixed Cys_CAM, variable Oxidation_M); minimal peptide z-score, 5; max p value, 0.01; and AC score of 5. Second search parameters were: parent error tolerance, 20 ppm; half cleaved mode with 4 missed cleavages; allowed amino-acid modifications (variable Cys_CAM, variable); Deamid, variable; phos, variable; Oxidation_M, variable pyrr; minimal peptide z-score, 5; max p value, 0.01. Protein identifications were only accepted with a score of 10, meaning when two different peptide sequences could be matched.

In silico analysis of NodD-binding sites in genes encoding exoproteins

Genes from the order *Rhizobiales* orthologous to the *R. etli* RHE_PD00275 and RHE_PD00316 *nodD* genes were obtained with the suite of programs in RSA tools [http://prokaryotes.rsat.eu/ (Thomas-Chollier et al. 2011)], used for defining transcriptional start sites. The list was manually curated to eliminate non-symbiotic strains. Sequences from −400 + 1 (transcriptional start site) of the genes obtained were submitted to the dyad-analysis program in RSA Tools to obtain the conserved dyads. Dyads were selected at a 1×10^{-4} threshold by scanning the upstream sequences of the corresponding orthologs. These matrices were also validated by matrix scanning of genes in the NCBI database (http://www.ncbi.nlm.nih.gov/gene/) that are known to be involved in Nod-factor synthesis in *R. etli* CE3 (results not shown). These matrices were used in a matrix scan of the data from this work and in the *R. etli* CE3 genome at a threshold of 1×10^{-4} and 1×10^{-3}.

Potential transcription binding sites for the RHE_PD00275 and RHE_PD00316 NodD transcriptional regulators were identified in the RSAT suit of programs (http://embnet.ccg.unam.mx/rsa-tools/) (Thomas-Chollier et al. 2011). Orthologous genes in the order Rhizobiales were obtained using the RSAT-get-orthologs program. These sequences were analyzed for spacing dyads using the RSAT-dyad-analysis program to generate a position specific scoring matrix (PSSM) (Medina-Rivera et al. 2011). Five matrices were obtained and used to run a matrix scan on the upstream sequences (−400 + 1) for orthologs of RHE_PD00275 at a p value 1e−4 in an RSAT-matrix scan quick and simple. Matrices 2 and 3, with sequences taGTAAAgtatatatTTTActa and caCGAaagaATCGgt, respectively (Figure S2), were specific for promoter RHE_PD00275 and are hereafter referred to as NodD1_2 and NodD1_3, respectively. For the RHE_PD00316 NodD transcriptional regulator, insufficient orthologous genes were found within the Rhizobiales order, so a matrix-scan analysis was done with matrices from RHE_PD00275 against the genes orthologous to RHE_PD00316 at a p value of 1e−4. Matrices 1, 4 and 5, corresponding to the sequences ggcAAAATcgatTGTTTGttg, caAACAaacgaTTTTtCCAcc and cGcAAAATCgaTTGtTTGgtg, respectively, (Figure S3) were specific for promoter RHE_PD00316 and are hereafter referred to as NodD2_1, NodD2_4 and NodD2_5. These matrices were used for a matrix scan of

the upstream sequences (−400 + 1) of known nod-factor synthesis genes from *R. etli* CFN 42 deposited in the NCBI data bank (https://www.ncbi.nlm.nih.gov/pmc/) (González et al. 2006). Of the 24 genes or operons for *R. etli* nod factor synthesis, comprised of a total of 40 genes, 19 gene promoters were detected with a matrix-scan analysis at *p* values of 1e−3 and/or 1e−4 (unpublished results). A matrix scan was run with these specific matrices for NodD1 and NodD2 transcriptional regulators against the upstream regions (−400 + 1) at a *p* value of 1e−4 (Table S3). Binding sites for NodD1 and NodD2 from *R. etli* (Table S3) were analyzed in the RSAT-Dyad-analysis program to produce a graphic of the five most highly representative binding sites for NodD1 (Figure S2) and NodD2 (Figure S3). For NodD1, the ATCG-n4-CGAT consensus binding site obtained (Figure S3) is similar to the NodD consensus of ATC-n9-GAT from *R. leguminosarum* (Feng et al. 2003) and *Sinorhizobium fredii* HH103 (Pérez-Montaño et al. 2016b). It should be noted that a third *R. etli nodD* gene transcriptional regulator, RHE_PD00318, having an intergenic region of 151 nucleotides, neighbors RHE_PD00319 as part of an operon. RHE_PD00318 has orthologs in *R. etli* CIAT 652 (uid59115), *Rhizobium tropici* CIAT 899 (uid185179 and GCA 000330885.1), which were insufficient to generate a PSSM.

Results and discussion

General characteristics of the *R. etli* exoproteome

By the criteria specified in "Methods", we identified a total of 693 proteins in the exoproteomes of *R. etli* cultures grown in the presence or absence of naringenin. The percentage of reproducibility for the two replicate experiments was 80.6% without naringenin and 73.4% with naringenin.

Proteins found exclusively in the control or naringenin-treated cultures each comprised about 14.5% of the total proteins (101 and 100 proteins, respectively) (Table 1). Four hundred ninety-two (71%) of the total proteins were produced by both the control and naringenin-treated cultures and are hereafter referred to as "common proteins" (S1 Table). For the 693 proteins identified in the exoproteomes, the functional classes representing more than 5% of the total were proteins with unknown function (18.6%), amino-acid transport and metabolism (13.6%), carbohydrate transport and metabolism (10.5%), translation (10.0%), energy production (7.9%), post-translational modification, protein turnover and chaperones (6.8%) and cell wall/envelope biogenesis (6.6%) (Table 1 and S1 Table).

Given the importance of flavonoids in the production of some symbiotically important proteins in rhizobia (Downie 2010; Pérez-Montaño et al. 2016a), we hypothesized that a high proportion of exoproteins exclusive to naringenin-treated cultures (Table 1) would also be present in *R. etli* bacteroids. More than one-third of the exoproteome proteins identified here (Table 1 and Table S1) were also found in *R. etli* bacteroids isolated from bean nodules (Resendis-Antonio et al. 2011). Of the exoproteome proteins also found in bacteroids, 14% were present in the naringenin-treated cultures, 9.3% in the control cultures and 77% in both cultures. This is similar to the total protein distribution found here in cells grown in culture (Table 1; Table S1), indicating that the proportion of exoproteins that are also present in bacteroids was not markedly increased by naringenin. In retrospect, this is perhaps not surprizing, since naringenin acts as a signal molecule early in the interaction, while the bacteroid proteome was determined in mature nodules (Resendis-Antonio et al. 2011). The major functional categories represented in the 33 proteins common to the naringenin-treated culture exoproteome and bacteroids were energy production and conversion (21.2%) and amino-acid transport and metabolism (30.3%), with the other 10 represented categories each having 3–9% of the total. For the bacteroid proteins common with the control culture exoproteome, the functional categories for energy production and conversion represented 22.7% of the total, lipid transport and metabolism 13.6% of the total and the other 9 categories each representing less than 10% of the total. For exoproteome common proteins that were also identified in bacteroids, the functional category representations were amino-acid transport and metabolism (20.4%), carbohydrate transport and metabolism (14.9%), energy production and conversion (10.5%), with the other 14 categories each representing less than 9%. In general, metabolic and energy generating processes were the most represented categories in both bacteroids and the exoproteomes of cultured cells.

The mechanism by which proteins arrive at their extracellular location is an essential consideration in evaluating bacterial exoproteomes. Proteins present in the *R. etli* exoproteome could result specifically from their secretion by a dedicated export system (Downie 2010) or non-specifically from cell lysis or leakage of cytoplasmic or periplasmic proteins (Krehenbrink and Downie 2008). Of 23 extracellular proteins identified in *R. leguminosarum* bv. *viciae* 3841 culture supernatants, over half were predicted to be localized to the periplasm and, because strain 3841 lacks a Type II secretion system that secretes periplasmic proteins across the outer membrane, these periplasmic proteins were proposed to have arisen mostly from non-specific leakage (Krehenbrink and Downie 2008). The same phenomenon could explain the presence of some of the periplasmic proteins, such as substrate binding proteins, in the *R. etli* exoproteome. However, in comparison to *R. leguminosarum*, lower percentages of periplasmic proteins were found in the exoproteomes of *R. etli* naringenin-treated, control or

Table 1 Exoproteome proteins differentially expressed in *R. etli* CE3 cultures grown with or without naringenin

Functional category and protein name (abbreviation)[a]	Accession number
Energy production	
Alcohol dehydrogenase	RHE_CH02884
Aldehyde dehydrogenase	RHE_CH03161*
Aldehyde dehydrogenase	RHE_CH03723*
Cytochrome-c-556 signal peptide (CycF)^{S}	RHE_CH01214
F_0F_1 ATP synthase α subunit (AtpA)	*RHE_CH03872**
Malic enzyme, NAD^+-dependent (Dme)	RHE_CH02355*
Malic enzyme, $NADP^+$-dependent (Tme)	RHE_CH00389*
Methylmalonate-semialdehyde dehydrogenase (acylating) (IolA)	*RHE_CH00731**
Phosphoglyceromutase (GpmA)	*RHE_CH00169**
Potassium channel, voltage-gated, ß subunit	RHE_CH01950*
Proline /pyrroline-5-carboxylate dehydrogenase (PutA)	RHE_PF00384
Quinone oxidoreductase (Qor)	RHE_CH02080*
S-(hydroxymethyl)glutathione dehydrogenase (AdhCch)	RHE_CH02227*
Ubiquinol-cytochrome-c reductase, iron-sulfur subunit (FbcF)^{T}	RHE_CH03038*
Amino-acid transport and metabolism	
2-Isopropylmalate synthase (LeuA2)	*RHE_CH03067**
3-Isopropylmalate dehydrogenase (LeuB)	RHE_CH04093*
3-Phosphoshikimate 1-carboxyvinyltransferase (AroA)	RHE_CH00884*
Acetyltransferase	RHE_CH00949
Amino-acid ABC transporter substrate-binding protein	RHE_CH01465
Amino-acid ABC transporter substrate-binding protein^{S}	RHE_PF00162
Argininosuccinate lyase (ArgH)	RHE_CH03796
Argininosuccinate synthase (ArgG2)	RHE_CH03924
Arylesterase	RHE_CH01936
Aspartate kinase (LysC)	RHE_CH03758
Chorismate synthase (AroC)	RHE_CH00935
Cysteine desulfurase	RHE_CH02249*
Dihydrodipicolinate synthase (DapAf1)	RHE_PF00094
Dihydrodipicolinate synthetase	RHE_PC00042
Dihydroxy-acid dehydratase (IlvDch2)	RHE_CH03160*
Dipeptide ABC transporter substrate-binding protein (DppA)^{S}	RHE_PF00239
Glutamate dehydrogenase, NAD-specific	RHE_CH04105*
Glutamate synthase large subunit (GltB)	RHE_CH03566
Glycine cleavage system protein H (GcvH)	RHE_CH02242
Glycine dehydrogenase (GcvP)	RHE_CH02243
GTP cyclohydrolase/3,4-dihydroxy-2-butanone 4-phosphate synthase, bifunctional (RibA)	RHE_CH00934
Homoserine dehydrogenase (ThrA)	RHE_CH01878*
Imidazole glycerol phosphate synthase subunit (HisH)	RHE_CH00045
Imidazoleglycerol-phosphate dehydratase (HisB)	RHE_CH00047
Leucyl aminopeptidase (PepA)	RHE_CH01456
***N*-methylhydantoinase (ATP-hydrolyzing) 5-oxoprolinase**	RHE_PE00300*
Oligoendopeptidase F (PepF)	RHE_CH03549*
Peptide ABC transporter, substrate-binding protein^{S}	RHE_CH00175*
Protease II (PtrB)	RHE_CH01226*
Sarcosine dehydrogenase	RHE_PE00084
Tryptophan synthase ß subunit (TrpB)	RHE_CH00021*
Zn^{2+}-dependent protease (TldD)	RHE_CH00950*
Carbohydrate transport and metabolism	
6-Phosphogluconate dehydrogenase (Gnd)	RHE_CH02468*

Table 1 (continued)

Functional category and protein name (abbreviation)[a]	Accession number
6-Phosphogluconate dehydrogenase-like protein	RHE_CH03488
Aldose-1-epimerase (GalM)	RHE_CH04034
C4-dicarboxylate ABC transporter substrate-binding protein[S]	RHE_PF00068
C4-dicarboxylate transport system, substrate-binding protein[S]	RHE_PC00213
Chitooligosaccharide deacetylase	RHE_CH03142
D-Ribose ABC transporter substrate-binding protein[S]	RHE_PF00217
Epimerase	RHE_PF00382
Glucose dehydrogenase B[S]	RHE_CH03002
Keto-hydroxyglutarate-aldolase/keto-deoxy-phosphogluconate aldolase (Eda)	RHE_CH03635
Monophosphatase	RHE_CH03573
Ribose ABC transporter, substrate-binding protein (RbsBch2)[S]	RHE_CH01787
ß-1, 2-Glucan production transmembrane protein (NdvB)[T]	*RHE_CH03997**
Sugar ABC transporter substrate-binding protein	RHE_PE00100
Sugar ABC transporter substrate-binding protein[S]	RHE_PC00149
Sugar ABC transporter substrate-binding protein[S]	RHE_PB00150
Sugar ABC transporter substrate-binding protein[S]	RHE_PF00374*
Sugar ABC transporter sugar-binding protein[S]	RHE_PE00245
Sugar ABC transporter, substrate-binding protein[S]	RHE_CH01210
Sugar ABC transporter, substrate-binding protein[S]	RHE_CH02068
Sugar ABC transporter, substrate-binding protein[S]	RHE_CH02361
Sugar ABC transporter, substrate-binding protein[S]	RHE_CH03844
Sugar kinase	RHE_CH00135*
Xylose ABC transporter, permease (GguB)[T]	RHE_CH03163
Lipid metabolism	
3-Oxoacyl-(acyl-carrier protein) synthase I (FabB)	*RHE_CH00108**
3-Oxoacyl-(acyl-carrier protein) synthase II (FabF1)	*RHE_CH01445*
3-Oxoacyl-(acyl-carrier protein) reductase	RHE_CH01048*
Enoyl-(acyl-carrier protein) reductase (FabI1)	RHE_CH00109*
Enoyl-CoA hydratase	RHE_CH03034
Esterase lipase thioesterase family protein	RHE_CH01714
Nucleotide metabolism	
Adenine phosphoribosyltransferase (Apt)	RHE_CH03035
Coenzyme metabolism	
CTP synthetase (PyrG)	RHE_CH02184
Glutamate-cysteine ligase (Gsh)	RHE_CH00803
Hypoxanthine phosphoribosyltransferase (Hpt)	RHE_CH03800
NAD synthetase (NadE)	RHE_CH01195*
Phosphoribosylaminoimidazole/carboxamide IMP cyclohydrolase formyltransferase, bifunctional (PurH)	RHE_CH04107*
Phosphoribosylformylglycinamidine synthase subunit (PurS)	RHE_CH02279
Ribose-phosphate pyrophosphokinase (PrsAch)	RHE_CH03023*
Phosphomethylpyrimidine synthase (ThiC)	RHE_PB00082
Thiamine-phosphate pyrophosphorylase (ThiE)	RHE_PB00079
Uracil phosphoribosyltransferase (Upp)	RHE_CH00196
Inorganic ion transport and metabolism	
Aliphatic sulfonate ABC transporter substrate-binding protein[S]	*RHE_PC00167**
Carbonic anhydrase	RHE_PD00192
Ferric uptake regulation protein (Fur)	RHE_CH00378
Taurine uptake ABC transporter substrate-binding protein (TauA)[S]	RHE_PC00019*
Zinc uptake ABC transporter, ATP-binding protein (ZnuC)	RHE_CH02711
Replication and repair	

Table 1 (continued)

Functional category and protein name (abbreviation)[a]	Accession number
DNA helicase, ATP-dependent (MgpS)	RHE_CH04022*
DNA polymerase I (PolA)	*RHE_CH00151**
DNA topoisomerase I (TopA)	RHE_CH01638
Integration host factor α subunit (IhfA)	RHE_CH01533
Integration host factor ß subunit (IhfB)	RHE_CH00399
Recombinase A (RecA)	RHE_CH02323
Transcription-repair coupling factor (TRCF) protein (Mfd)	RHE_CH02095
Cell cycle control	
ATP-binding protein (Mrp protein-like)	RHE_CH00859
TRANSLATION	
7-Cyano-7-deazaguanine reductase (QueF)	RHE_CH03071
30S ribosomal protein S12 (RpsL)	RHE_CH01670
30S ribosomal protein S20 (RpsT)	*RHE_CH00357**
50S ribosomal protein L1 (RplA)	*RHE_CH01664**
50S ribosomal protein L28 (RpmB)	*RHE_CH03816**
50 S ribosomal protein L7 L12 (RplL)	*RHE_CH01666**
Acetyltransferase	RHE_CH02834
Arginyl-tRNA synthetase (ArgS)	RHE_CH01818*
Histidyl-tRNA synthetase (HisS)	RHE_CH00821*
Ribonuclease PH (Rph)	RHE_CH00363
Ribosomal RNA small subunit methyltransferase H (RsmH)	RHE_CH02855
Seryl-tRNA synthetase (SerS)	*RHE_CH01826**
TRANSCRIPTION	
Cold shock protein (CspA4)	*RHE_CH02568*
MarF family transcriptional regulator	RHE_CH03623
N utilization substance protein B homolog (NusB)	RHE_CH01526
RNA polymerase sigma factor (RpoD)	RHE_CH02950
Transcription termination/antitermination protein (NusA)	*RHE_CH00118**
SECONDARY STRUCTURE	
2-Hydroxyhepta-2,4-diene-1,7-dioate isomerase/5-carboxymethyl-2-oxo-hex-3-ene-1,7-dioatedecarboxylase	RHE_CH00016
Fumarylpyruvate hydrolase	RHE_CH02701
Post-translational modification, protein turnover, chaperone functions	
10 kDa chaperonin (GroESch2)	*RHE_CH01238*
60 kDa chaperonin 2 (GroL2)	*RHE_CH01239*
60 kDa chaperonin 3 (GroL3)	*RHE_CH01271*
ATP-dependent Clp protease, ATP-binding subunit (ClpA)	RHE_CH01907
Chaperone protein DnaJ (DnaJ1)	RHE_CH00144
Glutaredoxin (GrlA)	RHE_CH02304
Glutathione peroxidase (BtuE)	RHE_CH01597*
Heat shock protein (GrpE)	RHE_CH00365*
Membrane protease subunit[T]	RHE_CH03557
Molecular chaperone small heat shock protein (Hsp)	RHE_CH00367
Nitrogen fixation protein (NifUch1)	RHE_CH00381
RNA-binding protein (Hfq)	RHE_CH01954
Urease accessory protein (UreE)	RHE_CH03302
Signal transduction	
cAMP-dependent kinase	RHE_CH03969
Serine protein kinase	RHE_CH02817*
Two-component response regulator	RHE_CH03010
Two-component response regulator, putative	RHE_CH03968*

Table 1 (continued)

Functional category and protein name (abbreviation)[a]	Accession number
Cell wall/membrane/ envelope biogenesis	
2-Dehydro-3-deoxyphosphooctonate aldolase (KdsA)	RHE_CH01930
Glycoside hydrolase (Lyc)[S]	RHE_CH02868
Glycosyltransferase[T]	RHE_CH01319
Ketal pyruvate transferase (PssM)	RHE_CH03215
LPS-assembly protein (LptD)[S]	*RHE_CH01452**
Outer membrane lipoprotein[S]	RHE_PF00471
Penicillin binding peptidoglycan synthetase (MrcA)[T]	RHE_CH01647
Polysaccharidase (PlyA1)	RHE_CH02605
UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase (MurA)	RHE_CH00579*
UDP-*N*-acetylmuramoyl-*l*-alanyl-*d*-glutamyl-*l*-lysyl-*d*-alanyl-*d*-alanine synthetase (MurF)	RHE_CH02851
undecaprenyldiphospho-muramoylpentapeptide beta-*N*-acetylglucosaminyltransferase (MurG)	RHE_CH02847
Intracellular trafficking and secretion	
Translocase subunit SecY, preprotein (SecY)[T]	RHE_CH01695[b]
Cell motility	
Chemotaxis motility protein (MotD)	RHE_CH00676
Defence mechanisms	
ß-lactamase (Bla)	RHE_CH01220
Glutathione-dependent formaldehyde-activating enzyme (Gfa)	RHE_PF00400
General function prediction only	
Chloride peroxidase	RHE_CH02571*
Hydrolase	RHE_CH00996
Oxidoreductase	RHE_CH02455
Oxidoreductase	RHE_CH03925*
UNKNOWN FUNCTION PROTEINS	
Unknown function protein[S]	RHE_CH00136
Unknown function protein[S]	RHE_CH00304
Unknown function protein[S]	RHE_CH00874
Unknown function protein	RHE_CH00940
Uncharacterized protein	RHE_CH01015
Unknown function protein	RHE_CH01383*
Unknown function protein	RHE_CH01421
Unknown function protein[S]	RHE_CH01650
Unknown function protein	RHE_CH02276
Unknown function protein	RHE_CH02902
Unknown function protein	RHE_CH03019
Unknown function protein[S]	RHE_CH03022
Unknown function protein	RHE_CH03336
Unknown function protein[T]	RHE_CH03343
Probable transcriptional regulatory protein	RHE_CH03475*
Unknown function protein[T]	RHE_CH03513
Unknown function protein	RHE_CH03724
Unknown function protein	RHE_CH03880
Unknown function protein	RHE_CH03908
Unknown function protein	RHE_PB00156*
Unknown function protein	RHE_PE00139
Unknown function protein	RHE_PE00320
Unknown function protein[S]	RHE_PF00470
Unknown function protein[S]	RHE_CH00050

Table 1 (continued)

Functional category and protein name (abbreviation)[a]	Accession number
Ribosome maturation factor (RimP)	RHE_CH00119
Nucleoid-associated protein	RHE_CH00125
Unknown function protein	*RHE_CH00721*
Unknown function protein	RHE_CH01051
Unknown function protein[T]	RHE_CH01405
Unknown function protein	RHE_CH01447
Unknown function protein	RHE_CH01833
Unknown function protein	RHE_CH01886
Unknown function protein	RHE_CH02191
Unknown function protein[S]	RHE_CH02285
Unknown function protein[S]	RHE_CH02333
Unknown function protein	*RHE_CH02379*[b]
Unknown function protein	*RHE_CH02456**
Unknown function protein	RHE_CH03460
Unknown function protein	RHE_CH03509
Unknown function protein	RHE_CH03634*
Unknown function protein	RHE_CH03813
Unknown function protein	RHE_CH03906*
Unknown function protein[S]	RHE_PD00276
Unknown function protein[S]	*RHE_PE00018*
Unknown function protein[S]	*RHE_PE00163*
Unknown function protein[S]	*RHE_CH00851**

Protein names in bold face indicate those found exclusively in naringenin-treated cultures and proteins in normal font those found only in control cultures. Protein accession numbers in italic face indicate proteins also found in our previous study of the *R. etli* exoproteome (Meneses et al. 2010) and in the *R. leguminosarum* bv. *viciae* exoproteome (Krehenbrink and Downie 2008). Protein accession numbers marked with asterisk were also found in *R. etli* bacteroids (Resendis-Antonio et al. 2011)

[a]Annotation from the Rhizobase or UniProtKB databases, also indicating the presence of signal peptide (superscript S) and transmembrane (superscript T) domains. Additional information on the majority of the hypothetical proteins is provided in Table S2, Supplemental material

[b]The GEP secretion system is predicted to export the protein based on data for *R. leguminosarum* bv. *viciae* secretome orthologs (Krehenbrink and Downie 2008)

both cultures (19, 43 and 35% of the totals, respectively). In addition, since *R. etli* encodes components of a Type II secretion system, it might specifically export some of these proteins.

Gram-negative bacterial proteins with N-terminal signal peptides may be destined for incorporation into the cytoplasmic or outer membrane, transport to the periplasm or export from the cell. Just over 27% of the 693 exoproteins identified in this study have signal peptides, less than 5% contain transmembrane domains indicative of a membrane localization (Table 1 and S1 and S2 Tables), while the remainder could result from leakage/cell lysis or secretion by non-classical pathways. To assess if cytosolic proteins originating from cell lysis contaminated the secretome, in this and our previous work (Meneses et al. 2010), we compared two-dimensional gel protein patterns obtained from the exoproteome with those of chemically lysed cells. In both cases, we observed substantial differences in the protein patterns and found that many proteins present at high levels in the lysed cell samples were absent from the exoproteome (Meneses et al. 2010) and (Fig. S1). From these data, we conclude that protein leakage or cell lysis is not major sources of the exoproteins found in the present work. We note that significantly fewer proteins with signal peptides were present in the exoproteins from the naringenin-treated cultures (10%) versus the control culture and common exoproteins (24.8 and 30.9%, respectively). The proportion of exoproteins with transmembrane domains in these categories were 6, 4, and 4.7%, respectively.

The previous proteomic analysis in *Escherichia coli* and Mycobacterium tuberculosis revealed that ribosomal and Ef-Tu proteins were also found in cell wall and membrane compartments (McBroom and Kuehn 2007; Mawuenyega et al. 2005), suggesting that these proteins are secreted through outer membrane vesicles (OMV). We suggest that in R. etli CE3, similar export mechanisms might be operating to allow the export of cytoplasmic, periplasmic, and OMV proteins.

In silico analysis of NodD-binding sites in genes encoding exoproteins

To determine the potential for NodD-dependent regulation of the expression of genes encoding exoproteins, we searched for possible binding sites for two NodD transcriptional regulators, NodD1 (RHE_PD00275) and NodD2 (RHE_PD00316) in the nucleotide regions upstream of predicted Open Reading Frames (ORFs) in the *R. etli* genome (see "Methods"). With a matrix scan threshold of 1×10^{-4}, we found 391 and 385 genes in the genome with a hypothetical binding site for NodD1 or NodD2, respectively (results not shown). These included genes encoding 17% of the naringenin-induced exoproteins, suggesting that their transcription may be controlled by NodD. However, we also found that 11% of genes encoding control culture proteins possessed these sites. Among the common exoproteins, 16% of the encoding genes had potential NodD1 and/or NodD2-binding sites (Table 2). Thus, genes with NodD1- and/or NodD2-binding sites are found in genes encoding proteins that are secreted in the presence or absence of naringenin, which suggests that naringenin regulates metabolic genes in addition to the *nod* genes. This supposition agrees with the flavonoid-induced metabolic proteins found in *R. leguminosarum* (Tolin et al. 2013) and *B. japonicum* (Da Silva Batista and Hungria 2012). The expression of the naringenin-induced metabolic genes in *R. etli* is likely also controlled by additional regulators. We found that nod factors were not produced in minimal medium in the absence of naringenin (unpublished data), and no other role for NodD apart from Nod-factor synthesis has been suggested in *R. etli* CE3. The results of our analysis of specific exoproteins are discussed in the following sections.

Proteins present in the exoproteomes of both naringenin-treated and control cultures

The *R. etli* exoproteins found in both naringenin-treated and control cultures (referred to here as "common proteins") that are probably specifically exported or present on the cell surface include PlyA2, an ortholog of the *R. leguminosarum* bv. *viciae* PlyB. PlyB is a ß-(1,4) glycanase that degrades *R. leguminosarum* EPS and also carboxymethylcellulose, the latter being an analog of plant cell wall cellulose (Johnsen and Krause 2014). *R. leguminosarum plyB* null mutants produce EPS with significantly higher molecular mass in comparison to the wild type and form less biofilm, but are not affected in symbiosis (Finnie et al. 1998; Russo et al. 2006; Zorreguieta et al. 2000). Cellulase activity is associated with the capsular polysaccharide layer of several rhizobia, including *R. leguminosarum* biovars *trifolii* and *phaseoli*, although whether these derive from PlyB-like enzymes has not been determined (Pérez-Montaño et al. 2016a; Mateos et al. 1992; Morales et al. 1984). Four Rhizobium-adhering proteins (Rap), which are cell surface proteins involved in autoaggregation and binding to host cell roots (Downie 2010; Mongiardini et al. 2008), were among the *R. etli* common proteins (S1 Table). While a role for the *R. etli* Rap proteins in root attachment remains to be demonstrated, these proteins could participate in the pronounced autoaggregation that occurs when strain CE3 cultures are grown in MM (Encarnación et al. 1995). Rhizobium outer membrane (Rop) proteins are major components of the outer membrane of some rhizobia (Foreman et al. 2010). Two RopB orthologs were present in the *R. etli* common exoproteome. In *S. meliloti* and *R. leguminosarum* bv. *viciae*, RopB mutants are more sensitive to detergents, hyperosmotic conditions, and low pH, indicating a role for RopB in maintaining membrane stability (Campbell et al. 2003; Foreman et al. 2010). Flavonoids can have a positive influence or negative influence on EPS production in different rhizobia (Janczarek 2011), but we did not find proteins involved in EPS synthesis that were exclusive to either the naringenin-treated or control culture exoproteomes. Because *R. etli* CE3 produces acidic EPS when grown in minimal medium (M. F. Dunn, unpublished observations), the presence of numerous proteins for EPS synthesis and modification in the common exoproteome was expected. The transcriptional regulators AniA and RosR are involved in controlling EPS synthesis in *R. etli* (Bittinger and Handelsman 2000; Encarnación et al. 2002) and were present in the common exoproteome (Table Sl).

Rhizobiocin (RzcA) is a rhizobial bacterocin present in the *R. etli* common exoproteome. In *R. leguminosarum* bv. *viciae*, bacteriocins of the RzcA type inhibit the growth of selected *R. leguminosarum* strains and nodulation competition tests show that RzcA is a determinant of nodulation competitiveness (Encarnación et al. 2002).

Orthologs of some of the presumably cytoplasmic proteins present in the *R. etli* common exoproteome (S1 Table) are known to have extracellular "moonlighting" roles in the interaction of various microbial pathogens with their hosts, where exported metabolic proteins can promote, invasion, and/or adherence to host cells (Henderson and Martin 2011). Given the significant similarities between the interaction of plant and animal pathogens with their hosts and the mutualistic symbiosis formed between rhizobia and legumes (Tóth and Stacey 2015; Soto et al. 2006), it is reasonable to suspect that some of the *R. etli* exoproteins described above could be playing moonlighting roles. This hypothesis remains to be tested.

Table 2 *R. etli* CE3 exoproteome proteins whose encoding genes contain potential NodD-binding sites in their promoter regions

Not COG	Accession	Exoproteome with protein product[a]	Binding site present in gene promoter
Unknown function protein	RHE_CH03513	Naringenin	NodD1
Unknown function protein	RHE_CH01015	Naringenin	NodD2
Unknown function protein	RHE_CH00851	Both	NodD1, NodD2
Polysaccharidase PlyA1	RHE_CH02605	Naringenin	NodD1
Porin Out RopAch1	RHE_CH01349	Both	NodD2
Porin Out RopAch2	RHE_CH02437	Both	NodD1, NodD2
Unknown function protein	RHE_CH00721	Control	NodD2
Unknown function protein	RHE_CH03336	Naringenin	NodD2
ENERGY PRODUCTION AND CONVERSION			
2-Oxoglutarate dehydrogenase E1 component SucA	RHE_CH03888	Both	NodD1
Bifunctional proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase PutA	RHE_PF00384	Naringenin	NodD2
Dihydrolipoamide dehydrogenase LpdAch1	RHE_CH01938	Both	NodD1, NodD2
Dihydrolipoamide dehydrogenase LpdAch2	RHE_CH03882	Both	NodD1
NADH dehydrogenase subunit G NuoG1	RHE_CH01611	Both	NodD2
Phosphoenolpyruvate carboxykinase PckA	RHE_CH00037	Both	NodD1, NodD2
Pyruvate carboxylase Pyc	RHE_CH04002	Both	NodD1, NodD2
Amino-acid transport and metabolism			
2-Isopropylmalate synthase LeuA2	RHE_CH03067	Naringenin	NodD2
Amino-acid ABC transporter, substrate-binding protein BraC1	RHE_CH03093	Both	NodD1, NodD2
Amino-acid ABC transporter, substrate-binding protein BraC2	RHE_CH03321	Both	NodD1, NodD2
Aspartate aminotransferase AatAch	RHE_CH02998	Both	NodD1, NodD2
Branched-chain amino-acid ABC transporter, substrate-binding protein	RHE_CH03445	Both	NodD1, NodD2
Chorismate synthase AroC	RHE_CH00935	Naringenin	NodD2
Cysteine synthase A CysK2	RHE_CH01775	Both	NodD1, NodD2
Cysteine synthase CysK1	RHE_CH00325	Both	NodD1, NodD2
NAD-specific glutamate Dehydrogenase	RHE_CH04105	Naringenin	NodD2
Peptide ABC transporter, substrate-binding protein	RHE_CH00175	Naringenin	NodD1
Xaa-Pro dipeptidase	RHE_CH03021	Both	NodD1, NodD2
Carbohydrate transport and metabolism			
Glyceraldehyde 3-phosphate dehydrogenase Gap	RHE_CH03496	Both	NodD1
Glycogen phosphorylase GlgP	RHE_CH03593	Both	NodD1, NodD2
sn-glycerol-3-phosphate ABC transporter substrate-binding protein UgpBc	RHE_PC00078	Both	NodD1, NodD2
Sugar ABC transporter substrate-binding protein	RHE_PF00217	Control	NodD1
Sugar ABC transporter substrate-binding protein	RHE_PF00374	Control	NodD1
Coenzyme transport and metabolism			
Glutamate-cysteine ligase Gsh	RHE_CH00803	Naringenin	NodD2
S-Adenosylmethionine synthetase MetK	RHE_CH00370	Both	NodD1, NodD2
Lipid transport and metabolism			
3-Hydroxybutyryl-CoA Dehydrogenase HbdA	RHE_CH03794	Both	NodD1, NodD2
Translation ribosomal structure and biogenesis			
30S ribosmal protein S3 RpsC	RHE_CH01681	Both	NodD2
30S ribosomal protein S16 RpsP	RHE_CH03959	Both	NodD1, NodD2
30S ribosomal protein S4 RpsD	RHE_CH02312	Both	NodD1
50S ribosomal protein L19 RplS	RHE_CH03962	Both	NodD1, NodD2
50S ribosomal protein L25/general stress protein Ctc RplY	RHE_CH03025	Both	NodD2
50S ribosomal protein L28 RpmB	RHE_CH03816	Control	NodD1
Elongation factor G FusA2	RHE_CH01672	Both	NodD2
Elongation factor Tu TufB	RHE_CH01658	Both	NodD1

Table 2 (continued)

Not COG	Accession	Exoproteome with protein product[a]	Binding site present in gene promoter
Phenylalanyl-tRNA synthetase subunit beta PheT	RHE_CH00264	Both	NodD1, NodD2
Polynucleotide phosphorylase/polyadenylase Pnp	RHE_CH00111	Both	NodD1
TRANSCRIPTION			
Cold shock protein CspA4	RHE_CH02568	Control	NodD2
DNA-directed RNA polymerase subunit beta RpoB	RHE_CH01667	Both	NodD2
DNA-directed RNA polymerase subunit beta' RpoC	RHE_CH01668	Both	NodD1
Replication, recombination and repair			
DNA gyrase subunit A GyrA	RHE_CH02110	Both	NodD1
DNA topoisomerase I topA	RHE_CH01638	Control	NodD1
Cell wall/envelope biogenesis			
Exopolysaccharide export protein PssN	RHE_CH03235	Both	NodD1, NodD2
Exopolysaccharide polymerization protein PssP	RHE_CH03233	Both	NodD1, NodD2
Mannose-1-phosphate guanylyltransferase (GDP) protein NoeJ	RHE_CH03244	Both	NodD1
Polysaccharide export system protein	RHE_CH01561	Both	NodD1, NodD2
Symbiotically induced surface protein	RHE_PE00373	Both	NodD1, NodD2
UTP-glucose-1-phosphate uridylyltransferase ExoN	RHE_CH03561	Both	NodD1, NodD2
Post-translational modification, protein turnover, chaperones			
Membrane protease subunit protein	RHE_CH03557	Control	NodD2
Thioredoxin protein TrxA1	RHE_CH00025	Both	NodD1, NodD2
Inorganic ion transport and metabolism			
Taurine uptake ABC transporter substrate-binding protein TauA	RHE_PC00019	Control	NodD1
Zinc uptake ABC transporter, ATP-binding protein ZnuC	RHE_CH02711	Naringenin	NodD1
General function prediction only			
Chloride peroxidase	RHE_CH02571	Naringenin	NodD1
Unknown function protein	RHE_PB00156	Naringenin	NodD1
RNA-binding protein Hfq	RHE_CH01954	Control	NodD2
Function unknown			
Unknown function protein	RHE_CH01383	Naringenin	NodD1
Unknown function protein	RHE_PF00470	Naringenin	NodD1
Unknown function protein	RHE_PE00320	Naringenin	NodD1
Unknown function protein	RHE_CH03509	Control	NodD2
Unknown function protein	RHE_CH03460	Control	NodD2
Signal transduction mechanisms			
General L-amino-acid ABC transporter, substrate-binding protein AapJ	RHE_CH01898	Both	NodD2
Serine protein kinase	RHE_CH02817	Naringenin	NodD1
Two-component response regulator TcrX	RHE_CH03275	Both	NodD1, NodD2
Intracellular trafficking, secretion, and vesicular transport			
ATP-dependent Clp protease proteolytic subunit Clp3	RHE_CH03069	Both	NodD1
Bifunctional preprotein translocase subunit SecD/SecF SecD1	RHE_CH00631	Both	NodD1
Inner membrane protein translocase component YidC	RHE_CH00437	Both	NodD1

[a]Control, culture without naringenin; Naringenin, culture with naringenin; both, present in both Control and Naringenin cultures

MAMPs proteins identified in naringenin-treated and control cultures

Studies on MAMPs (microbe-associated molecular patterns) and their perception by plants have been largely carried out with phytopathogenic microorganisms (Aslam et al. 2009). These analyses revealed that plants have perception systems for different bacterial MAMPs, including elongation factor Tu (EF-Tu), lipopolysaccharides, and flagellin (Gómez-Gómez and Boller 2000; Silipo et al. 2005; Zipfel et al. 2006). Plant reactions to MAMPs include promoting Ca^{2+} influx across the membrane,

induction of an oxidative burst, activation of calcium-dependent protein kinases and mitogen-activated protein kinase (MAPK) cascades, and the induction of defence-related genes (Aslam et al. 2009; Gómez-Gómez and Boller 2000). Interestingly, several of these responses occur in the epidermal cells of legume roots following the application of nod factors (Soto et al. 2009). In our analysis, we identified FlgD, FlgG, FlgEch, FlgLch (flagellin), FlaCe, FlaCch1, FlaCch2, FlaCch3, FlaCch4, FlaCch5, and EF-Tu proteins, which could potentially elicit one or more of the plant responses, described above. Flagellar proteins are often identified in extracellular proteomes, since flagella is easily disrupted from the cell surface (Süss et al. 2006), accounting for its presence in the exoproteomes of many bacteria, including *R. leguminosarum* bv. *viciae* (Krehenbrink and Downie 2008) and *R. etli* (Meneses et al. 2010) (S1 Table). In C. jejuni, however, these proteins are exported by the flagellar system and flaC null mutants show a significantly reduced level for the invasion of human cells (Song et al. 2004). In plant pathogens, flagellin, or the flg22 peptide derived from it, acts as an elicitor of host defense responses in a manner similar to that described for EF-Tu (Aslam et al. 2009; Zipfel et al. 2006). However, in rhizobia, the flg22 peptide is not conserved and has not been reported to elicit defense reactions. EF-Tu participates in translation in the bacterial cytoplasm but is commonly found in the exoproteomes of microbial pathogens (Cafardi et al. 2013; Jain et al. 2014; Kazemi-Pour et al. 2004; Wang et al. 2016; Zipfel et al. 2006), such as *Xanthomonas campestris* pv. *campestris*, where it affects calcium cycling and elicits defense responses in *Arabidopsis* (Aslam et al. 2009). In addition, EF-Tu plays an important role in cell shape maintenance in *Bacillus subtilis* and in the localization of MreB, an actin ortholog (Defeu Soufo et al. 2010).

Nucleoside diphosphate kinase (Ndk) is a cytoplasmic metabolic enzyme that, when secreted, is used by some pathogens to modulate extracellular ATP levels (Dar et al. 2011; Cao et al. 2014; Tanaka et al. 2010). In comparison to its intracellular levels in *R. etli* CE3 aerobic cultures, Ndk is downregulated and present at comparatively low levels during fermentative-like metabolism (Encarnación et al. 2003), although we do not know if this decrease results from reduced synthesis and/or increased degradation, or from its export. Ndk is also present in the exoproteome of *R. leguminosarum* bv. *viciae*, which appears to use a Type I general secretion system for its export (Krehenbrink and Downie 2008). In *P. aeruginosa*, Ndk secretion requires the C-terminal sequence DTEV (Kamath et al. 2000), and a similar sequence (DTEI) is present in the Ndks of both *R. leguminosarum* bv. *viciae* (Krehenbrink and Downie 2008) and *R. etli*.

Stress-related proteins shared in both culture conditions tested

Rhizobia use a variety of stress resistance mechanisms to contend with the adverse environments encountered in the soil and during infection (Fiebig et al. 2015; Zahran 1999). Several stress-related proteins were found in the *R. etli* common exoproteome, including chaperones, glutathione transferases, proteases, heat shock proteins, catalase (KatG), and superoxide dismutase (SodB) (Table S1). Several of these are found as non-classically secreted proteins in other bacteria (Wang et al. 2016).

As mentioned, the ability of bacteria to export "non-classical" metabolic proteins (i.e., those demonstrated or predicted to have a cytoplasmic localization) is increasingly being realized (Wang et al. 2016; Yang et al. 2011). For instance, pathogen-secreted glycolytic enzymes, such as glyceraldehyde 3-phosphate dehydrogenase (Gap), enolase (Eno), aldolase, and phosphoglucomutase (Pgm) are "moonlighting" proteins that act to bind the bacterial cells to those of the host (Chagnot et al. 2013; Henderson and Martin 2011). These proteins have been found in the exoproteomes of Gram-negative and Gram-positive animal pathogens (Wang et al. 2016; Rath et al. 2013; Gohar et al. 2005; Hansmeier et al. 2006; Pacheco et al. 2011; Silva et al. 2013) and in the plant-associated bacteria *X. campestris* pv. *Campestris* (Watt et al. 2005) and *Frankia* spp. (Mastronunzio et al. 2009). With the exception of aldolase, all of these proteins were present in the *R. etli* common exoproteome (S1 Table) and the *gap* gene was predicted to have a NodD1-binding site (Table 2).

A comparison to the *R. leguminosarum* bv. *viciae* 3841 exoproteome, obtained from the stationary phase culture supernatants of cells grown in minimal medium containing mannitol (Krehenbrink and Downie 2008), shows that over 80% of the 23 proteins identified have orthologs present in the *R. etli* exoproteome. These proteins and the predicted secretion system by which they might be exported are indicated in Table 1 and S1. All but one of the 19 proteins found in both studies were present among the common proteins in *R. etli*, consistent with the fact that the *R. leguminosarum* exoproteome was obtained from cultures grown without naringenin (Krehenbrink and Downie 2008). Similarly, of 52 proteins with annotated functions found in the *X. campestris* pv. campestris exoproteome obtained from cells grown in glucose minimal medium (Watt et al. 2005), over one-third were also present in the *R. etli* common (17 proteins) or control (2 proteins) exoproteomes.

In summary, a relatively large number of proteins having possible or proven roles in the rhizobia-legume symbiosis were found in the common exoproteome, suggesting that their production and/or excretion is not dependent on naringenin. However, because the mass spectrometric

method used in this study is qualitative, we do not know if the production and/or excretion of any of the common proteins was increased or decreased as a result of naringenin treatment.

Exoproteome proteins differentially expressed in response to naringenin

Because a relatively small number of proteins were found exclusively in the control or naringenin-treated cultures (Table 1), the representation of these exoproteins by functional categories can be misleading when only one to several proteins are present in a given category. If functional categories containing less than ten proteins are excluded, naringenin treatment significantly increased only those proteins for cell wall/envelope biogenesis (8 naringenin-exclusive proteins of 12 total), and significantly decreased those for carbohydrate transport and metabolism (7 naringenin-exclusive proteins of 24 total). In the latter category, eight of the proteins absent from the naringenin-treated cultures were substrate binding proteins for sugar transporters, while two other sugar binding proteins were present only in the naringenin-treated cultures.

Three studies have analyzed the exo- or intracellular proteomes of flavonoid-induced Rhizobia, namely, a secretome analysis genistein-induced proteins, in *B. japonicum* (Hempel et al. 2009) and two studies on naringenin-induced proteins in the intracellular proteome of *R. leguminosarum* bv. *viciae* (Tolin et al. 2013). Because of the close taxonomic relationship between *R. etli* and *R. leguminosarum* bv. *viciae* (Rogel et al. 2011), many of their responses to naringenin could be expected to be similar. However, a comparison of over 40 naringenin-induced proteins found in both *R. leguminosarum* studies (Arrigoni et al. 2013; Tolin et al. 2013) with the *R. etli* total exoproteome showed that 42% of the naringenin-induced *R. leguminosarum* proteins, including the four proteins with the highest naringenin induction ratio, were absent from the *R. etli* exoproteome. Forty-one percent of the *R. leguminosarum* proteins were found among the *R. etli* common proteins, while the percentages present in the *R. etli* control-only and naringenin-only exoproteomes were 7.9% and 9.2%, respectively. Thus, a significant proportion (58%) of the naringenin-induced *R. leguminosarum* intracellular proteins were also found in the *R. etli* exoproteome.

Nod proteins are found in the intracellular proteomes of flavonoid-treated cells of some rhizobia (Guerreiro et al. 1997) but were absent from the exoproteome of naringenin-treated *R. etli* cultures. Given their cytoplasmic functions, these proteins would not be expected to be exported from the cell. Nop (nodule outer proteins) proteins are symbiotic effectors secreted by rhizobial Type III secretion systems that deliver effectors directly to the host cell cytoplasm (Downie 2010; Gazi et al. 2012). The expression of genes encoding Type III secretion system is under control of NodD (Deakin and Broughton 2009; López-Baena et al. 2016). Contrary to expectations, we did not detect any Nop proteins in the *R. etli* naringenin-treated cultures (Table 2).

In addition to stimulating the production of lipochitooligosaccharides in rhizobia, *nod*-gene inducing flavonoids also cause changes in the cell surface, including the structural modification of exopolysaccharides (EPS) and lipopolysaccharides (LPS) (Dunn et al. 1992; Krishnan et al. 2003; Broughton et al. 2006; Simsek et al. 2009; Ardissone et al. 2011; Acosta-Jurado et al. 2016). Flavonoids also induce the expression of Type III and Type IV secretion systems in some rhizobia (Pappas and Cevallos 2011). In *R. etli*, EPS and LPS are important for resistance to coumestrans produced by bean roots and may also suppress plant defense responses (González-Pasayo and Martínez-Romero 2000). In the endophytic nitrogen-fixer *Herbaspirillum seropedicae* (beta-proteobacteria), analysis of cultures grown with or without naringenin revealed changes in the expression of several genes whose products determine cell surface architecture (Tadra-Sfeir et al. 2011). Similarly, several *R. etli* naringenin-induced exoproteins are involved in cell wall metabolism, including the glycoside hydrolase Lyc (a probable peptidoglycan hydrolase) and peptidoglycan synthesis proteins MurA, MurF, and MurG, consistent with a role for flavonoids in modifying rhizobial cell walls. MurA was among the 47 naringenin-induced intracellular proteome proteins found in *R. leguminosarum* bv. *viciae* 3841 (Tolin et al. 2013). PlyA1 is a polysaccharidase with 57% amino-acid identity to the PlyA2 protein found in the common exoproteome. This protein could act to reduce the molecular mass of *R. etli* EPS, similar to what occurs in *Sinorhizobium fredii* genistein-treated cultures (Dunn et al. 1992; Acosta-Jurado et al. 2016). The *R. etli plyA1* promoter contains a putative NodD1-binding site (Table 2). Deacetylated forms of EPS from *R. leguminosarum* bv. *trifolii* and *S. meliloti* are more susceptible to cleavage by glycanases (York and Walker 1998). The *R. etli* exoprotein annotated as a putative chitooligosaccharide deacetylase (RHE_CH03142) has a domain (IPR017625) found in polysaccharide deacetylases and has 97% sequence identity to a putative polysaccharide deacetylase of *R. leguminosarum* bv. *viciae* 3841. PssK is an exopolysaccharide polymerase that in *R. etli, it* is negatively regulated by the RosR transcriptional regulator (Bittinger and Handelsman 2000). In *R. leguminosarum* bv. *trifolii*, PssK appears to be localized to the inner face of the outer membrane and so is not exposed to the cell surface (Król et al. 2006).

Several metabolic enzymes with probable or demonstrated cytoplasmic roles in rhizobia were found exclusively in the naringenin-treated culture exoproteome, for example, two aldehyde dehydrogenases that could act in

the catabolism of aldehydes, which may serve as a carbon source for Bradyrhizobia in nodules (Peterson and LaRue 1982). Proline dehydrogenase (PutA) catalyzes the production of proline in the final step of the rhizobial arginine degradation pathway and is required for nodulation or nodulation competitivity in various rhizobia (Dunn 2015). High concentrations of *myo*-inositol are present in nodules and soil and the ability of *R. leguminosarum* bv. *viciae* to catabolize this compound is required for nodulation competitiveness but not nitrogen fixation (Fry et al. 2001), while a *S. fredii myo*-inositol catabolic mutant is deficient in both of these parameters (Jiang et al. 2001). The *R. etli* naningenin-induced exoproteome contained the first and last enzymes of the *myo*-inositol catabolic pathway, namely myo-inositol dehydrogenase (IdhA) and methylmalonate-semialdehyde dehydrogenase (IolA).

As mentioned, metabolic proteins often play moonlighting roles in bacteria–eukaryote interactions (Henderson and Martin 2011). The *R. etli* 6-phosphogluconate dehydrogenase (Gnd) along with an additional Gnd-like protein was present in the naringenin-induced exoproteome (Table 1). In *Streptococcus suis*, surface-localized Gnd functions in adhesion to animal cells (Tan et al. 2008). Gnd is also present in strain CFN42 bacteroids, indicative of an operational pentose phosphate pathway during symbiosis (Resendis-Antonio et al. 2011).

Although the great majority of *R. etli* exoproteins involved in stress resistance were found among the common proteins, the transmembrane ß (1,2) glucan biosynthesis protein NdvB was present exclusively in the naringenin-induced exoproteome (Table 1). In *Pseudomonas aeruginosa*, glucans are able of bind the aminoglycoside antibiotic kanamycin, thus linking cyclic glucan production to antibiotic resistance (Sadovskaya et al. 2010). In *R. leguminosarum* bv. *viciae*, NdvB is present in the naringenin-induced intracellular proteome (Arrigoni et al. 2013). Synthesis of β-(1,2)-glucan is important for osmotic adaptation and NdvB is required for the hypoosmotic growth in *S. meliloti* and *A. tumefaciens* (Ingram-Smith and Miller 1998). An *ndvB* mutant of *Sinorhizobium fredii* NGR234 lacking ß (1,2) glucans was deficient in motility, root attachment, and legume infection (Gay-Fraret et al. 2012), and essential early events in the establishment of symbiosis. In *S. fredii* HH103, *ndvB* mutants lacking cyclic ß-glucans but overproducing EPS formed only pseudonodules on two different hosts (Crespo-Rivas et al. 2009). To fact that NdvB could only be found in the *R. etli* naringenin-induced exoproteome is remarkable, given the essential symbiotic role of cyclic β (1–2) glucans in different rhizobia (Breedveld and Miller 1994).

Glutamate-cysteine ligase (Gsh), which catalyzes the first reaction of the two-step pathway for the synthesis of the tripeptide glutathione, was found exclusively in the naringenin-induced exoproteome. Glutathione is essential for stress resistance in many rhizobia and for an efficient symbiosis in *R. etli* and *S. meliloti* (Dunn 2015). We predict that the *gsh* gene contains a NodD2 binding site in its promoter region (Table 2). Glutathione synthetase (GshB), the second enzyme of the glutathione synthesis pathway, was present in the common proteome (Table S1). The exclusive presence of the chaperones DnaJ1, GroEL, and GroES in the naringenin-treated cultures could indicate a flavonoid-induced stress response, since these proteins are involved in the refolding of stress-damaged proteins, either in the cytoplasm or in association with membranes (Horváth et al. 2008).

Proteins differentially secreted by *R. etli* grown in MM without naringenin

One hundred unique proteins were identified in the *R. etli* control cultures (Table 2). Similar to the proteins specifically induced in the naringenin-treated cultures, the control exoproteome contained several metabolic enzymes. For instance, AdhAch, formaldehyde (glutathione)/alcohol dehydrogenase, and RHE_CH02884, a putative alcohol dehydrogenase, may be involved in fermentative metabolism. Malate dehydrogenase and NAD^+-dependent malic enzyme (Dme) were present only in the control exoproteome, while the $NADP^+$-dependent malic enzyme (Tme) was present only in the naringenin-induced exoproteome in *S. meliloti*; Dme is required for nitrogen fixation in alfalfa, while Tme is not required and studies aimed at determining its physiological importance have been inconclusive (Driscoll and Finan 1996; Dunn 1998; Ye Zhang 2013). Thus, the selective induction of the enigmatic Tme by naringenin is intriguing.

Control culture exoproteomes, also contained several proteins involved in transport or amino-acid metabolism, including LeuB for leucine and isoleucine biosynthesis, in *R. leguminosarum*, these amino acids are not synthesized by the bacteriods but are supplied by the plant during symbiosis (Prell et al. 2009). The control exoproteome also contained GcbH and GcvP (glycine metabolism), and LysC and 2 DapA orthologs (lysine biosynthesis). Similar to *R. leguminosarum* bv. *viciae* 3841 cultured in the presence of naringenin (Tolin et al. 2013), *R. etli* decreased its growth rate in the presence of naringenin as compared with the control (data not shown). While *R. etli* grown in MM must synthesize all 20 amino acids, this is not required during symbiosis, where the bacteroid is unable to synthesize several amino acids that are apparently supplied by the plant (Resendis-Antonio et al. 2011). This metabolic condition could be similar in *R. etli* when is cultured in MM plus naringenin, which could induce the first steps on bacterial differentiation previous to symbiosis, in our oppinion, this

is supported by the differences in the expression of proteins involved in metabolic pathways for amino-acid biosynthesis, and justified the different capacity to growth of *R.etli* in MM plus naringenin in comparison with the control. However, further experiments are needed to prove these assumptions.

ThiC (phosphomethylpyrimidine synthase), required for the synthesis of the thiamine pyrophosphate (TPP) which is the cofactor of pyruvate dehydrogenase, and several other enzymes, was secreted only by cells grown in MM without naringenin, suggesting a possible differential demand for TPP in the presence and absence of the flavonoid.

Several lipid biosynthesis proteins were secreted by cells in control cultures, including FabI1 (enoyl-acyl-carrier protein (ACP) reductase) and FabB (3-oxoacyl-ACP synthase I), RHE_CH01048 (3-oxoacyl-ACP reductase), RHE_CH01714 (putative lipase/esterase protein), and FabG (3-oxyacyl-ACP reductase). In *R. etli*, fatty acid metabolism is predicted to play a significant role in nitrogen fixation (Resendis-Antonio et al. 2011), in contrast to the drastic reduction of lipid biosynthesis observed in B. japonicum bacteroids (Sarma and Emerich 2006). The different patterns of fatty acid metabolic enzymes exported in cultures grown with or without naringenin suggest that the flavonoid influences a diversity of processes, such as the synthesis of membrane fatty acids, lipopolysaccharides, and vitamins, such as lipoic acid and biotin. The testing of this hypothesis and its relevance to nitrogen fixation are subjects for future experimental investigation.

Concluding remarks

In this work, we identified proteins present in the culture supernatants late-log phase *R. etli* cultures grown in the presence or absence of the *nod*-gene inducer naringenin. We did not find a significantly higher proportion of symbiotically relevant exoproteins present in naringenin-treated cultures (this work) versus bacteroids (Resendis-Antonio et al. 2011). Instead, many proteins proven or suspected to be involved in symbiosis were present in both the naringenin-treated and control culture exoproteomes. These exoproteins include a number of predicted cell surface- or membrane-localized proteins that may modify the rhizobial cell surface (ß-glycanase) or interact with host root cells (Rap and Rop proteins). Other exoproteins found in both control and naringenin-treated cultures, such as several glycolytic enzymes, EF-Tu, flagellin, and Ndk, could have "moonlighting" roles that modulate the interaction of the symbionts or protect against stress conditions generated during infection (chaperones, catalase, and superoxide dismutase).

Thus, our expectation that the exoproteome of naringenin-treated cultures would be comprised of a higher proportion of symbiotically relevant proteins, relative to control cultures, was not borne out by the experimental results. Naringenin treatment did, however, provoke a modest increase in the number of proteins involved in cell wall/envelope biogenesis and a significant decrease in proteins for sugar transport. Modifications in the rhizobial cell surface, particular in EPS and LPS structure or composition, occur in response to flavonoids and are important for the formation of an effective symbiosis. Two sugar substrate-binding proteins were present only in the naringenin-treated culture exoproteome, while eight others of the type were present only in the control cultures. This might indicate that naringenin causes a general downregulation of sugar transport capacity in *R. etli*, but the significance of this result is uncertain, since we do not know the substrate specificities of the binding proteins affected by the flavonoid. In addition, the identification of *R. etli* exoproteins having potential moonlighting roles in symbiosis is of particular interest and provides a basis for more detailed studies of their possible roles in the symbiotic interaction.

Acknowledgements Part of this work was supported by CONACyT Grant 220790 and DGAPA-PAPIIT Grant IN213216. Thanks to Jaime A. Castro Mondragón for contributing to the Dyad analysis and Omar Alejandro Aguilar for bioinformatics assistance. The authors would like to thank the anonymous reviewers for their valuable comments and suggestions to improve the quality of the review.

References

Acosta-Jurado SS, Navarro-Gómez P, Murdoch PDS et al (2016) Exopolysaccharide production by *Sinorhizobium fredii* HH103 is repressed by genistein in a NodD1-dependent manner. PLoS One 11:1–16. doi:10.1371/journal.pone.0160499

Afroz A, Zahur M, Zeeshan N, Komatsu S (2013) Plant-bacterium interactions analyzed by proteomics. Front Plant Sci 4:21. doi:10.3389/fpls.2013.00021

Ardissone S, Noel KD, Klement M et al (2011) Synthesis of the flavonoid-induced lipopolysaccharide of *Rhizobium* Sp. strain NGR234 requires rhamnosyl transferases encoded by genes *rgpF* and *wbgA*. Mol Plant Microbe Interact 24:1513–1521. doi:10.1094/MPMI-05-11-0143

Arrigoni G, Tolin S, Moscatiello R et al (2013) Calcium-dependent regulation of genes for plant nodulation in *Rhizobium leguminosarum* detected by iTRAQ quantitative proteomic analysis. J Proteome Res 12:5323–5330. doi:10.1021/pr400656g

Aslam SN, Erbs G, Morrissey KL et al (2009) Microbe-associated molecular pattern (MAMP) signatures, synergy, size and charge: Influences on perception or mobility and host defence responses. Mol Plant Pathol 10:375–387. doi:10.1111/j.1364-3703.2009.00537.x

Bittinger M a., Handelsman J (2000) Identification of genes in the RosR regulon of *Rhizobium etli*. J Bacteriol 182:1706–1713. doi:10.1128/JB.182.6.1706-1713.2000

Breedveld MW, Miller KJ (1994) Cyclic beta-glucans of members of the family Rhizobiaceae. Microbiol Rev 58:145–161

Broughton WJ, Hanin M, Relic B et al (2006) Flavonoid-inducible modifications to rhamnan O antigens are necessary for *Rhizobium* sp. strain NGR234-legume symbioses. J Bacteriol 188:3654–3663. doi:10.1128/JB.188.10.3654-3663.2006

Cafardi V, Biagini M, Martinelli M et al (2013) Identification of a novel zinc metalloprotease through a global analysis of *Clostridium difficile* extracellular proteins. PLoS One 8:1–14. doi:10.1371/journal.pone.0081306

Campbell GRO, Sharypova LA, Scheidle H et al (2003) Striking complexity of lipopolysaccharide defects in a collection of *Sinorhizobium meliloti* mutants. J Bacteriol 185:3853–3862

Cao Y, Tanaka K, Nguyen CT, Stacey G (2014) Extracellular ATP is a central signaling molecule in plant stress responses. Curr Opin Plant Biol 20:82–87. doi:10.1016/j.pbi.2014.04.009

Chagnot C, Zorgani MA, Astruc T, Desvaux M (2013) Proteinaceous determinants of surface colonization in bacteria: bacterial adhesion and biofilm formation from a protein secretion perspective. Front Microbiol 4:303. doi:10.3389/fmicb.2013.00303

Cosme AM, Becker A, Santos MR et al (2008) The outer membrane protein TolC from *Sinorhizobium meliloti* affects protein secretion, polysaccharide biosynthesis, antimicrobial resistance, and symbiosis. Mol Plant Microbe Interact 21:947–957. doi:10.1094/MPMI-21-7-0947

Crespo-Rivas JC, Margaret I, Hidalgo A et al (2009) *Sinorhizobium fredii* HH103 *cgs* mutants are unable to nodulate determinate- and indeterminate nodule-forming legumes and overproduce an altered EPS. Mol Plant Microbe Interact 22:575–588. doi:10.1094/MPMI-22-5-0575

Dar HH, Prasad D, Varshney GC, Chakraborti PK (2011) Secretory nucleoside diphosphate kinases from both intra- and extracellular pathogenic bacteria are functionally indistinguishable. Microbiology 157:3024–3035. doi:10.1099/mic.0.049221-0

Deakin WJ, Broughton WJ (2009) Symbiotic use of pathogenic strategies: rhizobial protein secretion systems. Nat Rev Microbiol 7:312–320. doi:10.1038/nrmicro2091

Defeu Soufo HJ, Reimold C, Linne U et al (2010) Bacterial translation elongation factor EF-Tu interacts and colocalizes with actin-like MreB protein. Proc Natl Acad Sci U S A 107:3163–3168. doi:10.1073/pnas.0911979107

Dou D, Zhou J-MM (2012) Phytopathogen effectors subverting host immunity: Different foes, similar battleground. Cell Host Microbe 12:484–495. doi:10.1016/j.chom.2012.09.003

Downie JA (2010) The roles of extracellular proteins, polysaccharides and signals in the interactions of rhizobia with legume roots. FEMS Microbiol Rev 34:150–170. doi:10.1111/j.1574-6976.2009.00205.x

Driscoll BT, Finan TM (1996) NADP+-dependent malic enzyme of *Rhizobium meliloti*. J Bacteriol 178:2224–2231

Dunn MF (1998) Tricarboxylic acid cycle and anaplerotic enzymes in rhizobia. FEMS Microbiol Rev 22:105–123

Dunn MF (2015) Key roles of microsymbiont amino acid metabolism in rhizobia-legume interactions. Crit Rev Microbiol 41:411–451. doi:10.3109/1040841X.2013.856854

Dunn MF, Pueppke SG, Krishnan HB (1992) The nod gene inducer genistein alters the composition and molecular mass distribution of extracellular polysaccharides produced by *Rhizobium fredii* USDA193. FEMS Microbiol Lett 97:107–112. doi:10.1016/0378-1097(92)90372-U

Dunn MF, Araíza G, Encarnación S et al (2002) Effect of *aniA* (Carbon Flux Regulator) and and *phaC* (poly-beta-hydroxybutirate synthase) mutations on Pyruvate Metabolism in *Rhizobium etli* 1–5. doi:10.1128/JB.184.8.2296-2299.2002.

Emerich DW, Krishnan HB (2014) Symbiosomes: temporary moonlighting organelles. Biochem J 460:1–11. doi:10.1042/BJ20130271

Encarnación S, Dunn M, Willms K et al (1995) Fermentative and aerobic metabolism in *Rhizobium etli*. J Bacteriol 177:3058–3066

Encarnación S, Del Carmen Vargas M, Dunn MF et al (2002) Ania regulates reserve polymer accumulation and global protein expression in *Rhizobium etli*. J Bacteriol 184:2287–2295. doi:10.1128/JB.184.8.2287-2295.2002

Encarnación S, Guzmán Y, Dunn MF et al (2003) Proteome analysis of aerobic and fermentative metabolism in *Rhizobium etli CE3*. Proteomics 3:1077–1085. doi:10.1002/pmic.200300427

Fauvart M, Michiels J (2008) Rhizobial secreted proteins as determinants of host specificity in the *rhizobium*-legume symbiosis. FEMS Microbiol Lett 285:1–9. doi:10.1111/j.1574-6968.2008.01254.x

Feng J, Li Q, Hu HL et al (2003) Inactivation of the nod box distal half-site allows tetrameric NodD to activate *nodA* transcription in an inducer-independent manner. Nucleic Acids Res 31:3143–3156. doi:10.1093/nar/gkg411

Fiebig A, Herrou J, Willett J, Crosson S (2015) General stress signaling in the alphaproteobacteria. Annu Rev Genet 49:603–625. doi:10.1146/annurev-genet-112414-054813

Finnie C, Hartley NM, Findlay KC, Downie J a (1997) The *Rhizobium leguminosarum prsDE* genes are required for secretion of several proteins, some of which influence nodulation, symbiotic nitrogen fixation and exopolysaccharide modification. Mol Microbiol 25:135–146

Finnie C, Zorreguieta A, Hartley NM, Downie JA (1998) Characterization of *Rhizobium leguminosarum* exopolysaccharide glycanases that are secreted via a type I exporter and have a novel heptapeptide repeat motif. J Bacteriol 180:1691–1699

Foreman DL, Vanderlinde EM, Bay DC, Yost CK (2010) Characterization of a gene family of outer membrane proteins (ropB) in *Rhizobium leguminosarum* bv. *viciae* VF39SM and the role of the sensor kinase ChvG in their regulation. J Bacteriol 192:975–983. doi:10.1128/JB.01140-09

Fry J, Wood M, Poole PS (2001) Investigation of myo-inositol catabolism in *Rhizobium leguminosarum* bv. *viciae* and its effect on nodulation competitiveness. Mol Plant Microbe Interact 14:1016–1025. doi:10.1094/MPMI.2001.14.8.1016

Gay-Fraret J, Ardissone S, Kambara K et al (2012) Cyclic-β-glucans of *Rhizobium* (*Sinorhizobium*) sp. strain NGR234 are required for hypo-osmotic adaptation, motility, and efficient symbiosis with host plants. FEMS Microbiol Lett 333:28–36. doi:10.1111/j.1574-6968.2012.02595.x

Gazi AD, Sarris PF, Fadouloglou VE et al (2012) Phylogenetic analysis of a gene cluster encoding an additional, rhizobial-like type III secretion system that is narrowly distributed among *Pseudomonas syringae* strains. BMC Microbiol 12:188. doi:10.1186/1471-2180-12-188

Gohar M, Gilois N, Graveline R et al (2005) A comparative study of *Bacillus cereus, Bacillus thuringiensis* and *Bacillus anthracis* extracellular proteomes. Proteomics 5:3696–3711. doi:10.1002/pmic.200401225

Gómez-Gómez L, Boller T (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. Mol Cell 5:1003–1011

González V, Santamaría RI, Bustos P et al (2006) The partitioned *Rhizobium etli* genome: genetic and metabolic redundancy in seven interacting replicons. Proc Natl Acad Sci USA 103:3834–3839. doi:10.1073/pnas.0508502103

González-Pasayo R, Martínez-Romero E (2000) Multiresistance genes of *Rhizobium etli* CFN42. Mol Plant Microbe Interact 13:572–577. doi:10.1094/MPMI.2000.13.5.572

Guerreiro N, Redmond JW, Rolfe BG, Djordjevic M a (1997) New *Rhizobium leguminosarum* flavonoid-induced proteins revealed by proteome analysis of differentially displayed

proteins. Mol Plant Microbe Interact 10:506–516. doi:10.1094/MPMI.1997.10.4.506

Gunasekera K, Wüthrich D, Braga-Lagache S et al (2012) Proteome remodelling during development from blood to insect-form *Trypanosoma brucei* quantified by SILAC and mass spectrometry. BMC Genomics 13:556. doi:10.1186/1471-2164-13-556

Hansmeier N, Chao TC, Kalinowski J et al (2006) Mapping and comprehensive analysis of the extracellular and cell surface proteome of the human pathogen *Corynebacterium diphtheriae*. Proteomics 6:2465–2476. doi:10.1002/pmic.200500360

Hempel J, Zehner S, Göttfert M, Patschkowski T (2009) Analysis of the secretome of the soybean symbiont *Bradyrhizobium japonicum*. J Biotechnol 140:51–58. doi:10.1016/j.jbiotec.2008.11.002

Henderson B, Martin A (2011) Bacterial Virulence in the Moonlight: Multitasking Bacterial Moonlighting Proteins Are Virulence Determinants in Infectious Disease. Infect Immun 79:3476–3491. doi:10.1128/IAI.00179-11

Horváth I, Multhoff G, Sonnleitner A, Vígh L (2008) Membrane-associated stress proteins: more than simply chaperones. Biochim Biophys Acta 1778:1653–1664. doi:10.1016/j.bbamem.2008.02.012

Ingram-Smith C, Miller KJ (1998) Effects of ionic and osmotic strength on the glucosyltransferase of *Rhizobium meliloti* responsible for cyclic beta-(1,2)-glucan biosynthesis. Appl Environ Microbiol 64:1290–1297

Jain S, Kumar S, Dohre S et al (2014) Identification of a protective protein from stationary-phase exoproteome of *Brucella abortus*. Pathog Dis 70:75–83. doi:10.1111/2049-632X.12079

Janczarek M (2011) Environmental signals and regulatory pathways that influence exopolysaccharide production in rhizobia. Int J Mol Sci 12:7898–7933. doi:10.3390/ijms12117898

Jiang G, Krishnan AH, Kim YW et al (2001) A functional myo-inositol dehydrogenase gene is required for efficient nitrogen fixation and competitiveness of *Sinorhizobium fredii* USDA191 to nodulate soybean (*Glycine max* [L.] Merr.). J Bacteriol 183:2595–2604. doi:10.1128/JB.183.8.2595-2604.2001

Johnsen HR, Krause K (2014) Cellulase activity screening using pure carboxymethylcellulose: application to soluble cellulolytic samples and to plant tissue prints. Int J Mol Sci 15:830–838. doi:10.3390/ijms15010830

Kamath S, Chen ML, Chakrabarty AM (2000) Secretion of nucleoside diphosphate kinase by mucoid *Pseudomonas aeruginosa* 8821: involvement of a carboxy-terminal motif in secretion. J Bacteriol 182:3826–3831

Kazemi-Pour N, Condemine G, Hugouvieux-Cotte-Pattat N (2004) The secretome of the plant pathogenic bacterium *Erwinia chrysanthemi*. Proteomics 4:3177–3186. doi:10.1002/pmic.200300814

Krehenbrink M, Downie JA (2008) Identification of protein secretion systems and novel secreted proteins in *Rhizobium leguminosarum* bv. *viciae*. BMC Genomics 9:55. doi:10.1186/1471-2164-9-55

Krishnan HB, Lorio J, Kim WS et al (2003) Extracellular proteins involved in soybean cultivar-specific nodulation are associated with pilus-like surface appendages and exported by a type III protein secretion system in *Sinorhizobium fredii* USDA257. Mol Plant Microbe Interact 16:617–625. doi:10.1094/MPMI.2003.16.7.617

Król JE, Mazur A, Marczak M, Skorupska A (2006) Syntenic arrangements of the surface polysaccharide biosynthesis genes in *Rhizobium leguminosarum*. doi:10.1016/j.ygeno.2006.08.015

López-Baena FJ, Ruiz-Sainz JE, Rodríguez-Carvajal MA, Vinardell JM (2016) Bacterial Molecular Signals in the *Sinorhizobium fredii*-Soybean Symbiosis. Int J Mol Sci 17:755. doi:10.3390/ijms17050755

Mastronunzio JE, Huang Y, Benson DR (2009) Diminished exoproteome of *Frankia spp*. in culture and symbiosis. Appl Environ Microbiol 75:6721–6728. doi:10.1128/AEM.01559-09

Mateos PF, Jimenez-Zurdo JI, Chen J et al (1992) Cell-associated pectinolytic and cellulolytic enzymes in *Rhizobium leguminosarum* biovar *trifolii*. Appl Environ Microbiol 58:1816–1822

Mawuenyega KG, Forst CV, Dobos KM et al (2005) *Mycobacterium tuberculosis* functional network analysis by global subcellular protein profiling. Mol Biol Cell 16:396–404. doi:10.1091/mbc.E04-04-0329

McBroom AJ, Kuehn MJ (2007) Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response. Mol Microbiol 63:545–558. doi:10.1111/j.1365-2958.2006.05522.x

Medina-Rivera A, Abreu-Goodger C, Thomas-Chollier M et al (2011) Theoretical and empirical quality assessment of transcription factor-binding motifs. Nucleic Acids Res 39:808–824. doi:10.1093/nar/gkq710

Meneses N, Mendoza-Hernández G, Encarnación S (2010) The extracellular proteome of *Rhizobium etli* CE3 in exponential and stationary growth phase. Proteome Sci 8:51. doi:10.1186/1477-5956-8-51

Mongiardini EJ, Ausmees N, Pérez-Giménez J et al (2008) The rhizobial adhesion protein RapA1 is involved in adsorption of rhizobia to plant roots but not in nodulation. FEMS Microbiol Ecol 65:279–288. doi:10.1111/j.1574-6941.2008.00467.x

Morales VM, Martinez-Molina E, Hubbell DH (1984) Cellulase production by *Rhizobium*. Plant Soil 80:407–415

Pacheco LGC, Slade SE, Seyffert N et al (2011) A combined approach for comparative exoproteome analysis of *Corynebacterium pseudotuberculosis*. BMC Microbiol 11:12. doi:10.1186/1471-2180-11-12

Pappas KM, Cevallos MA (2011) Plasmids of the Rhizobiaceae and Their Role in Interbacterial and Transkingdom Interactions. In: Witzany G (ed) Biocommunication in soil microorganisms, soil biology 23. Berlin Heidelberg, pp 295–338

Pérez-Montaño F, del Cerro P, Jiménez-Guerrero I et al (2016a) RNA-seq analysis of the *Rhizobium tropici* CIAT 899 transcriptome shows similarities in the activation patterns of symbiotic genes in the presence of apigenin and salt. BMC Genomics 17:1–11. doi:10.1186/s12864-016-2543-3

Pérez-Montaño F, Jiménez-Guerrero I, Acosta-Jurado S, et al (2016b) A transcriptomic analysis of the effect of genistein on *Sinorhizobium fredii* HH103 reveals novel rhizobial genes putatively involved in symbiosis. Sci Rep 6:31592. doi:10.1038/srep31592

Peterson JB, LaRue TA (1982) Soluble aldehyde dehydrogenase and metabolism of aldehydes by soybean bacteroids. J Bacteriol 151:1473–1484

Pickering BS, Yudistira H, Oresnik IJ (2012) Characterization of the twin-arginine transport secretome in *Sinorhizobium meliloti* and evidence for host-dependent phenotypes. Appl Environ Microbiol 78:7141–7144. doi:10.1128/AEM.01458-12

Poupot R, Martinez-Romero E, Gautier N, Promé JC (1995) Wild type *Rhizobium etli*, a bean symbiont, produces acetylfucosylated, N-methylated, and carbamoylated nodulation factors. J Biol Chem 270:6050–6055

Prell J, White JP, Bourdes A et al (2009) Legumes regulate *Rhizobium* bacteroid development and persistence by the supply of branched-chain amino acids. Proc Natl Acad Sci USA 106:12477–12482. doi:10.1073/pnas.0903653106

Rath P, Huang C, Wang TT et al (2013) Genetic regulation of vesiculogenesis and immunomodulation in *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA 110:E4790–E4797. doi:10.1073/pnas.1320118110

Resendis-Antonio O, Hernández M, Salazar E et al (2011) Systems biology of bacterial nitrogen fixation: high-throughput technology and its integrative description with constraint-based modeling. BMC Syst Biol 5:120. doi:10.1186/1752-0509-5-120

Rogel MA, Ormeño-Orrillo E, Martinez Romero E (2011) Symbiovars in rhizobia reflect bacterial adaptation to legumes. Syst Appl Microbiol 34:96–104. doi:10.1016/j.syapm.2010.11.015

Russo DM, Williams A, Edwards A et al (2006) Proteins exported via the PrsD-PrsE type I secretion system and the acidic exopolysaccharide are involved in biofilm formation by *Rhizobium leguminosarum*. J Bacteriol 188:4474–4486. doi:10.1128/JB.00246-06

Sadovskaya I, Vinogradov E, Li J et al (2010) High-level antibiotic resistance in *Pseudomonas aeruginosa* biofilm: the *ndvB* gene is involved in the production of highly glycerol-phosphorylated—(1→3)-glucans, which bind aminoglycosides. Glycobiology 20:895–904. doi:10.1093/glycob/cwq047

Sánchez B, Urdaci MC, Margolles A (2010) Extracellular proteins secreted by probiotic bacteria as mediators of effects that promote mucosa-bacteria interactions. Microbiology 156:3232–3242. doi:10.1099/mic.0.044057-0

Sarma AD, Emerich DW (2006) A comparative proteomic evaluation of culture grown vs nodule isolated *Bradyrhizobium japonicum*. Proteomics 6:3008–3028. doi:10.1002/pmic.200500783

Silipo A, Molinaro A, Sturiale L et al (2005) The elicitation of plant innate immunity by lipooligosaccharide of *Xanthomonas campestris*. J Biol Chem 280:33660–33668. doi:10.1074/jbc.M506254200

Silva WM, Seyffert N, Santos A V et al (2013) Identification of 11 new exoproteins in *Corynebacterium pseudotuberculosis* by comparative analysis of the exoproteome. doi:10.1016/j.micpath.2013.05.004

Da Silva Batista JS, Hungria M (2012) Proteomics reveals differential expression of proteins related to a variety of metabolic pathways by genistein-induced *Bradyrhizobium japonicum* strains. J Proteomics 75:1211–1219. doi:10.1016/j.jprot.2011.10.032

Simsek S, Ojanen-Reuhs T, Marie C, Reuhs BL (2009) An apigenin-induced decrease in K-antigen production by *Sinorhizobium* sp. NGR234 is *4gM*- and *nodD1*-dependent. Carbohydr Res 344:1947–1950. doi:10.1016/j.carres.2009.07.006

Song YC, Jin S, Louie H et al (2004) FlaC, a protein of *Campylobacter jejuni* TGH9011 (ATCC43431) secreted through the flagellar apparatus, binds epithelial cells and influences cell invasion. Mol Microbiol 53:541–553. doi:10.1111/j.1365-2958.2004.04175.x

Soto MJ, Sanjuán J, Olivares J (2006) Rhizobia and plant-pathogenic bacteria: common infection weapons. Microbiology 152:3167–3174. doi:10.1099/mic.0.29112-00

Soto MJ, Domínguez-Ferreras A, Pérez-Mendoza D et al (2009) Mutualism versus pathogenesis: the give-and-take in plant-bacteria interactions. Cell Microbiol 11:381–388. doi:10.1111/j.1462-5822.2008.01282.x

Süss C, Hempel J, Zehner S et al (2006) Identification of genistein-inducible and type III-secreted proteins of *Bradyrhizobium japonicum*. J Biotechnol 126:69–77. doi:10.1016/j.jbiotec.2006.03.037

Tadra-Sfeir MZ, Souza EM, Faoro H et al (2011) Naringenin regulates expression of genes involved in cell wall synthesis in *Herbaspirillum seropedicae*. Appl Environ Microbiol 77:2180–2183. doi:10.1128/AEM.02071-10

Tan C, Fu S, Liu M et al (2008) Cloning, expression and characterization of a cell wall surface protein, 6-phosphogluconate-dehydrogenase, of *Streptococcus suis* serotype 2. Vet Microbiol 130:363–370. doi:10.1016/j.vetmic.2008.02.025

Tanaka K, Gilroy S, Jones AM, Stacey G (2010) Extracellular ATP signaling in plants. Trends Cell Biol 20:601–608. doi:10.1016/j.tcb.2010.07.005

Thomas-Chollier M, Defrance M, Medina-Rivera A et al (2011) RSAT 2011: Regulatory sequence analysis tools. Nucleic Acids Res 39:86–91. doi:10.1093/nar/gkr377

Tolin S, Arrigoni G, Moscatiello R et al (2013) Quantitative analysis of the naringenin-inducible proteome in *Rhizobium leguminosarum* by isobaric tagging and mass spectrometry. Proteomics 13:1961–1972. doi:10.1002/pmic.201200472

Tóth K, Stacey G (2015) Does plant immunity play a critical role during initiation of the legume-*rhizobium* symbiosis? Front Plant Sci 6:401. doi:10.3389/fpls.2015.00401

Udvardi M, Poole PS (2013) Transport and metabolism in legume-rhizobia symbioses. Annu Rev Plant Biol 64:781–805. doi:10.1146/annurev-arplant-050312-120235

von Tils D, Blädel I, Schmidt MA, Heusipp G (2012) Type II secretion in *Yersinia*-a secretion system for pathogenicity and environmental fitness. Front Cell Infect Microbiol 2:160. doi:10.3389/fcimb.2012.00160

Wang G, Xia Y, Song X, Ai L (2016) Common non-classically secreted bacterial proteins with experimental evidence. Curr Microbiol 72:102–111. doi:10.1007/s00284-015-0915-6

Watt SA, Wilke A, Patschkowski T, Niehaus K (2005) Comprehensive analysis of the extracellular proteins from *Xanthomonas campestris* pv. *campestris* B100. Proteomics 5:153–167. doi:10.1002/pmic.200400905

Yang C-K, Ewis HE, Zhang X et al (2011) Nonclassical protein secretion by *Bacillus subtilis* in the stationary phase is not due to cell lysis. J Bacteriol 193:5607–5615. doi:10.1128/JB.05897-11

Ye Zhang MS (2013) The roles of malic ensymes in *Rhizobium* carbon metabolism. McMaster University, Hamilton, Ontario

York GM, Walker GC (1998) The succinyl and acetyl modifications of succinoglycan influence susceptibility of succinoglycan to cleavage by the *Rhizobium meliloti* glycanases ExoK and ExsH. J Bacteriol 180:4184–4191

Zahran HH (1999) *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. Microbiol Mol Biol Rev 63:968–989

Zanotti G, Cendron L (2014) Structural and functional aspects of the *Helicobacter pylori* secretome. World J Gastroenterol 20:1402–1423. doi:10.3748/wjg.v20.i6.1402

Zipfel C, Kunze G, Chinchilla D et al (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. Cell 125:749–760. doi:10.1016/j.cell.2006.03.037

Zorreguieta A, Finnie C, Downie JA (2000) Extracellular glycanases of *Rhizobium leguminosarum* are activated on the cell surface by an exopolysaccharide-related component. J Bacteriol 182:1304–1312