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Original Article

Effect of actinorhizal root exudates on the proteomes of *Frankia soli* NRRL B-16219, a strain colonizing the root tissues of its actinorhizal host via intercellular pathway

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ABSTRACT

Frankia and actinorhizal plants exchange signals in the rhizosphere leading to specific mutual recognition of partners and nitrogen-fixing nodule organogenesis. *Frankia soli* strain NRRL B-16219, from the *Elaeagnus* specificity group, colonizes the root tissues of its actinorhizal host through direct intercellular penetration of root epidermis cells and cortex. Here, we studied the early proteogenomic response of strain NRRL B-16219 to treatment with root exudates from compatible *Elaeagnus angustifolia*, and incompatible *Ceanothus thyrsiflorus* and *Coriaria myrtifolia*, host plants grown in nitrogen depleted hydroponic medium. Next-generation proteomics was used to identify the main *Frankia* proteins differentially expressed in response to the root exudates. No products of the *nod* genes present in B-16219 were detected. Proteins specifically upregulated in presence of *E. angustifolia* root exudates include those connected to nitrogen fixation and assimilation (glutamate synthetase, hydrogenase and squalene synthesis), respiration (oxidative phosphorylation and citric acid cycle pathways), oxidative stress (catalase, superoxide dismutase, and peroxidase), proteolysis (proteasome, protease, and peptidase) and plant cell wall degrading proteins involved in the depolymerization of celluloses (endoglucanase, glycosyltransferase, beta-mannanases, glycoside hydrolase and glycosyl hydrolase). Proteomic data obtained in this study will help link signaling molecules/factors to their biosynthetic pathways once those factors have been fully characterized.

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1. Introduction

The genus *Frankia* encompasses symbiotic and non-symbiotic actinobacterial endophytes of root nodules of dicotyledonous plant species designated actinorhizal plants [27]. Most *Frankia* strains are nitrogen-fixing; it is generally agreed that this ability

increases the fitness of their plant partners to serve as pioneer species in nitrogen-poor, disturbed, and marginal soils [7].

Frankia strains generally fall within one of four host specificity groups (HSG) initially proposed by [4]. HSG1 comprises strains infecting *Alnus* (*Betulaceae*), *Comptonia*, *Morella* and *Myrica* (*Myricaceae*) species; HSG2 infects *Casuarina* and *Allocasuarina* (*Casuarinaceae*) and *Morella*; HSG3 strains infect members of the *Elaeagnaceae*, members of the Tribe *Colletieae* in the *Rhamnaceae*, *Gymnostoma* (*Casuarinaceae*) and *Morella* (*Myricaceae*); HSG4 strains nodulate members of the *Elaeagnaceae* but not the promiscuous hosts in the *Myricaceae* or *Gymnostoma* (*Casuarinaceae*). An additional but less characterized group, HSG5, occurs and its

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strains infect actinorhizal *Coriariaceae*, *Datisceae*, *Dryadoideae* (*Rosaceae*) and *Ceanothus* (*Rhamnaceae*) [26,32,41,54].

Frankia strains infect their host plants via one of two paths dictated by the host plants [40,49]. Root hair infection characterizes *Alnus*, *Casuarina*, *Comptonia* and *Myrica* [8,13,55] and direct inter-cellular penetration of root epidermis cells and cortex occurs in *Ceanothus*, *Elaeagnus* and *Shepherdia* species [35,39,49].

From mutual recognition in the rhizosphere to functional root nodules, developmental programs coordinated by the two partners imply cross-regulation of their genes through symbiotic signals. The numerous common developmental features shared between actinorhizal and legume root nodules initially suggested conserved molecular signals. Unfortunately, and unlike the legume symbioses, progress in understanding molecular interactions between *Frankia* and actinorhizal plants has been hampered by an inability to inactivate or to complement genes of interest using traditional bacteriological techniques [6]. In common with legumes, we know that the secretion of flavonoids [1,2] and the implementation of LysM-receptor-like kinases (LysM-RLKs) [24,51] are likely to be involved in actinorhizal root nodule development. In rhizobia, the products of the *nodABC* genes [38] synthesize lipo-chito-oligosaccharides or Nod factors (NFs), which are sensed by LysM-receptor-like kinases leading to a signal transduction cascade via the common, rhizobial, frankial and arbuscular mycorrhizal, symbiotic signaling pathway resulting in the genesis of functional nodules [22,37,43]).

The initial signaling molecules in the actinorhizal symbiosis are likely different from the rhizobial symbiosis. Studies have failed to detect NFs similar to those in rhizobia in culture supernatants; purified rhizobial NFs fail to trigger a plant symbiotic response [14,15,21,52]; and rhizobial *nod* mutants fail to be complemented with libraries of *Frankia* DNA [14]. Indeed, sequencing of the first *Frankia* genomes [42] followed by several other genomes covering most host specificity groups [53] indicate the absence genes similar to rhizobial *nod* genes, with the exception of some *nodB* homologues, members of which can also be found in many other bacteria [42]. Recent characterization of signaling factors of *Frankia casuarinae*, including root hair deforming factor (CgRHDF) and *NIN* activating factor (CgNINA) indicated that, unlike rhizobial NFs or mycorrhizal Myc-LCOs, CgRHDF and CgNINA factors share low molecular weight, thermoresistance, hydrophobicity and resistance to chitinase digestion, [15,17].

A different situation, regarding *nod* genes, may be observed for uncultured members of HSG4. While HSG4 *Frankia coriariae* strain BMG5.1 [26] and BMG5.30 [28], do not contain *nod* genes, genomes of two uncultured *Frankia* strains, *Candidatus Frankia datisciae* Dg1 and *Candidatus Frankia californiensis* Dg2, do have canonical *nodABC* genes [45]; Nguyen et al., 2016). These genes were expressed in symbiosis with *Datisca glomerata* [45] and in *Ceanothus thyrsoflorus* root nodules [50]. Furthermore, rhizobial *nod* gene orthologs have been detected in a large collection of *Frankia* strains in pure cultures and in root nodules of field collected *Ceanothus americanus* strains suggesting the widespread occurrence of highly homologous *nodA* and *nodB* gene fragments in microsymbionts from HSG4 [34].

To date only one *Frankia* strain has been isolated that has two *nod* clusters. The first cluster contains genes encoding the *nodA1*, *nodC*, ABC-2 type transport system ATP-binding protein (*nodJ*), ABC-2 transporter efflux protein, *DrrB* family (*nodI*) and *nodH*. The second cluster contains *nodA*, *nodB* and *nodH* genes. This strain is designated NRRL B-16219 *Frankia soli* [25], from HSG4, and was directly isolated on solid medium from the soil rhizosphere of *Ceanothus jepsonii* [5]. NRRL B-16219 is able to nodulate *Elaeagnaceae* but not *Ceanothus* spp [25,34].

Our understanding of the earlier steps of the symbiotic interaction between *Frankia* strains and actinorhizal plants has been enhanced by examining proteomes upon exposure to root exudates from different plant species for two *Frankia* species; *F. coriariae* from HSG5 [33] and *Frankia alni* from HSG1 [48] In this study, we extend our proteomics database to *F. soli* strain NRRL B-16219, a member of the HSG4 *Colletieae-Elaeagnaceae-Gymnostoma* host specificity group, upon exposure to root exudates from compatible, *Elaeagnus angustifolia*, and incompatible *C. thyrsoflorus* and *Coriaria myrtifolia* species.

2. Materials and methods

2.1. Production of root exudates

E. angustifolia, *C. thyrsoflorus* and *C. myrtifolia* seedlings were grown axenically in Broughton and Dilworth solution supplemented with 5 mM KNO₃ as nitrogen sources (BD + N) [12]. Fifteen days later, BD + N medium was replaced by BD medium without nitrogen (BD-N). Root exudates were collected after two weeks of plant growth, filter sterilized through a 0.22 µm polycarbonate membrane and freshly added to strain NRRL B-16219 cultures.

2.2. Bacterial growth conditions and proteome preparation

F. soli strain NRRL B-16219 [25] was grown in 125 ml bottles containing BD-N supplemented with 5 mM pyruvate as carbon source, at 28 °C and without shaking. Five-day-old exponentially growing cultures were supplemented with one volume (v/v) of root exudate from each actinorhizal species. As controls, one volume of BD-N was added to NRRL B-16219 cultures grown under the same condition. All experiments were conducted with four biological replicates (Fig. 1). After three days exposure to exudates, cellular proteomes of NRRL B-16219 were determined as previously described [33].

Root exudates were also analyzed for potential interference of plant proteins with bacterial proteome as previously described [33]. Proteins from 40 ml root exudates were precipitated with trichloroacetic acid and the resulting pellets were preceded for further proteomic analysis. For *Frankia* proteome, harvested cells were resuspended in 90 µl lithium dodecyl sulfate β-mercaptoethanol protein gel sample buffer (Invitrogen, Carlsbad, CA, USA) and incubated at 99 °C for 5 min and then were subjected to a short SDS-PAGE migration on a NuPAGE 4–12% gel (Invitrogen). Polyacrylamide gel bands (volume about 100 µl) containing the entire cellular proteome—one band per proteome—were cut and processed for in-gel proteolysis with Sequencing Grade Trypsin (Roche, Meylan, France) in the presence of 0.01% Protease Max reagent (Promega, Madison, WI, USA), as described previously [18,33].

2.3. Nano liquid chromatography–MS/MS analysis

Peptides from the trypsin digestion were analyzed using an Ultimate 3000 LC system (Thermo-Scientific, Villebon-sur-Yvette, France) and MS/MS measurements were carried out with a Q-Exactive HF tandem mass spectrometer implementing an ultra-high-field Orbitrap analyzer (Thermo-Scientific). LC system operating conditions were as reported by [31]. The Q-Exactive HF tandem mass spectrometer was operated in data-dependent mode comprising a scan cycle initiated with a full scan of peptide ions in the ultra-high-field Orbitrap analyzer, followed by selection of the precursor, high energy collisional dissociation and MS/MS scans on the 20 most abundant precursor ions (Top20). Full scan mass spectra were acquired with an Automatic Gain Control Target set at 3 × 10⁶ ions from *m/z* 350 to 1800 at a resolution of 60,000. MS/MS scan was

initiated at a resolution of 15,000 when the AGC target reached 10E5 ions with a threshold intensity of 83,000 and potential charge states of 2+ and 3+. A 10 s dynamic exclusion was activated throughout the gradient. MS/MS spectra were processed and interpreted with the MASCOT 2.3.02 search engine (Matrix Science, London, UK), using standard parameters as described previously [29]. The draft genome of *Frankia* NRRL B-16219 (GenBank/EMBL/DDBJ accession number MAXA00000000.1) composed of 6223 annotated proteins was used as a guide for peptide identification. Peptide matches with a score above their peptidic identity threshold were filtered at $P < 0.05$. Proteins were validated based on the assignment of a least two peptides according to the principle of parsimony. Protein abundance was assessed from shotgun analysis using MS/MS spectral counts as previously described [36,58]. Normalized spectral count abundance factors were calculated following the formula used by [44] and were given in percentages of total signal.

2.4. Data analysis

Functional enrichment analysis for interpreting high-throughput proteomics data was processed using the online bioinformatics platform BLAST2GO (<https://www.blast2go.com/>) [19]. Differentially expressed proteins were mapped to gene ontology (GO) terms. The proteins were classified into three functional categories: CC (cellular components), BP (biological processes) and MF (molecular functions). Statistics related to the enzyme code mapping results were also performed using Omixbox 1.2.4. All mapped sequences were further subjected to KEGG (Kyoto Encyclopedia of Genes and Genomes) Pathway/BRITE/Module mapping tools.

2.5. Data repository

Mass spectrometry proteomic data obtained in this study were deposited at the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository [56] with the dataset identifiers PXD015186 and 10.6019/PXD015186.

3. Results

3.1. Next-generation proteomic coverage of NRRL B-16219

Fig. 1 summarizes the experimental pipeline used in the present study for root exudate production, treatment of NRRL B-16219, protein extraction and NanoLC-MS/MS analysis. Mass spectrometry analysis generated 246,352 high quality MS/MS peptide reads for strain NRRL B-16219 when summing results of all the samples. Plant proteins from 40 ml RE obtained by acid precipitation were quantitatively insignificant, as shown by SDS-PAGE together with the very low level of MS/MS spectra recorded by our shotgun procedure and the low confidence for exudates spectrum assignment (Supplementary Table S1). Thus the MS/MS spectra allocations were entirely derived from bacterial peptides without substantial interference of plant proteins derived from RE. A total of 20,832 peptides from the whole experimental dataset were attributed to 2150 *Frankia* proteins (each validated by at least two different peptides) for the cellular proteome (Supplementary Table S2).

3.2. Differentially expressed proteins of *Frankia* strain NRRL B-16219 in presence of actinorhizal root exudates

To be counted as differentially expressed, either up or down regulated, a cut-off of \pm fold (≥ 1.5) with a p -value ≤ 0.05 was used (Supplementary Tables S3 and S4). A total of 874, 697 and 146 proteins were identified as differentially expressed in the presence of *E. angustifolia*, *C. myrtifolia* and *C. thyrsoiflorus* root exudates, respectively. The proteome of strain NRRL B-16219 was more substantially altered in the presence of root exudates from compatible *E. angustifolia*, than incompatible *C. myrtifolia* and *C. thyrsoiflorus* actinorhizal plants. The respective counts of up/down regulated proteins was 211/663, 138/559 and 16/130 (Fig. 2).

We looked for proteins encoded by genes in the two gene clusters containing rhizobial common nodulation gene orthologs, *nodACIJH* and *nodABH*. We concluded that no proteins encoded by

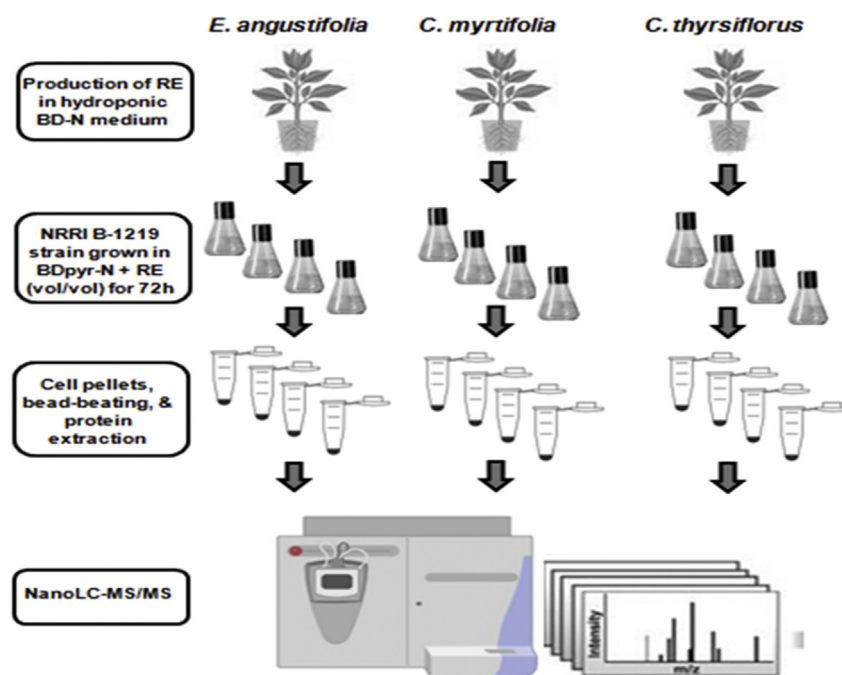


Fig. 1. Experimental pipeline used in the present study for root exudate production, treatment of NRRL B-16219, protein extraction and NanoLC-MS/MS analysis.

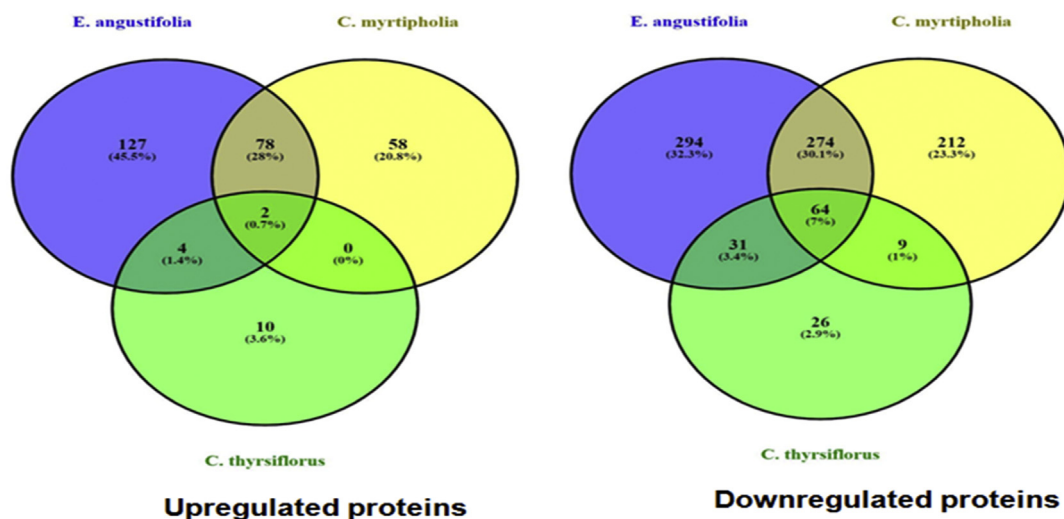


Fig. 2. Venn Diagrams showing the distribution of up- and downregulated proteins of NRRL B-16219 grown in BDpyr-N and in the presence of root exudates from the indicated plant species.

any of the genes from the two clusters could be detected by our approach, except for the “transcriptional regulator *LuxR* family (WP_071059928.1)” which was detected but below the validation threshold.

Only two proteins were commonly upregulated (transposase; WP_071059963.1 and cell division protein FtsK; WP_071062310.1) and 64 proteins were downregulated by all three root's exudates (Fig. 2). Most of the downregulated proteins were associated with metabolism such as enolase (WP_071067211.1), dihydrolipoamide dehydrogenase (WP_083390934.1), xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (WP_071066382.1), beta-glucosidase (WP_071067228.1), starch synthase (maltosyl-transferring) (WP_071065998.1), maleate isomerase (WP_020461250.1) and lipid II isoglutaminyl synthase (glutamine-hydrolysing) (WP_020463940.1).

Pairwise comparison of proteins differentially expressed in the presence of the root exudates showed that *E. angustifolia* and *C. myrtifolia* shared the highest number of upregulated proteins ($n = 78$) than the other pairs; four were shared between *E. angustifolia* and *C. thyrsoflorus* and none were shared between *C. myrtifolia* and *C. thyrsoflorus* root exudates. Similarly, downregulated proteins in this category count 274, 31 and 9 proteins shared between *E. angustifolia* x *C. myrtifolia*, *E. angustifolia* x *C. thyrsoflorus*, and *C. myrtifolia* x *C. thyrsoflorus* root exudates. A total of 127 proteins were specifically upregulated in the presence of *E. angustifolia* root exudates while fewer, 58 and 10, were specifically detected in the presence of root exudates from incompatible actinorhizal species *C. myrtifolia* and *C. thyrsoflorus* respectively.

3.3. Functional annotation and classification of the differentially expressed proteins

Gene Ontology (GO) enrichment of downregulated (Fig. 3) and upregulated proteins revealed three GO sets ranked according to their enrichment scores (Fig. 4), biological processes (BP), molecular functions (MF) and cellular components (CC). Biological processes ontology was mainly “metabolic process”, “cellular process”, “biological regulation”, “regulation of biological process” and “response to stimulus” for up- and downregulated proteins. While molecular functions were mainly “catalytic activity” and “binding” for downregulated proteins, they included also “transporter

activity”, “structural molecular activity” and “antioxidant activity” for upregulated proteins.

Cellular component (CC) downregulated proteins were dominated by “cell” and “cell part” then “membrane” and “membrane part.” Upregulated proteins were mapped to “membrane part”, “membrane”, “cell”, “cell part” and “protein-containing complex.” Downregulated and upregulated proteins' enrichment scores were the highest for *E. angustifolia* followed by *C. myrtifolia* then *C. thyrsoflorus* root exudates (Fig. 4). Unique downregulated proteins responding to *E. angustifolia* root exudates were involved in “tetrahydrofolate interconversion”, “proteasomal protein catalytic process,” and “methionine biosynthetic process” for biological process ontology. Molecular functions ontology included proteins involved in “NADH dehydrogenase (ubiquinone) activity,” “methylmalonyl-CoA mutase activity,” “3-hydroxyacyl-CoA dehydrogenase activity,” “phosphoglucomutase activity” and “antioxidant activity.” Cellular component ontology included proteins involved in “proteasome complex” and “ATP-binding cassette (ABC) transporter.”

Upregulated proteins unique to *E. angustifolia* root exudates grouped in “translation,” “cellular iron ion homeostasis,” “iron ion transport” and “cellular oxidant detoxification” for biological process ontology; “structural constituent of ribosome”, “ferric iron binding”, “NADH dehydrogenase (ubiquinone) activity”, “transferase activity” and “methyltransferase activity” for molecular function ontology; “ribosome,” “fatty acid synthase complex,” “proteasome core complex” and “beta-subunit complex” for cellular component ontology. Summary of the distribution of orthologous clusters and protein data is provided in Fig. 5.

Enzymatic code classification of downregulated proteins mainly involved “transferases,” then “hydrolases,” “lyases,” “isomerases,” and then “oxidoreductases” with clearly higher scores under *C. myrtifolia* followed by *E. angustifolia* root exudates (Fig. 6). For upregulated proteins they were mainly “oxidoreductases”, “hydrolases,” “transferases,” and “isomerases” then “ligases” and “lyases” with higher scores for *E. angustifolia* than for *C. myrtifolia* root exudates (Fig. 7).

To gain insight into biological implications, differentially expressed proteins have been further subject to pathway enrichment analysis based on the KEGG database (supplementary Fig. S1 and Fig. S2). The main functional sets were connected to “metabolism” and “genetic information processing” for downregulated proteins and “metabolism” for upregulated proteins.

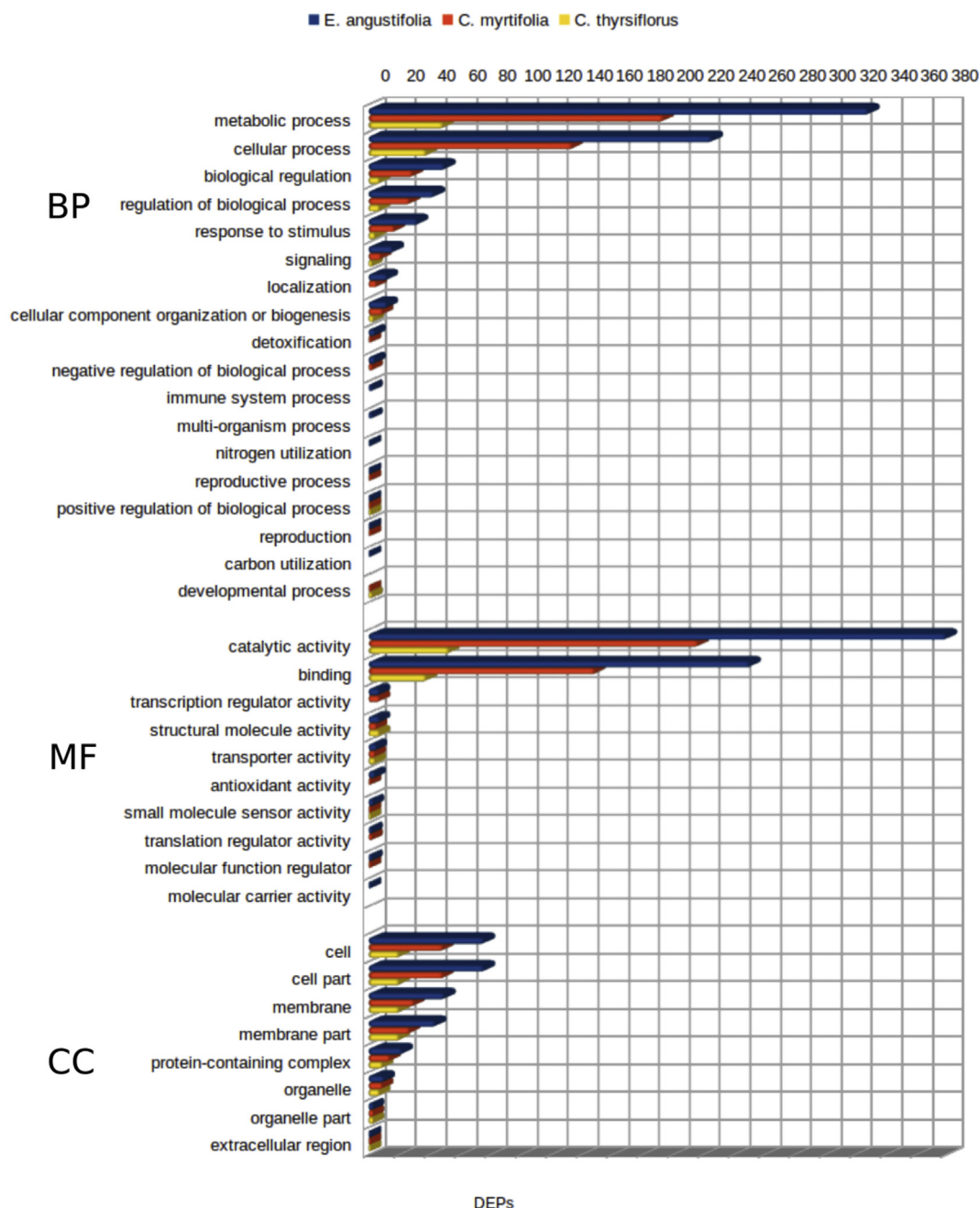


Fig. 3. GO analysis of downregulated proteins of NRRL B-16219 strain grown in presence of *E. angustifolia*, *C. myrtifolia* and *C. thyrsoiflorus* root exudates; Biological processes (BP), molecular function (MF) cellular component (CC).

Downregulated proteins in the “carbohydrate metabolism” category included “starch and sucrose metabolism”, “glycolysis/gluconeogenesis” and “pyruvate metabolism.” Upregulated proteins on this category included those associated with “glyoxylate and dicarboxylate metabolism”, “citrate cycle (TCA cycle)”, “fructose and mannose metabolism” and “pentose and glucuronate interconversions”. In the “Energy metabolism” category, “oxidative phosphorylation”, “nitrogen metabolism” and “sulfur metabolism” were upregulated. “Valine, leucine and isoleucine degradation”, “tryptophan metabolism”, “alanine, aspartate and glutamate metabolism” and “lysine metabolism” were among those upregulated for *E. angustifolia* compared with *C. myrtifolia* root exudates. “Cysteine and methionine metabolism” and “valine, leucine and

isoleucine metabolism” were solely upregulated for *E. angustifolia* root exudates. In the “metabolism of cofactors and vitamins” category, “thiamine metabolism” was upregulated for *E. angustifolia* and *C. myrtifolia* root exudates and “pantothenate and CoA biosynthesis” for *E. angustifolia* root exudates. “Benzoate degradation” and “Nitrotoluene degradation” were upregulated for *E. angustifolia* and *C. myrtifolia* root exudates.

4. Discussion

The main objective of the present study was to assess proteome alteration of *F. solii* NRRL B-16219 when exposed to root exudates of compatible, *E. angustifolia*, and incompatible, *C. myrtifolia* and

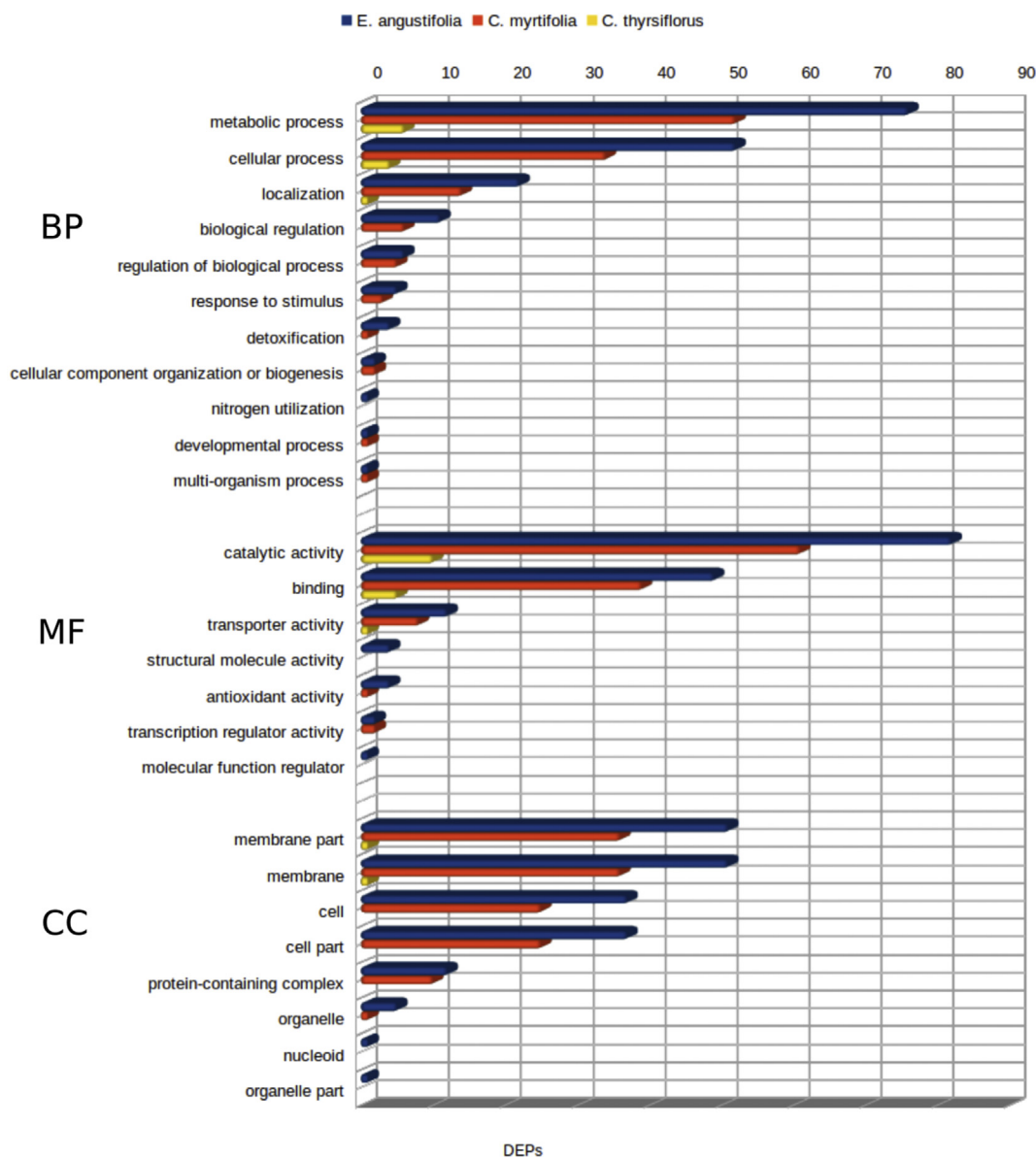


Fig. 4. GO analysis of upregulated proteins of NRRL B-16219 strain grown in presence of *E. angustifolia*, *C. myrtifolia* and *C. thyrsoflorus* root exudates; Biological processes (BP), molecular function (MF) cellular component (CC).

C. thyrsoflorus. Using a high-throughput shotgun proteomic approach we detected 211 up- and 663 downregulated cellular proteins in the presence of *E. angustifolia* root exudates, versus cells without added exudate. The lowest numbers of differentially expressed proteins were obtained in the presence of *C. thyrsoflorus* root exudates with 16 up- and 214 downregulated proteins even though NRRL B-16219 was originally isolated from rhizosphere soil beneath a related *Ceanothus*, *C. jepsonii* (D. D. Baker as described in [25]. Root exudates of *C. myrtifolia* elicited 138 and 559 proteins that were up- and downregulated respectively. In concordance with data published by [33]; the number of differentially expressed proteins is highest in presence of root exudates from compatible than from incompatible actinorhizal plants. Root exudates provide the first opportunity for the microsymbiotic partner to recognize, and respond to, the host plant [33]. reported that *Frankia* proteome dynamics, in the presence of root exudates, were mainly driven by host compatibility with one set of proteins matching root colonization and a second one corresponding to symbiotic recruitment.

No Nod proteins made by B-16219 were detected here either because their level was low, or they are not involved in the initial stages of infection.

In other studies that used the same approach, fewer differentially expressed proteins were observed for *F. alni* ACN14a and *F. coriariae* BMG5.1 in the presence of their respective *Alnus glutinosa* and *C. myrtifolia* root exudates. For ACN14a, a total of 180 upregulated cellular proteins were found [48]. For BMG5.1, 53 upregulated cellular proteins were found [33]. Both studies reported small numbers of downregulated proteins when grown in the presence of root exudates.

It has been shown previously that “metabolism” pathways dominate in *Frankia* during exposure to root exudates [33]. This observation has been explained by the presence of carbon compounds and energy content in root exudates [3]. Several upregulated proteins in this study were linked to “carbohydrate metabolism” and “energy metabolism”. These include membrane electron transport complexes NADH-quinone oxidoreductase

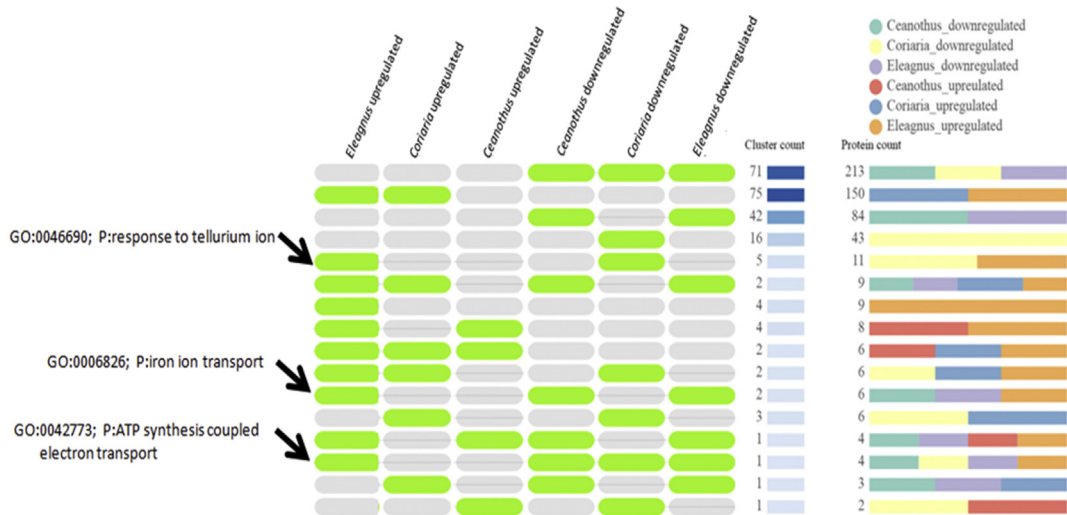


Fig. 5. Summary of the distribution of differentially expressed proteins of NRRL B-16219 strain grown in presence of *E. angustifolia*, *C. myrtifolia* and *C. thyrsoflorus* root exudates OrthoVenn2.

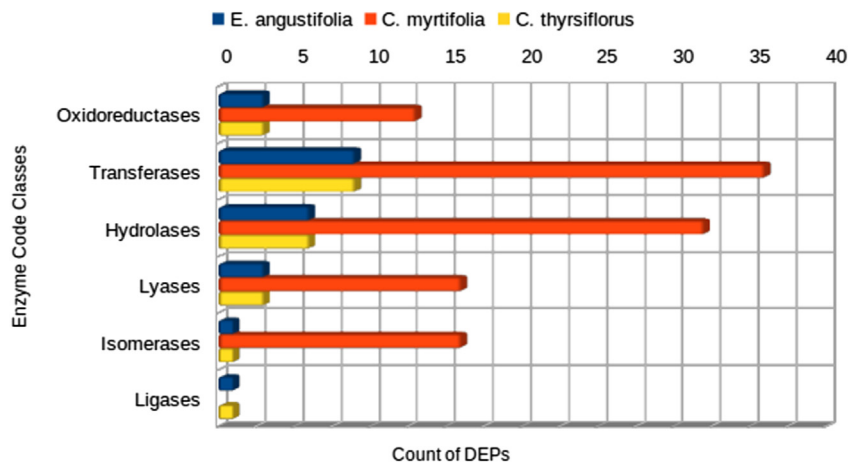


Fig. 6. Enzyme code statistic of downregulated of NRRL B-16219 strain grown in presence of *E. angustifolia*, *C. myrtifolia* and *C. thyrsoflorus* root exudates.

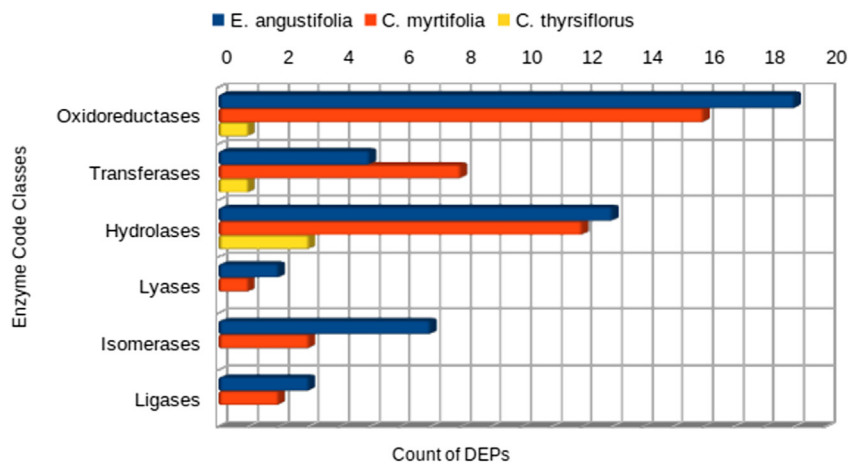


Fig. 7. Enzyme code statistic of upregulated of NRRL B-16219 strain grown in presence of *E. angustifolia*, *C. myrtifolia* and *C. thyrsoflorus* root exudates.

subunits NuoK (WP_020463509.1), NuoL (WP_071066452.1), NuoM (WP_071066451.1) and NuoN (WP_071066450.1); succinate dehydrogenase/fumarate reductase subunits FsdA (WP_071061295.1), FsdB (WP_020458904.1), and cytochrome *b* subunit FsdC (WP_083390835.1); ubiquinol-cytochrome *c* reductase cytochrome *b* subunit (WP_071061932.1); cytochrome *c* oxidase subunit I CtaD (WP_071059328.1 and WP_071061944.1), subunit II CtaC (WP_071061947.1); and F-type H⁺-transporting ATPase and subunit *b* (WP_020458658.1). This pattern suggests that upregulated proteins connected to respiratory complexes are required for metabolizing dicarboxylates contained in, or derived from, root exudates and to satisfy additional energy demand required for the upcoming symbiotic recruitment.

Other proteins connected to “carbohydrate metabolism” and linked to sources in root exudates include glucuronate isomerase (WP_071066409.1), xylose isomerase (WP_071065073.1), galactonate dehydratase (WP_071066403.1) and (1→4)- α -D-glucan 1- α -D-glucosylmutase (WP_083390868.1). “Pyruvate metabolism” was downregulated although it was the only carbon source provided in the *Frankia* growth medium.

Also detected were proteins potentially linked to plant cell wall degradation including an annotated endoglucanase (WP_071062082.1), beta-mannanases (WP_083391114.1 WP_083390847.1 and WP_071066004.1), glycoside hydrolase (WP_071060868.1), glycosyl hydrolase (WP_083391211.1) glycosyltransferase (WP_071067004.1) and a cellobiose transport system substrate-binding protein (WP_071062082.1) [33,48]. reported similar potential plant cell wall degrading *Frankia* proteins among upregulated in the presence of compatible root exudates [48]. detected cellulose synthase activity at the tip of *Frankia* hyphae in the presence of *Alnus* root exudates. The authors attributed the disrupted or clumped ultrastructure in the root hair at the site of *Frankia* entry to bacterial endoglucanase activity reported by [9]. The detection of the toolbox of plant cell wall degrading and remodeling enzymes in the present study may play a similar role during the infection of *Elaeagnus* through natural openings in outer root tissues (crack entry) or intact epidermis via the apoplastic interface between adjacent host cells (intercellular entry). Fournier et al. (2018) reported a deeper alteration in both size and composition of the intercellular apoplast, which occurs during the early infection stage to accommodate colonization of the root epidermis and outer cortex of *Discaria trinervis* microsymbiont. The authors demonstrated that the protease subtilase is among the many symbiosis-related enzymes that are responsible for wall cell remodeling at the site of entry. We suggest that several of the proteins detected here result in cell wall degradation/remodeling activities of *Frankia* microsymbionts.

Among other proteins known to be related to nitrogen fixation and assimilation (Alloisio et al., 2010; Mastrorunzio and Benson, 2010), we detected the nickel-dependent hydrogenase (WP_071065876.1 and WP_071065877.1), proteins linked to squalene synthesis including acetyl-CoA C-acetyltransferase (WP_071065991.1) and squalene-hopene/tetraprenyl-beta-curcumene cyclase (WP_071060384.1), and glycerophosphoryl diester phosphodiesterase (WP_071065777.1). Squalenes are intermediates in hopane biosynthesis, which help protect nitrogenase from oxygen in *Frankia* vesicles in symbiosis and free-living cultures [9]. [48] found Suf system proteins (Fe-S cluster assembly) but did not detect nitrogenase, hydrogenase and squalene biosynthesis peptides under *A. glutinosa* root exudates growth in nitrogen depleted conditions. Proteins related to nitrogen fixation and assimilation were similarly not detected by [33]. Contrary to what is expected for the latter and the present studies, and despite the depletion of nitrogen sources in both bacterial and plant growth media, the expression of nitrogen fixation genes is controversial.

Some of the proteins related to nitrogen fixation such as NifD (WP_071067111.1), NifK (WP_071067112.1) and NifH (WP_020464282.1), glutamine synthetase (WP_071065378.1), squalene synthesis; 1-deoxy-D-xylulose-5-phosphate synthase WP_071061644.1 (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate synthase (WP_071060580.1 and 4-hydroxy-3-methylbut-2-en-1-yl diphosphate reductase (WP_071060385.1) were only upregulated in strain NRRL B-16219 in the presence of root exudates of incompatible host *C. myrtifolia* suggesting that such cells perceived suboptimal nitrogen nutrition in such exudates.

Results obtained in this study for “amino acid metabolism” under the different root exudates tested joined the model proposed by [30] for the regulation of N-metabolism in *Frankia* in free-living cells and in symbiosis within actinorhizal root nodules. According to this model, amino acids may act differently depending on metabolic status. Glutamate, glutamine and aspartate are used by *Frankia* as nitrogen sources, alanine, valine and asparagine stimulate nitrogen fixation and respiration, while amino acids such as arginine or histidine may be toxic on *Frankia* strains. The differentially expressed proteins in this category in compatible and incompatible root exudations may reflect a mix of amino acids used for symbiotic recruitment by compatible host or potential subsistence in the rhizosphere of an incompatible host plant and thus co-regulated together with proteins related to nitrogen fixation and assimilation.

As was reported previously [33,48] proteins related to oxidative stress were upregulated in the presence of compatible root exudates and include nickel superoxide dismutase (WP_018502291.1), Cu-Zn superoxide dismutase (WP_071059896.1), catalase (WP_071059582.1) and porphyrinogen peroxidase (WP_071062182.1), together with proteolytic related proteins Zn-dependent membrane protease (WP_071063752.1), proteasome alpha subunit (WP_071063554.1) and beta subunit (WP_071063556.1), leucyl aminopeptidase (WP_071059318.1 and WP_071065370.1), peptidase S8 (WP_071059193.1) and peptidoglycan endopeptidase (WP_071063732.1). In addition, tyrosinase (WP_071059133.1) was upregulated in our study in presence of compatible root exudates. This enzyme is associated with melanin synthesis and has been shown to be involved in nodulation efficiency and symbiosis-associated stress resistance for rhizobia [47].

Proteins that have been suggested as specifically upregulated in presence of compatible root exudates [33] – scramblase (WP_071065217.1) preprotein translocase subunit SecY (WP_071066138.1), acetyl-CoA C-acetyltransferase (WP_071065991.1) and GlcNAc-PI de-N-acetylase (WP_020458433.1) – were also upregulated for NRRL B-16219 strain grown in presence of *E. angustifolia*. The link between GlcNAc-PI de-N-acetylase product (GlcNAc-PI) and the chitinase-resistant short oligomer able to activate the conserved symbiosis-signaling pathway (CSSP) deserves further investigation [16]. For the Prokaryotes, N-acetylglucosamine (GlcNAc) is a building block for peptidoglycan, biofilm exopolysaccharides, and for the N-glycan decoration of some proteins. It is also found as part of several signaling components such as chitin-oligosaccharide (Nod) factors in rhizobia [20] and in a variety of virulence factors [11,57]. If the receptors-mediated nodulation factors are similar in legume and actinorhizal plants, then these proteins may play a role in symbiosis.

The fact that none of the proteins encoded by *nod* genes was detected in our study even with the root exudates of the compatible host *E. angustifolia*, is reminiscent of what was found for the *nod*-dependent versus the *nod*-independent symbiotic interaction used by photosynthetic *Bradyrhizobium* strain ORS285 in nodulating *Aeschynomene afraspera* and *A. indica* respectively [10]. Interestingly the infection of *Elaeagnus* by *Frankia* occurs through direct

intercellular penetration of root epidermis cells and cortex [39], as also reported for *A. indica* during nodulation by *Bradyrhizobium* strain ORS285. Thus we may conclude that *nod* genes in NRRL B-16219 strain are not expressed even in presence of *E. angustifolia* root exudates because they are not essential for infecting this host plant.

Based on BRITE analysis (data not shown), the set of upregulated proteins also included many membrane transporters and signaling process (Ktari et al., 2017a; Pujic et al., 2019). In the present study we found mainly phosphate transporters; phosphate transport system substrate-binding protein (WP_071065787.1) and phosphate transport system permease protein (WP_071065815.1), cobalt/nickel transport system permease protein (WP_071065352.1), MFS transporter, NNP family, nitrate/nitrite transporter (WP_071064708.1), and Na⁺:H⁺ antiporter, NhaA family (WP_071066714.1). Proteins connected to signaling process were as previously reported [33,48] among those upregulated in the presence of root exudates and include two-component systems (WP_071061844.1, WP_071065787.1, WP_071063277.1, WP_020463624.1, WP_020459379.1, WP_071065991.1, WP_071065877.1 and WP_071065876.1); Phosphatidylinositol signaling system; phosphatidate cytidyltransferase (WP_071060588.1); tellurite resistance protein TerD (WP_020458691.1) and tellurite resistance protein TerC (WP_071065523.1); and cyclic-di-GMP-binding protein (WP_020463499.1).

5. Conclusion

Based on the early stage of symbiosis investigated through proteogenomics in this study and other recently published studies, the symbiotic signalling systems in actinorhizal symbiosis are quite complex and tightly regulated. Based on the three proteogenomic datasets for *Elaeagnus* compatible strain NRRL B-16219 obtained in this study, *Alnus* compatible *Frankia* strain ACN14a [48] and *Coriaria* compatible strain BMG5.1 very few candidate proteins seem related to any signalling factor that will be sensed by LysM-receptor-like kinases leading to a signal transduction cascade in actinorhizal plant. A role for an oligosaccharide containing N-acetylglucosamine (GlcNAc) that may fit the low molecular weight, thermoresistance, hydrophobicity and resistance to chitinase digestion [15,17] profile, of a *Frankia* signaling factor may still be possible. The presence of proteins shared between *Elaeagnus* and *Coriaria* compatible strains, but absent in *Alnus* compatible strain, suggests the presence of distinct signaling routes mediating intracellular or intercellular *Frankia* root colonization.

Availability of data and material

Mass spectrometry proteomic data obtained in this study were deposited at the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository (Vizcaíno et al., 2016) with the dataset identifiers PXD015186 and 10.6019/PXD015186. (Editors and reviewers are able to access the data using the following username/password; <https://www.ebi.ac.uk/pride/archive>: Username: reviewer06715@ebi.ac.uk Password: bdf1BW51).

Authors' contributions

MG conceived of the project. AG and FGG was responsible for the experimental work, JA for the proteomic analysis, IS and AS for bioinformatic analysis. MG and DRB analysed the data. MG drafted the manuscript which was critically reviewed by all of the authors.

Ethical approval

This article does not include any studies involving human participants or animals.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.resmic.2021.103900>.

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