The *Frankia alni* Symbiotic Transcriptome

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The actinobacteria *Frankia* spp. are able to induce the formation of nodules on the roots of a large spectrum of actinorhizal plants, where they convert dinitrogen to ammonia in exchange for plant photosynthates. In the present study, transcriptional analyses were performed on nitrogen-replete free-living *Frankia alni* cells and on *Alnus glutinosa* nodule bacteria, using whole-genome microarrays. Distribution of nodule-induced genes on the genome was found to be mostly over regions with high synteny between three *Frankia* spp. genomes, while nodule-repressed genes, which were mostly hypothetical and not conserved, were spread around the genome. Genes known to be related to nitrogen fixation were highly induced, *nif* (nitrogenase), *hup2* (hydrogenase uptake), *suf* (sulfur-iron cluster), and *shc* (hopanoids synthesis). The expression of genes involved in ammonium assimilation and transport was strongly modified, suggesting that bacteria ammonium assimilation was limited. Genes involved in particular in transcriptional regulation, signaling processes, protein drug export, protein secretion, lipopolysaccharide, and peptidoglycan biosynthesis that may play a role in symbiosis were also identified. We also showed that this *Frankia* symbiotic transcriptome was highly similar among phylogenetically distant plant families Betulaceae and Myricaceae. Finally, comparison with rhizobia transcriptome suggested that *F. alni* is metabolically more active in symbiosis than rhizobia.

Frankia spp. are among the very few soil microbes able to modify the plant hormonal balance, induce tissue differentiation, and establish nitrogen-fixing structures on the roots of higher plants belonging to eight dicotyledonous families collectively called actinorhizals (Benson and Silvester 1993). Despite marked morphological differences in nodules between actinorhizal and leguminous plants (Pawlowski and Bisseling 1996), the molecular phylogeny of these two groups of plants that engage in root symbioses showed they all belong to a single lineage, the Rosid I clade (Soltis et al. 1995), suggesting the existence of conserved determinants, such as the SymRK kinase that is essential to establish nodulation with *Frankia* spp. (Gherbi et al. 2008; Markmann et al. 2008).

Microarray data are available in the National Center for Biotechnology Information Gene Expression Omnibus database under accession number GSE18190.

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Although both bacteria establish root nodule symbiosis with plants, *Frankia* spp. show many physiological features different from rhizobia. *Frankia* spp. fix nitrogen under free-living (FL) conditions (Benson and Silvester 1993), while most rhizobia do not. Under FL nitrogen (N)-fixing conditions and under symbiotic conditions with most hosts, *Frankia* spp. differentiate into unique structures called vesicles (Parsons et al. 1987). Nitrogen fixation occurs in these vesicle cells, in which nitrogenase is protected from oxygen by multilayered hopanoid lipid envelopes surrounding the cell. This is in marked contrast to rhizobia, in which leghemoglobin supplied by the host plant controls oxygen concentration (Ott et al. 2005).

The genomes of three *Frankia* strains have recently been determined and were shown to have undergone multiple lateral transfers, duplications, and losses of genes (Normand et al. 2007b). These genomes have, nevertheless, retained a core of about 3,000 genes representing 35 to 51% of identified open reading frames, depending on the strain, some of which are expected to be responsible for symbiosis (Normand et al. 2007a). However, no significant symbiotic island or canonical *nod* gene homologs of rhizobia were identified in the genomes, confirming the results of Ceremonie and associates (1998), who had found no genetic complementation by *Frankia alnia* DNA of *Rhizobium nodB* and *nodC* mutants, leaving unanswered the question of which genes were involved in the symbiosis establishment and functioning.

In rhizobia, genes involved in symbiosis were identified by mutagenesis and most of them are clustered either on symbiotic plasmids (Galibert et al. 2001; Gonzalez et al. 2003; Mavingui et al. 2002; Young et al. 2006) or in symbiotic islands on the chromosome (Gottfert et al. 2001; Kaneko et al. 2002). Genes contained in the clusters have significantly lower GC content than those in other parts of the genome, suggesting a foreign origin. Symbiotic plasmids occur in bacteria belonging to genera *Rhizobium* (Mavingui et al. 2002; Gonzalez et al. 2003; Young et al. 2006) and *Sinorhizobium* (Galibert et al. 2001) and vary in size from 0.4 to 1.4 Mb. Symbiotic islands are found in species of genera *Mesorhizobium* (Kaneko et al. 2000), *Bradyrhizobium* (Kaneko et al. 2002) and *Azorhizobium* (Lee et al. 2008), which are around 0.6 Mb in size except that of *Azorhizobium caulinodans*, which is quite small (0.09 Mb). Symbiotic islands are flanked by tRNA sequences, suggesting that they have been inserted in the chromosome by site-specific integration with phage integrase. These structures are reminiscent of what is known to occur in bacterial pathogens and may arise due to lateral gene transfer (LGT) or due to protein synthesis optimized by gene clustering (Bloch and Rode 1996; Claros et al. 2006; Kers et al. 2005; Nano et al. 2004). Most often, the symbiotic plasmid or island region comprises the *nif* and *nod* genes, the dicarboxylate transporter *dctA*, as well as a few hypothetical and strain-spe-

cific genes (Perret et al. 1991); it can also be transferred laterally as a symbiotic island (Sullivan and Ronson 1998). Other *Rhizobium* genes that have been shown or suspected to play a role in nodulation are those involved in the synthesis of exopolysaccharides (Gonzalez et al. 1996), lipopolysaccharides (LPS) (Lerouge and Vanderleyden 2002), capsular polysaccharides (KPS) (Parada et al. 2006), cytokinins (Cooper and Long 1994), and 5-aminoimidazole-4-carboxamide riboside, a purine synthesis intermediate (Newman et al. 1994). In any case, a Nod factor-based mechanism may not be universal, as it was recently shown in photosynthetic *Bradyrhizobium* strains that no canonical *nod* genes were present in the genome (Giraud et al. 2007).

Some *Frankia* genes are known or at least postulated to be involved in symbiotic interactions. They are those coding for nitrogenase, the enzyme at the core of nitrogen fixation (Lundquist and Huss-Danell 1991), uptake hydrogenase (Sellstedt and Mattsson 1994), and hopanoid lipids biosynthesis genes (Berry et al. 1993). Some rare metabolites have been heralded as *Frankia* species specific, such as the sugar 2-O-methyl-D-mannose found in all strains assayed (Mort et al. 1983) and the aromatic phenylacetate found in *F. alni* (Hammad et al. 2003). They may be a part of the molecular dialogue between *Frankia* spp. and their hosts in parallel with other plant-active compounds. The first such compounds described were the plant hormones cytokinin (Stevens and Berry 1988) and three auxin-like indole derivatives (Berry et al. 1989). Later, a small (molecular weight <3,000) heat-stable water-soluble compound present in the supernatant of *Frankia* culture medium that induced root-hair branching was described (Van Ghelue et al. 1997) and shown to probably contain *N*-acetyl-D-glucosamine and to be susceptible to enzymatic attack (Ceremonie et al. 1999). Finally, phenylacetate also detected in the supernatant of *Frankia* culture medium (Hammad et al. 2003) has auxinic activity as well as cell-differentiating activity and is also an antimicrobial and antifungal molecule (Hwang et al. 2001).

DNA arrays have been used to identify modifications of gene-expression patterns such as those occurring in *Sinorhizobium* (Barnett et al. 2004; Becker et al. 2004; Capela et al. 2006), *Mesorhizobium* (Uchiumi et al. 2004), *Bradyrhizobium* (Chang et al. 2007; Hauser et al. 2007; Pessi et al. 2007) and *Azorhizobium* (Tsukada et al. 2009) species as they enter symbiosis. Such studies typically detect those genes previously known through classical genetic approaches to be involved as well as other structural or regulatory genes. However, this technique is especially promising in nongenetically workable bacteria such as *Mycobacterium leprae*, in which it allowed confirmation of the catabolic utilization of fatty acids (Williams et al. 2004) or the study of the regulatory role for noncoding pseudogenes (Suzuki et al. 2006) and holds promise for the study of numerous *Mycoplasma* and *Rickettsia* species and other unisolated microbial pathogens and symbionts.

Given the present lack of classical genetic tools to identify symbiotic genes, *Frankia* genomes were sequenced and annotated to determine their structure, to identify putative genes in-

involved in symbiosis, and to allow the investigation of symbiosis by large-scale tools such as proteomics or transcriptomics. In the present study, we report for the first time on expression profiling of 6,607 predicted genes of *F. alni* ACN14a (ACN). Transcript levels of these genes were monitored under N-replete FL conditions and in *Alnus glutinosa* nodule bacteria to identify genes differentially expressed in symbiosis. Two similar array experiments performed independently in our two laboratories were first compared to identify strongly significant differential genes. Then, these results were further strengthened by transcriptomic analysis in three other tree species, *A. nepalensis*, *Morella rubra*, and *Myrica gale*. We also tried to distinguish nitrogen fixation-related genes from nodulation-related genes by comparing these results with transcriptomic results of cells grown under N-fixing FL conditions. Finally, an in silico comparison of the transcriptomic analysis with rhizobia was performed.

RESULTS AND DISCUSSION

Gene expression pattern in *A. glutinosa* nodule as compared with FL bacteria.

Analysis overview. To address gene expression changes of *F. alni* ACN in the symbiotic state, we compared transcript levels between young nodules (3 to 4 weeks old) and cultured cells grown in N-replete minimal medium. Two data sets (A and B), obtained in our two laboratories with similar experimental conditions, were analyzed using a stringent statistical test. In data set A, 871 genes showed a differential expression in symbiosis vs. in vitro N-replete FL cells (Supplementary Table 1) compared with 457 genes in data set B (Supplementary Table 2). The lower number of differential genes in data set B was related to lower fold change (FC), up to 396 and 149 for data sets A and B, respectively. From these two differential expression gene lists, we extracted i) 292 'common' genes (Supplementary Table 3), with 166 genes upregulated and 126 genes downregulated in both data sets (Table 1) and ii) two genes displaying opposite regulation in the two data sets (FRAAL0302, encoding an antioxidant protein, and FRAAL1882, encoding a putative small heat-shock protein), which were discarded. Interestingly, these two discordant regulations result essentially from differences between fluorescent array signals under N-replete FL conditions but not in nodules, illustrating that the reference condition has a major effect on differential expression results (Supplementary Table 4). We concluded that the expression change in nodule of these 292 common genes was strongly significant and that they were very likely to play some roles in symbiosis. In this paper therefore, we will review and discuss mainly the putative symbiotic role of the common genes and consider genes differentially expressed in only one data set if they reinforce the claim made by common genes, e.g., six out of eight genes belonging to the ATP synthase cluster (FRAAL5930 to 5937) were up-regulated in the two data sets, whereas the two remaining genes FRAAL5934 and 5937 were up-regulated only in one of the two data sets, A or

Table 1. Overview of *Frankia alni* ACN14a symbiotic transcriptome analyses

Comparison	Differential ^a	Upregulated ^b	Downregulated ^c
<i>Alnus glutinosa</i> nodules vs. N-replete free-living (FL) cells (data set A)	871	415	456
<i>A. glutinosa</i> nodules vs. N-replete FL cells (data set B)	457	265	192
Genes differential in data sets A and B	292	166	126
Genes differential in the four tree species ^d	223	117	106

^a Total number of differentially expressed genes (up- and downregulated).

^b Number of genes with increased expression in nodules.

^c Number of genes with decreased expression in nodules.

^d *A. glutinosa*, *A. nepalensis*, *Myrica gale*, and *Morella rubra*.

B. Indeed, it must be kept in mind that those genes were judged as nondifferential in one data set, but FC or *P* value could be on the borderline.

Chromosomal distribution of the genes that are up-regulated in nodules was evaluated (Fig. 1). Nodule-induced genes are separately clustered in several small chromosomal regions, showing that *F. alni* does not contain a symbiotic island consisting of numerous nodule-induced genes, which was found in rhizobia such as *Bradyrhizobium japonicum* (Pessi et al. 2007) and *Mesorhizobium loti* (Uchiumi et al. 2004). However, they are mostly located in the first and last thirds of the linearized genome, which correlates with the high synteny in these regions, suggesting that those genes are highly conserved in the three sequenced *Frankia* genomes (ACN, EAN1pec (EAN), and CcI3) (Normand et al. 2007a). In contrast, downregulated genes are more homogenously spread around the chromosome. Table 2 shows correlation between conservation of genes and differential expression. About 40% of ACN genes contained in genome or microarray were conserved in the other two *Frankia* genomes (EAN and CcI3), thus these genes constitute

the *Frankia* conserved core. More than 66% of genes were conserved in upregulated genes, whereas the percentage in downregulated genes was similar to that in the genome ($\leq 44\%$), confirming the results shown in Figure 1 that nodule-activated genes are mostly conserved. Genes crucial for symbiosis would be contained in the regions with high conservation and induction.

Assignment of functional clusters of orthologous gene (COG) classes (Tatusov et al. 2001) on ACN genes showed that differentially expressed genes were overrepresented in classes J (translation, ribosomal structure, and biogenesis) and C (energy production and conversion) and were mostly up-regulated (Fig. 2). Differentially expressed genes without COG function account for about 25%, a proportion similar to that of the whole genome, and are predominantly down-regulated. This last feature suggests that most downregulated genes encode nonessential functions.

Nitrogen fixation and closely related functions. Among the 292 common genes showing a differential expression in the two data sets, 53 genes had FC values >10 in one or both data

Fig. 1. Expression values in nodules vs. N-replete free-living (FL) cells for each gene plotted against gene number FRAAL order on the linearized *Frankia alni* genome with the chromosome replication origin (ori) at both ends. We used weighted average difference (WAD) values (y axis), which is a $\log_{10}$ -transformed fold change weighted by the absolute expression level (Kadota et al. 2008) to improve visualization of regions with differential gene expression. Data from experiments A and B are indicated by blue and pink dots, respectively. The positions of seven upregulated clusters are shown. The *rib*-encoding ribosomal proteins (FRAAL1064 to 1115; FRAAL5779 to 5812), the *shc1*-encoding hopanoid biosynthesis enzymes (FRAAL1426 to 1434), the *hup2*-encoding uptake hydrogenases (FRAAL1822 to 1832), the *suf*-encoding sulfur-iron cofactor assembly proteins (FRAAL4557 to 4563), the *cyt*-encoding cytochrome c oxidases (FRAAL5109 to 5123), the *atp*-encoding ATP synthases (FRAAL5930 to 5937), and the *nif*-encoding nitrogenases (FRAAL6797 to 6814). Syntenic regions are indicated by gray dots at the top and defined by the presence of at least five consecutive genes in the three strains (ACN, EAN, and CcI3) showing 50% amino acid identity over 80% of the length of the smaller gene.

Table 2. Absolute number and percentage of the conserved genes in various subsets of genes^a

Genes	Total	Conserved	%	Upregulated			Downregulated		
				Total	Conserved	%	Total	Conserved	%
ACN genome	6,723	2,824	42	...	...	...	...	...	...
Microarray	6,607	2,815	43	...	...	...	...	...	...
Differential (A) ^b	871	451	52	415	274	66	456	177	39
Differential (B) ^c	457	279	61	265	200	75	192	79	41
Common genes ^d	292	183	63	166	127	77	126	56	44

^a Number of genes conserved in three *Frankia* genomes (ACN, CcI3, and EAN) at a threshold level of 40% amino acid identity over 80% of the length of the smaller gene. Bold characters indicate higher percentage of conserved genes.

^b Genes differentially expressed in data set A.

^c Genes differentially expressed in data set B.

^d Genes differentially expressed in both data sets A and B.

sets (Table 3). As expected, the most highly induced genes were the *nif* genes, especially the structural *nifHDK* genes encoding nitrogenase (FC up to 396), which confirms the usefulness of the transcriptome approach. The cluster of *nif* genes containing 15 genes (FRAAL6800 to 6814), three of which are hypothetical, behaved coherently as all genes were up-regulated (Fig. 3A). This operon is bordered on its left side by two genes encoding ferredoxin oxidoreductase subunits and a gene encoding ferredoxin (FRAAL6797 to 6799) which are all highly upregulated (Table 3; Fig. 3A), suggesting a role in the electron transport pathway to nitrogenase, as it was shown in *Bradyrhizobium japonicum* (Hauser et al. 2007). Synteny of those genes observed in the genomes of two other *Frankia* strains supports this hypothesis (Table 3; Fig. 3A). A second conserved ferredoxin gene (*fdx1*, FRAAL3855), localized away from the *nif* operon, was also strongly induced, being the fifth top-scoring gene (Table 3). This gene could also contribute to nitrogen fixation, since several ferredoxins in *B. japonicum* are involved in nitrogen fixation (Hauser et al. 2007). On the other hand, three other ferredoxin genes (FRAAL1469, FRAAL1995, FRAAL6126) were not induced in nodule. FRAAL1673, encoding a putative molybdenum-binding protein, was induced in nodules (Table 3) and has orthologs in EAN and Cc13. Its gene product could supply molybdenum cofactor for nitrogenase complex biosynthesis.

The second upregulated cluster (FC up to 21.5) encodes uptake hydrogenase 2 (Hup2) (FRAAL1822 to 1832), which is assumed to convert hydrogen produced by nitrogenase into protons and electrons (Table 3; Fig. 3B) (Sellstedt and Mattsson 1994). Conversely, another hydrogenase cluster, *hup1* (FRAAL2388 to 2400), showed no significant upregulation. Similar results were reported by Leul and associates (2007), who analyzed transcript level of uptake Hup1 and Hup2, using quantitative real-time polymerase chain reaction (PCR), and found that the latter one is more-expressed in symbiosis whereas the former is more-expressed in FL cells. Like the *nif* cluster, the *hup2* cluster is present in the other two *Frankia* strains, even though it has been reshuffled in EAN through

LGT (Fig. 3B) (Leul et al. 2009). These results support the suggestion that uptake Hup2 is related to nitrogen fixation.

The third upregulated cluster (FC up to 11.1) contained *sufBCDESU* genes (FRAAL4557 to 4563) that are involved in iron-sulfur (Fe-S) cluster formation (Fig. 3C). This fits the strong requirement for Fe-S clusters of key nitrogen fixation-related enzymes such as nitrogenase and uptake Hup2. In an *Azorhizobium caulinodans* mutant with a disruption in the *sufS* gene, nodules produced lacked nitrogen-fixing ability (Suzuki et al. 2007).

Vesicle hopanoid membrane. Formation of vesicles is a unique feature in the microbial world. These specialized nitrogen-fixing cells, which differentiate under nitrogen-limiting conditions, provide a barrier for up to 80% oxygen (Parsons et al. 1987) and, thus, prevent the fragile nitrogenase from oxygen inactivation. Oxygen diffusion is tightly controlled by the vesicle envelope made up of several tens of hopanoid lipid layers consisting of the relatively common bacteriohopanetetrol (BHT) and its unique phenylacetate ester (PAA-BHT) (Berry et al. 1993). Hyphae, on the contrary, are not surrounded by this multilayered lipid envelope and do not contain the PAA-BHT; however, they contain BHT, although probably less than in large symbiotic vesicles (Nalin et al. 2000). In that sense, genes involved in biosynthesis of BHT and BHT-PAA can be essential for nitrogen fixation and, thus, to symbiosis. These genes encode enzymes belonging to the methyl-erythritol (nonmevalonate) isopentenyl diphosphate and hopanoid pathways. Several of them were up-regulated, *dxs* (FRAAL2088) and *ispG* (FRAAL5772) involved in isopentenyl diphosphate biosynthesis as well as *idi* (FRAAL6504) and a putative squalene/phytoene dehydrogenase (FRAAL1430) involved in hopanoid biosynthesis. The last gene is localized in the *shc1* cluster (FRAAL1428-1433) containing the major genes leading to hopanoid biosynthesis such as those encoding squalene synthase and squalene-hopene cyclase (Shc) 1; all these genes were up-regulated (Fig. 3D). In contrast, the second *shc* gene (*shc2*, FRAAL2491), which is not contained in the *shc1* cluster, did not show differential expression change. This result

Fig. 2. Differentially expressed genes in nodules vs. N-replete free-living (FL) cells categorized by functional clusters of orthologous genes (COG) classes (Tatusov et al. 2001). Percentages are calculated by dividing the number of up- (white) or downregulated (gray) genes in each class by the total number of differentially expressed genes. For comparison, black columns indicate the percentage of genes of ACN14a genome in each COG class. Differentially expressed genes without COG function (*) account for about 25%, as in the genome, and are predominantly down-regulated. In contrast, differentially expressed genes belonging to J and C classes are overrepresented and mostly upregulated. COG identification: J = translation, ribosomal structure, and biogenesis; K = transcription; L = replication, recombination, and repair; D = cell cycle control, cell division, chromosome partitioning; V = defense mechanisms; T = signal transduction mechanisms; M = cell wall/membrane/envelope biogenesis; N = cell motility; U = intracellular trafficking, secretion, and vesicular transport; O = posttranslational modification, protein turnover, chaperones; C = energy production and conversion; G = carbohydrate transport and metabolism; E = amino acid transport and metabolism; F = nucleotide transport and metabolism; H = coenzyme transport and metabolism; I = lipid transport and metabolism; P = inorganic ion transport and metabolism; Q = secondary metabolites biosynthesis, transport, and catabolism; R = general function prediction only; S = function unknown.

suggests that the *shc2* gene is not a gene essential for nitrogen fixation as previously mentioned, based on its absence in the CcI3 genome. Furthermore, we found that two genes (FRAAL1426 and 1427) preceding the *shc1* cluster and encoding a hypothetical protein and a putative short-chain dehydro-

genase/oxydoreductase (Fig. 3D) were among the most highly induced genes (Table 3). Interestingly, this short-chain dehydrogenase and *Shc1* belong to the core *Frankia* secretome, which consists of 161 secreted proteins conserved in the three *Frankia* strains (Mastrorunzio et al. 2008). Moreover, this pro-

Table 3. The 53 most highly induced genes in *Alnus glutinosa* nodules (fold change >10 in data sets A or B)

Gene no. ^a	Gene name	Product description	Fold change ^b		COG ^d	Core ^e	Spe. ^f	Syn. ^g	Secr. ^h
			A ^c	B ^c					
FRAAL6813	<i>nifH</i>	Nitrogenase reductase	396.3	103.8	DP	+	+	+	–
FRAAL6812	<i>nifD</i>	Nitrogenase alpha subunit	390.3	149.3	C	+	+	+	–
FRAAL6811	<i>nifK</i>	Nitrogenase beta subunit	375.3	44.0	C	+	+	+	–
FRAAL6814	<i>nifV</i>	Homocitrate synthase	207.3	19.0	CE	+	+	–	–
FRAAL3855	<i>fdxI</i>	Ferredoxin	161.8	35.2	C	+	–	–	–
FRAAL6806		Conserved hypothetical protein	134.7	19.7		+	+	+	–
FRAAL6807		Conserved hypothetical protein	116.9	14.1		+	+	+	–
FRAAL6808	<i>nifX</i>	NifX protein	99.3	13.2		+	+	+	–
FRAAL6805	<i>nifW</i>	Nitrogenase stabilizing/protective protein	98.2	14.4		+	+	+	–
FRAAL6810	<i>nifE</i>	Nitrogenase FeMo Co biosynthesis protein	86.6	9.5	C	+	+	+	–
FRAAL6799		Ferredoxin oxidoreductase alpha subunit	62.8	5.0	C	+	–	+	–
FRAAL6797		Ferredoxin	60.2	28.4	C	+	–	+	–
FRAAL6803	<i>nifB</i>	FeMo Co biosynthesis protein	51.2	6.4	R	+	+	+	–
FRAAL6801		Putative molybdopterin biosynthesis protein HesA	48.1	5.9	H	+	+	+	+
FRAAL6486	<i>groL</i>	Chaperone Hsp60	40.3	7.2	O	+	–	–	–
FRAAL6804	<i>nifZ</i>	NifZ protein	39.7	4.7	C	+	+	+	–
FRAAL3254		Hypothetical protein	38.8	14.4	S	+	+	–	–
FRAAL6816		Hypothetical protein	37.2	14.5	E	+	+	+	–
FRAAL6798		Ferredoxin oxidoreductase beta-subunit	28.2	4.6	C	+	–	+	–
FRAAL3287		Conserved hypothetical protein	24.9	8.5		–	–	–	–
FRAAL4419		Hypothetical protein	22.4	3.6		–	–	–	–
FRAAL4403		Putative RNA helicase	22.3	3.9	LRJK	+	–	+	–
FRAAL1830	<i>hupS2</i>	[NiFe] uptake hydrogenase small subunit	21.5	3.4	C	–	–	–	–
FRAAL3922		Putative export protein	20.6	5.2	RU	–	–	–	–
FRAAL5232		Putative integration host factor-like protein	18.2	6.1	L	+	–	+	–
FRAAL1829	<i>hupL2</i>	[NiFe] uptake hydrogenase large subunit	18.0	2.8	C	–	–	+	–
FRAAL1673		Putative Mo binding protein	17.8	3.8	GR	+	–	–	–
FRAAL4897		1-Acylglycerol-3-phosphate <i>O</i> -acyltransferase	16.1	9.2	I	+	–	–	–
FRAAL1426		Hypothetical protein	15.3	17.6		–	–	–	–
FRAAL1831	<i>hypA2</i>	Hydrogenase nickel incorporation protein	15.0	2.9	R	–	–	+	–
FRAAL4147	<i>ctaD</i>	Cytochrome c oxidase	14.3	6.2	C	+	–	–	–
FRAAL5516		Putative AAA family Cell division control ATPase	14.2	3.4	OKR	–	–	–	–
FRAAL4858		Putative LuxR-family transcriptional regulator	13.7	8.3	K	+	–	–	–
FRAAL1156	<i>sucC</i>	Succinyl-CoA synthetase beta subunit	13.6	10.5	CI	+	–	+	–
FRAAL4896		Putative multidrug efflux transporter	13.3	9.8	RUV	+	–	+	–
FRAAL5724		Putative RNA polymerase ECF sigma factor	12.2	9.1	K	+	–	+	–
FRAAL6541		Hypothetical protein	12.1	16.0		–	–	–	–
FRAAL1155		Putative serine/threonine protein kinase	12.0	3.7	KLRT	+	+	+	–
FRAAL1427		Putative short-chain dehydrogenase/oxydoreductase	12.0	4.0	IQR	+	–	+	+
FRAAL0774		Hypothetical protein	11.8	4.3		–	–	–	–
FRAAL1832	<i>hypB2</i>	Hydrogenase nickel incorporation protein	11.8	3.4	EKO	+	–	+	–
FRAAL2240		Hypothetical protein	11.7	3.0		–	–	–	–
FRAAL4471		Hypothetical protein	11.6	17.0	NT	–	–	–	–
FRAAL4559	<i>sufS</i>	Cysteine desulfurase/selenocysteine lyase	11.1	3.7	ER	+	–	+	–
FRAAL2921		Hyp. protein (repressor-like DNA-binding domain)	10.7	12.0		–	–	+	–
FRAAL2922		Hypothetical protein	10.7	14.3		–	–	–	–
FRAAL4195		Conserved hypothetical protein	10.6	3.5	FE	–	–	–	–
FRAAL1157	<i>sucD</i>	Succinyl-CoA synthetase alpha subunit	10.5	6.7	C	+	–	+	–
FRAAL5231	<i>gmk</i>	Guanylate kinase	10.5	4.7	F	+	–	+	–
FRAAL6657		<i>N</i> -acetylmuramoyl-L-alanine amidase (autolysin)	10.5	10.5	M	+	–	+	+
FRAAL4560	<i>sufC</i>	Fe-S cluster assembly transport protein	10.4	4.5	EPV	+	–	+	–
FRAAL4473		Hypothetical protein	9.4	15.3		–	–	–	–
FRAAL0111	<i>gltA</i>	Citrate synthase	9.4	10.1	C	+	–	+	–

^a Gene numbers are ordered according to decreasing fold changes of data set A. Gene number, gene name, and product description according to the Genoscope database.

^b Fold change comparing nodules vs. N-replete free-living cells.

^c Data sets A and B refer to similar experiments performed in our two laboratories, respectively.

^d Clusters of orthologous genes (COG) classes (Tatusov et al. 2001).

^e Core genes are conserved genes in the three strains (ACN, EAN, and CcI3) at a threshold level of 40% amino acid identity over 80% of the length of the smaller gene. + = present; – = absent.

^f Specific genes are core genes not conserved in *Acidothermus cellulosilyticus* (NC_008578), *Streptomyces avermitilis* (NC_003155, NC_004719), and *S. coelicolor* (NC_003888, NC_003903, NC_003904) at a threshold level of 40% amino acid identity over 80% of the length of the smaller gene.

^g Synteny is the presence of a group of genes in the three strains (ACN, EAN, and CcI3) having 30% amino acid identity over 80% of the length of the smaller gene, separated by no more than five intervening sequences.

^h Core secretome encompasses 161 conserved secreted proteins in the three strains (ACN, EAN, and CcI3) (Mastrorunzio et al. 2008).

tein is homologous to the RkpH protein involved in KPS production in *Sinorhizobium fredii*. Soybean inoculated with the *S. fredii* *rkpH* mutant showed reduced nodulation and severe symptoms of nitrogen starvation (Parada et al. 2006). On the

other hand, genes involved in PAA metabolism were not up-regulated in nodules. The FRAAL1434 gene coding for a putative PAA thioesterase did not show differential expression change, whereas the FRAAL3103 gene, which encodes a puta-

Fig. 3. Four symbiosis-related clusters upregulated in *Alnus glutinosa* nodules. Genes showing synteny (amino acid identity above 30% over 80% of the length of the smaller gene, separated by no more than five intervening sequences) with ACN14a and located within the corresponding cluster in each genome (EAN1pec and Ccl3) are indicated by red arrows. Sets of genes that showed synteny with ACN14a but located outside of the corresponding cluster in the EAN1pec genome are blue and yellow arrows. Unfilled arrows indicate a gene whose homolog is present but outside of the cluster in each genome. In boxes below the arrows, gene names are given (top) together with their FRAAL identity number (middle) and their fold-change value (bottom). These rectangles are green when significantly upregulated in nodule, hatched when not related to the function of the cluster, and gray when not upregulated. For genes without a gene name, an abbreviation of the gene product description is given: hyp = hypothetical protein, fdxR = ferredoxin oxidoreductase, glyt = glycosyl transferase, msb = metal-sulfur cluster biosynthetic enzyme, mexp = membrane export protein, reg = TetR-family transcriptional regulator, sdr = short-chain dehydrogenase/oxidoreductase, phs = phytoene synthase, spd = squalene/phytoene dehydrogenase, and pte = phenylacetic acid thioesterase.

tive PAA degradation protein (PaaA) and localizes away from the *shc1* cluster, was down-regulated. All the above genes are conserved in the three *Frankia* genomes, except FRAAL1426 and 3103.

Ammonium assimilation and transport. Ammonium generated from nitrogen fixation is assimilated into glutamate with glutamine synthase (GS), glutamate synthase (GOGAT), and glutamate dehydrogenase (GDH). In nodules, the *glnA* gene (FRAAL5161) encoding GSI was strongly repressed (FC down to 0.061; Tables 4 and 5), whereas the *glnII* gene (FRAAL5163) encoding GSII was only slightly induced (about twofold; Table 5). Four other GS-encoding genes (FRAAL0678, 1887, 5165, and 6426) did not show differential expression changes (Table 5). The *gdhA* gene (FRAAL6693)

encoding GDH displayed pronounced induction in nodule (5.4-fold; Table 5). In FL N-fixing conditions, strong repression of GSI (*glnA*) was also observed (0.2-fold; Table 5). Induction of GSII (*glnII*) expression also occurred but at a much higher level (15.1-fold; Table 5) than that occurring in nodule. Induction of GDH (*gdhA*) was not observed under N-fixing FL conditions (Table 5). Such a pattern of regulation in GSI, GSII, and GDH under N-fixing FL conditions was also reported in *Frankia* ACN and CpI1, using enzymatic and proteomic analyses (Alloisio et al. 2007; Schultz and Benson 1990). These results suggest that regulation of ammonium assimilation is different between symbiotic and FL N-fixing conditions. In addition, two out of four genes (FRAAL4964 and 4965) coding for GOGAT and one of the two ammonium transporter

Table 4. The 43 most highly repressed genes in *Alnus glutinosa* nodules (fold change <0.1 in data sets A or B)

Gene no. ^a	Gene name	Product description	Fold change ^b		COG ^d	Core ^e	Spe. ^f	Syn. ^g	Secr. ^h
			A ^c	B ^c					
FRAAL1946		Hypothetical protein	0.009	0.018		–	–	–	–
FRAAL3765		Putative sugar ABC transporter	0.016	0.011	G	–	–	–	–
FRAAL3078		Transposase	0.016	0.062	L	+	–	+	–
FRAAL0504		Hypothetical protein	0.022	0.037	R	–	–	–	–
FRAAL6024		Putative glutamate-1-semialdehyde aminotransferase	0.025	0.096	EH	+	+	+	–
FRAAL3764		Putative sugar ABC transporter	0.029	0.011	G	–	–	–	–
FRAAL5525		Putative acyl-CoA ligase	0.038	0.047	QI	–	–	–	–
FRAAL6577	<i>hpaA</i>	Pyrophosphate-energized proton pump	0.039	0.042	CEGPR	+	–	+	–
FRAAL1014		Hypothetical protein	0.043	0.054	K	+	+	+	–
FRAAL3310		Conserved hypothetical protein	0.043	0.057		+	–	+	–
FRAAL0879		Hypothetical protein	0.048	0.056	Q	–	–	–	–
FRAAL0300		Putative S-adenosylmethionine transferase	0.049	0.106	QR	–	–	–	–
FRAAL0514		Hypothetical protein	0.051	0.037		+	+	+	–
FRAAL1344		Hypothetical protein	0.057	0.127		–	–	–	–
FRAAL0891		Hypothetical protein	0.058	0.184		–	–	–	–
FRAAL0629		Putative acetyltransferase	0.060	0.106	J	+	+	–	–
FRAAL5161	<i>glnA</i>	Glutamine synthetase I	0.061	0.279	E	+	–	+	–
FRAAL6224		Conserved hypothetical protein	0.061	0.039		+	–	+	–
FRAAL3311		Conserved hypothetical protein	0.062	0.079		+	–	+	–
FRAAL6548		Putative WhiB-family transcriptional regulator	0.062	0.112		+	–	+	–
FRAAL2150		Hypothetical protein	0.064	0.067	K	+	+	+	–
FRAAL3570		Conserved hypothetical protein	0.068	0.257		–	–	–	–
FRAAL0373		Putative acetyltransferase	0.070	0.054	R	–	–	–	–
FRAAL0515		Hypothetical protein	0.070	0.042		–	–	–	–
FRAAL2313		Putative metallo-phospho-hydrolase	0.073	0.157	R	+	–	–	–
FRAAL1286		Hypothetical protein	0.076	0.039		–	–	–	–
FRAAL4030		Hypothetical protein	0.085	0.042		–	–	–	–
FRAAL2387		Hypothetical protein	0.085	0.078		+	+	–	–
FRAAL4029		Conserved hypothetical protein	0.086	0.042	QO	–	–	–	–
FRAAL3149	<i>atoA</i>	Acetoacetyl-CoA transferase beta subunit	0.093	0.134	I	+	–	+	–
FRAAL6022		Putative aldehyde dehydrogenase	0.093	0.213	C	+	–	+	–
FRAAL3763		Putative sugar ABC transporter	0.097	0.021	P	–	–	–	–
FRAAL0140		Putative helix-turn-helix-type transcriptional regulator	0.101	0.089	K	–	–	–	–
FRAAL6325		Hypothetical protein	0.127	0.064		–	–	–	–
FRAAL4664		Hypothetical protein	0.130	0.052		–	–	–	–
FRAAL0094		Putative serine protease	0.137	0.070	O	+	–	+	+
FRAAL2120		Conserved hypothetical protein	0.139	0.051	O	+	–	+	–
FRAAL4031	<i>hsp18</i>	Chaperone Hsp18	0.156	0.065	O	–	–	–	–
FRAAL6787		Conserved hypothetical protein	0.180	0.075		–	–	–	–
FRAAL4321		Hypothetical protein	0.207	0.086	O	+	+	+	–
FRAAL3762		Putative sugar ABC transporter	0.220	0.028	EPG	+	–	–	–
FRAAL0953		Hypothetical protein	0.230	0.098		–	–	–	–
FRAAL0021		Hypothetical protein	0.295	0.094		–	–	–	–

^a Gene numbers arbitrary ordered according to increasing fold changes of data set A (the lowest fold changes were similar in the two data sets: 0.009 and 0.011). Gene number, gene name and product description according to the Genoscope database.

^b Fold change comparing nodules vs. N-replete free-living cells.

^c Data sets A and B refer to similar experiments performed in our two laboratories.

^d Clusters of orthologous genes (COG) classes (Tatusov et al. 2001).

^e Core genes are conserved genes in the three strains (ACN, EAN, and CcI3) at a threshold level of 40% amino acid identity over 80% of the length of the smaller gene. + = present; – = absent.

^f Specific genes are core genes not conserved in *Acidothermus cellulolyticus* (NC_008578), *Streptomyces avermitilis* (NC_003155, NC_004719), and *S. coelicolor* (NC_003888, NC_003903, NC_003904) at a threshold level of 40% amino acid identity over 80% of the length of the smaller gene.

^g Synteny is the presence of a group of genes in the three strains (ACN, EAN, and CcI3) having 30% amino acid identity over 80% of the length of the smaller gene, separated by no more than five intervening sequences.

^h Core secretome encompasses 161 conserved secreted proteins in the three strains (ACN, EAN and CcI3) according to Mastrorunzio et al (2008).

genes were significantly up-regulated under both symbiotic and N-fixing FL conditions (Table 5). If GDH and GOGAT are absent in ACN vesicles as previously reported in CpII (Schultz and Benson 1990), the very strong repression of GSI and the slight induction of GSII could result in reduction of total ammonium assimilation in vesicle cells of ACN in nodules. As a result, we hypothesize that ammonium would be accumulated in vesicles and, consequently, might diffuse to host plant cells to be assimilated by the host GS (Guan et al. 1996) and to hyphae, in which more highly expressed GDH was observed. Thus, it can be suggested that the host plant tightly limits bacterial ammonium assimilation through inhibition of *glnII* activation in order to increase ammonium diffusion from the vesicles to host infected cells. To support our hypothesis, it is necessary to determine localization of those enzymes, because nodule RNA used for the microarray analysis was isolated from a mixture of hyphae and vesicles.

Transporters, secretion system, and export proteins. Transporters and molecules secreted by *Frankia* spp. could provide information about interactions between the symbiont and its host. In the ACN genome, there are 426 genes labeled as involved in transport, export, or efflux, 229 of which are ABC transporters. Surprisingly, only a dozen were differentially expressed, suggesting that nutrients and secondary metabolites exchanged between the two partners may be reduced to the bare essentials, in order to focus bacterial activity on nitrogen fixation. As energy sources for nitrogen fixation, *F. alni* obtains photosynthetic products presumably released through the plant transporter DCTA1, which was identified as a nodule-specific transcript and was shown to transport malate, succinate, fumarate, and oxaloacetate (Jeong et al. 2004). In rhizobia, the *dctA* gene product is involved in the uptake of the photosynthetic products (C₄-dicarboxylates). The corresponding *Frankia* ACN *dctA* gene (FRAAL1390) was not differentially expressed in symbiosis. However, as no *dctA* homolog is present in the CcI3 genome, we can assume that another transporter could take up photosynthetic products. Indeed, several poorly characterized ATP-binding ABC transporters (FRAAL2212, 2213, and 1843) were two- to threefold up-regulated but only in one data set. Thus, the *Frankia* carbon uptake transporter remains

elusive. It is also possible that the reference for the arrays, that is *F. alni* growing on propionate in basal medium with propionate (BAP), uses the same transporter, and thus, no upregulation could be obtained. Transporter genes exhibiting the remarkable expression change were those encoding putative sugar ABC transporter (FRAAL3762 to 3765), which were highly down-regulated in nodules (Table 4). Strong repression of sugar transporter clusters in nodules was also observed in *B. japonicum*, although its function remains unknown (Pessi et al. 2007).

Three genes encoding putative protein drug exporters of the RND (resistance, nodulation, cell division) superfamily (FRAAL3922, 4896, and 1424) were significantly up-regulated, especially the first two (FC = 20.6, 13.3, and 2.5, respectively). Finally, a gene (FRAAL0792) encoding a putative high-affinity branched-chain amino acid ATP-binding ABC transporter was 2.5-fold up-regulated, as were other poorly characterized transporters (FRAAL0725 and 1482).

It is possible that bacterial proteins are secreted to interact with the host, as happens in pathogens such as *Mycobacterium tuberculosis* (Raghavan et al. 2008) and in rhizobia (Kambara et al. 2009). Protein secretion in *F. alni* appears to be accomplished by the Tat and the Sec secretory systems (Mastrorunzio et al. 2008). Two *tat* genes were two- to threefold down-regulated (*tatA*, FRAAL2883; *tatB*, FRAAL6088), while one *sec* gene (*secG*, FRAAL4584) was threefold up-regulated. The potential secretome of the ACN strain comprised 279 proteins, of which 161 orthologous proteins form the core *Frankia* secretome (Mastrorunzio et al. 2008). Ten differentially expressed genes (FRAAL0094, 0725, 1097, 1427, 1432, 1482, 4553, 6657, 6658, and 6801) encode products belonging to the core *Frankia* secretome; their roles are discussed elsewhere.

Energy production and conversion. Given the high energetic need of the conversion of nitrogen to ammonia, upregulation of genes involved in energy production was expected. Indeed, genes encoding the eight enzymes of the tricarboxylic acid (TCA) cycle were up-regulated, with the highest scores (8.7- to 13.6-fold) for succinyl-CoA synthetase (FRAAL1156, 1157), succinate dehydrogenase (FRAAL1187 to 1190), and citrate synthase (FRAAL0111). Also, cluster FRAAL5930 to 5937 encoding ATP synthase subunits was highly induced (up

Table 5. Expression of genes involved in ammonium assimilation and transport

Gene no. ^a	Gene name ^a	Product description ^a	Fold change ^b		
			A ^c	B ^c	N–
With differential expression change					
FRAAL5161	<i>glnA</i>	Glutamine synthetase I	0.1	0.3	0.2
FRAAL5163	<i>glnII</i>	Glutamine synthetase II	1.9	2.1	15.1
FRAAL4964	<i>gltD</i>	Glutamate synthase small subunit	4.0	3.0	3.7
FRAAL4965	<i>gltB</i>	Glutamate synthase large subunit	4.5	2.7	2.9
FRAAL6693	<i>gdhA</i>	Glutamate dehydrogenase	5.4	3.7	1.7
FRAAL0904	<i>glnB1</i>	Regulatory protein PII for nitrogen assimilation	7.2	2.6	11.8
FRAAL0905	<i>amtB1</i>	Ammonium transport protein	6.7	2.4	9.8
Without differential expression change					
FRAAL0678	<i>glnA2</i>	Glutamine synthetase	1.0	1.1	0.9
FRAAL1887	<i>glnIII</i>	Glutamine synthetase III	1.1	1.1	1.1
FRAAL5165	<i>glnA1</i>	Glutamine synthetase 2	0.6	1.3	1.2
FRAAL6426		Putative glutamine synthetase	1.2	1.4	0.9
FRAAL5164	<i>glnE</i>	Glutamine-synthetase adenyllyltransferase	1.1	1.1	1.1
FRAAL1888		Putative glutamine amidotransferase	1.1	1.4	1.4
FRAAL1889	<i>glxC</i>	Glutamate synthase alpha subunit	1.1	1.0	1.0
FRAAL1890	<i>glxD</i>	Glutamate synthase large subunit	0.9	1.3	0.9
FRAAL0903	<i>glnD</i>	PII uridylyl-transferase	1.3	1.1	1.0
FRAAL0032	<i>glnB2</i>	Regulatory protein PII for nitrogen assimilation	1.4	1.3	1.4
FRAAL1969	<i>amtB2</i>	Ammonium transport protein	1.3	1.4	1.2

^a Gene number, gene name and product description according to the Genoscope database.

^b Fold change comparing nodules vs. N-replete free-living (FL) cells (A and B) and N-fixing FL cells vs. N-replete FL cells (N–). Bold characters indicate significant differential expression change.

^c Data sets A and B refer to similar experiments performed in our two laboratories.

to 8.8-fold) as well as genes encoding cytochrome c or bd oxidase subunits (FRAAL0598, 0599, 4147, 5122, 5123), in particular *ctaD* and *cydA* (up to 14.6-fold). Interestingly, *Rhizobium phaseoli* cytochrome c-deficient mutant induced empty nodules on *Phaseolus vulgaris*, supporting a role for c-type cytochrome in nodule infection (Soberon et al. 1993). In addition, cytochrome bd may play an important role in microaerobic nitrogen fixation since, in the enteric bacterium *Klebsiella pneumoniae*, it is expressed under all conditions that permit diazotrophy (Juty et al. 1997), and in *Azotobacter vinelandii*, disruption of the gene resulted in the loss of nitrogen-fixing ability (Kelly et al. 1990). The *ppa* gene (FRAAL6584) encoding inorganic pyrophosphatase was up-regulated. All these results fit with the high energy requirement of the nitrogen fixation process and explain that genes belonging to COG class C (energy production and conversion) are overrepresented and mostly upregulated in nodules (Fig. 2).

DNA metabolism, transcription, translation, and chaperones. Expression changes in fundamental cellular processes as well as in specific transcriptional regulators could give insight into *Frankia* physiology inside nodules. We found only a few genes involved in DNA metabolism (replication, recombination, and repair) showing differential expression change, among them a highly upregulated (FC = 18.2) gene encoding a putative integration host factor-like protein (FRAAL5232) and two upregulated genes involved in repair of DNA damaged by oxidation (*mutM*, FRAAL5802 and putative *mutT*, FRAAL2463). In contrast, another putative *mutT* gene (FRAAL4301) was down-regulated. The role of integration host factors in stimulating transcription from the σ^{54} -dependant *nifH* promoter in a variety of bacteria has been reported (Hervas et al. 2009; Santero et al. 1992; Wasseem et al. 2000). More genes involved in transcription and its related functions were induced, beta and sigma subunits of RNA polymerase, transcription terminator Rho, ribonuclease III, antitermination protein NusG, and DEAD-box RNA helicase. Seven genes encoding transcriptional regulators showed increased expression in symbiosis, and all were conserved or syntenic in the three *Frankia* strains (Table 6). They contained two LuxR- and a MarR-family members. LuxR-type helix-turn-helix (HTH) regulators control a wide variety of activities in various biological processes,

among them is the Rhizobiaceae FixJ regulator that induces expression of nitrogen-fixation genes under microaerobic conditions (Birck et al. 2002). Regulators with the MarR-type HTH domain control stress-related biological functions, such as resistance to oxidative stress and regulation of the virulence factor synthesis in pathogens of humans and plants, and respond to aromatic compounds (Aleksun and Levy 1999; Eglund and Harwood 1999). In addition, a putative *fur* (ferric uptake regulator) gene (FRAAL5117) was up-regulated. The regulation is as expected, since iron is a key element for the activity of nitrogenase and many other related iron-sulfur enzymes (uptake hydrogenase, ferredoxins, IspG, etc.). Six genes encoding transcriptional regulators were down-regulated. The most downregulated one (FRAAL6548) coded for a putative transcriptional regulator of the WhiB family, conserved in EAN and CcI3 (Tables 4 and 6). WhiB is a transcription factor of actinobacteria required for differentiation and sporulation. The process of aerial mycelia formation in *Streptomyces aureofaciens*, which occurs in response to nutrient limitation, is controlled by a number of *whi* genes (Kormanec and Homerova 1993). The mycobacterium *whmD* gene, which encodes a homolog of WhiB, is required for proper septation and division (Gomez and Bishai 2000). The major cellular rearrangements members of genus *Frankia* spp. undergo as they switch from a FL lifestyle to a symbiotic intercellular one presumably entails such regulators; thus, we expected to see important up- or downregulation in differentiation-related regulators.

COG class J (translation, ribosomal structure, and biogenesis) was the second class overrepresented by upregulated genes (Fig. 2), including two clusters encoding ribosomal proteins (FRAAL1064 to 1115 and 5779 to 5812), numerous genes involved in translation machinery and four genes involved in the synthesis of aminoacyl tRNAs (*gatABC*, FRAAL5853 to 5856 and *pth*, FRAAL6266), whereas *cysS2* (FRAAL2858) was repressed. A *groL* gene (FRAAL6486) encoding the chaperone Hsp60 was strongly induced in nodule (FC = 40.3), suggesting a predominant role of this chaperone under these conditions (Table 3). In contrast, clusters of chaperone-encoding genes *dnaK-grpE-dnaJ-hsrP-clpB* (FRAAL6639 to 6643) and *clpB-dnaJ-grpE-dnaK* (FRAAL2601 to 2605) were not differentially expressed and *hsp18* (FRAAL4031) was highly down-

Table 6. Transcriptional regulator genes differentially expressed in nodules in the two data sets

Gene no. ^a	Product description ^a	Fold change ^b		Core ^c	Spe. ^d	Syn. ^e
		A	B			
Upregulated genes						
FRAAL4858	Putative LuxR-family transcriptional regulator	13.7	8.3	+	–	–
FRAAL2921	Hypothetical protein (repressor-like DNA-binding domain)	10.7	12.0	–	–	+
FRAAL0711	Putative MarR-family transcriptional regulator	8.9	3.0	+	–	+
FRAAL1544	Putative LuxR-family transcriptional regulator	8.7	5.5	+	+	+
FRAAL1491	Putative helix-turn-helix (HTH)-type transcriptional regulator	5.1	4.6	+	+	+
FRAAL5117	Putative Fur-family transcriptional regulator	3.3	3.1	+	–	+
FRAAL5617	Putative AbaA-like regulator protein	3.0	3.9	+	–	+
Downregulated genes						
FRAAL1768	Putative CrP/Fnr-family transcriptional regulator	0.4	0.3	–	–	–
FRAAL5839	Putative CrP/Fnr-family transcriptional regulator	0.3	0.3	+	+	+
FRAAL6525	Putative CarD-like transcriptional regulator	0.2	0.3	+	–	+
FRAAL0198	Putative transcriptional regulator	0.2	0.1	–	–	–
FRAAL0140	Putative HTH-type transcriptional regulator	0.1	0.1	–	–	–
FRAAL6548	Putative WhiB-family transcriptional regulator	0.1	0.1	+	–	+

^a Gene number and product description according to the Genoscope database.

^b Fold change comparing nodules vs. N-replete free-living cells. Data sets A and B refer to similar experiments performed in our two laboratories.

^c Core genes are conserved genes in the three strains (ACN, EAN, and CcI3) at a threshold level of 40% amino acid identity over 80% of the length of the smaller gene. + = present; – = absent.

^d Specific genes are core genes not conserved in *Acidothermus cellulolyticus* (NC_008578), *Streptomyces avermitilis* (NC_003155, NC_004719), and *S. coelicolor* (NC_003888, NC_003903, NC_003904) at a threshold level of 40% amino acid identity over 80% of the length of the smaller gene.

^e Synteny is the presence of a group of genes in the three strains (ACN, EAN, and CcI3) having 30% amino acid identity over 80% of the length of the smaller gene and separated by no more than five intervening sequences.

regulated (Table 4). The high number of induced genes in COG class J could be necessary for the biosynthesis of nitrogen-fixation machinery such as vesicles.

Stress defense and detoxification. Maybe due to the adaptation to a protected biotope, symbiosis does not seem to be a stressful condition, except for oxidative stress, since several heat-shock proteins genes involved in adaptation to stress (FRAAL0094, 1053, and 4243) were highly down-regulated, whereas genes involved in detoxification of reactive oxygen species such as the *bcp* gene (FRAAL5045) encoding a peroxiredoxin that detoxifies H₂O₂ and alkyl hydroperoxides (Barr and Gedamu 2001) (FC = 4.2) and *sodF* (FRAAL4337) encoding superoxide dismutase 1 (FC = 2.9) were up-regulated in nodules. Interestingly, peroxiredoxin and SodF were reported to be induced upon addition of root exudates (Hammad et al. 2001). In contrast, the *kata* gene (FRAAL3167) encoding catalase A, which catalyzes the conversion of H₂O₂ to molecular oxygen was down-regulated in data set B. However, we noticed that *kata* as well as *sodF* showed very high microarray signals (among the 100 highest) under symbiotic conditions as well as under FL conditions. Barnett and associates (2004) reported that the probe sets with highest average signal values (within the top 100) correlated well with computer-based predictions of highly expressed genes in *Sinorhizobium meliloti*. Therefore, the *kata* gene would be highly expressed and play a role in nodule even though it is down-regulated. In addition, previous reported studies further sustain this idea, since they showed that ACN catalase has a very high constitutive activity under nitrogen-replete FL conditions (Santos et al. 2007). Incidentally, we observed that the second *kat* gene, *katB* (FRAAL3889), which was not significantly down-regulated, showed low microarray signals in FL condition and in symbiosis, i.e., >30-fold smaller than the *kata* gene, suggesting a minor contribution of KatB to catalase activity, at least under these conditions. We also noticed that the gene neighboring *sod*, the *bfrA* gene (FRAAL4338) encoding an iron-storage protein bacterioferritin, was up-regulated (FC = 3.5) and, like *sodF* and *kata*, among the 100 highest microarray signals under FL and symbiotic conditions, suggesting a need for coordinated expression.

Lipopolysaccharides and cell-wall peptidoglycans. Surface polysaccharides such as LPS play important roles in promoting the rhizobial infection of root nodules. In particular, *Sinorhizobium meliloti* mutants of the *lpsB* gene, which encodes a protein of the glycosyl transferase 1 family participating in the biosynthesis of the LPS core, failed to effectively colonize *Medicago* nodules (Campbell et al. 2002). Moreover the *lpsB* gene was induced in *S. meliloti* bacteroids (Becker et al. 2004). In the ACN genome, several dozens of putative genes related to biosynthesis of surface polysaccharide are found. Interestingly, among them, a gene encoding a glycosyl transferase related to the GT1 family (FRAAL5287) was strongly up-regulated. However, it was a stark contrast to the rhizobial transcriptome, in which 17 genes related to polysaccharide synthesis showed significant expression changes in nodule (Becker et al. 2004).

Most genes coding for peptidoglycan synthesis were not differentially expressed, except for four genes, *murI* (FRAAL1505) encoding glutamate racemase (FC = 5.9), *rfe* (FRAAL5941) encoding undecaprenyl *N*-acetylglucosaminyl transferase (FC = 3), and two neighboring genes (FRAAL6657 and 6658) encoding *N*-acetylmuramoyl-L-alanine amidase (autolysin) (FC = 10.5; Table 3) and a hypothetical protein with a lysozyme-like domain (FC = 3.9) coherent with either cell-wall remodeling in relation to vesicle synthesis or possibly with senescing cells. These two last genes belong to the core *Frankia* secretome (Mastrorunzio et al. 2008). It is interesting that two lytic transglycosylases were highly induced, which raises the question of their contribution to effective invasion of

F. alni to plant cells as reported in an animal pathogen (Cabanes et al. 2004).

Nod factors and signaling processes. The *Rhizobium nod* genes encode the enzymes that synthesize lipo-chito-oligosaccharides, called Nod factors, which are the signal molecules initiating symbiosis. In genomes studied so far, the genes homologous to *nodB*, *nodC*, *nodD*, and *nodQ* are present but, as in many organisms, at low levels of sequence identity, and they are not clustered or organized in operons (Normand et al. 2007a). Our results showed that the expression of neither of these genes was changed between FL conditions and symbiosis, so the question of their putative role in nodulation remains open.

On the other hand, two genes involved in signaling processes were up-regulated, a gene (FRAAL1155) encoding a putative serine and threonine kinase (FC = 12) and *rshA* gene (FRAAL5991) encoding an anti-sigma factor (FC = 4.8). In contrast, a gene (FRAAL5664) encoding a putative tyrosine-specific protein phosphatase was down-regulated (FC = 0.1). This gene labeled as specific is highly conserved in the three *Frankia* strains. Tyrosine-specific protein phosphatases are key regulatory components in signal transduction pathways and are important in the control of cell cycle, cell growth, proliferation, differentiation, and transformation (Denu and Dixon 1998).

Secondary metabolism. In the genome, most genes categorized in COG class Q (secondary metabolites biosynthesis, transport, and catabolism), such as those encoding nonribosomal peptide synthetases and polyketide synthases, did not show differential expression change. Those differentially expressed were mostly down-regulated, which again suggests that ex-changes of metabolites with host plant are reduced to the bare essentials.

Hypothetical proteins. A total of 49% of the genes in the ACN genome encode hypothetical proteins. Interestingly, when located in a gene cluster with known function, these genes often displayed similar regulation with surrounding known genes, suggesting a similar role for them. For instance, FRAAL6806 and 6816 in the *nif* cluster may have a role in nitrogen fixation (Fig.3A). Hypothetical genes showing highly differential expression changes are worthy of attention, in particular, those belonging to the core gene subset such as FRAAL3254 (FC = 38.8) (Table 3) and FRAAL1014 (FC = 0.043) (Table 4).

Comparison of *Frankia* transcriptomes between *Alnus* nodules and N-fixing FL cells.

In the absence of an organic source of nitrogen, FL *Frankia* cells can fix nitrogen, whereas most rhizobia cannot. However, vesicle formation of *F. alni* in N-starved FL conditions differs from that in nodules. Briefly, in nodules, vesicles are more numerous and have a markedly different shape, being much larger, less spherical, and more septated than in N-fixing FL conditions (Benson and Silvester 1993). Nevertheless, it was decided to compare transcriptomes between N-fixing FL cells and symbiotic cells, in order to try to clarify which genes are associated with nitrogen fixation and which are associated with nodule symbiotic interactions. Indeed, 96 (33%) of the 292 genes differentially expressed in symbiosis vs. N-replete FL cells showed significant expression change in N-fixing FL vs. N-replete FL cells (Supplementary Table 5). Remarkably, all of these genes displayed similar up- or downregulation in the two conditions, however, often not in the same proportion. In particular, *nif* genes (FRAAL6811 to 6814) showed much lower FC (about 13-fold) in FL N-fixing cells, and furthermore, all other genes of the *nif* cluster did not display significant differential expression. These results reveal much lower N-fixation under N-fixing FL conditions than in symbiosis, which correlates with morphologi-

cal differences of vesicles described above. It is quite likely that in N-fixing FL conditions, gene expression of vesicles was hidden behind that of hyphae, which are much more predominant. One consequence of this observation is that we cannot conclude that all the genes showing differential expression only in nodules are related to nodulation. On the other hand, most of the 96 genes that were differentially expressed under both symbiotic and N-fixing FL conditions could be associated with nitrogen fixation. In addition to *nif* genes, we found upregulated genes likely involved in nitrogen fixation-related functions or associated to vesicle formation, such as *fdxI* (ferredoxin), *sufCE* (Fe-S clusters), *ispG* (isopentenyl diphosphate synthesis), as well as many genes classified into COG class C, reflecting the high energy requirements for nitrogen fixation (ATP synthase, TCA cycle, cytochrome c), and genes classified into COG classes K (RNA polymerases, LuxR, and AbaA-like transcriptional regulators) and J (ribosomal proteins, glutamyl-tRNA, chaperone GroL), which, as suggested for symbiosis results, could be related to vesicle biosynthesis. Thus, these comparative results allowed us to clearly assign nitrogen fixation-related function to some genes and highlighted the difficulties and limits of such transcriptome analyses with heterogeneous multicellular organisms.

Comparison of *Frankia* transcriptomics data from two *Alnus* and two *Myricaceae* species.

Unlike rhizobia, which nodulate only *Fabaceae* plants, *Frankia* spp. establish symbiosis with a wide variety of plants belonging to different families, and *F. alni*, in particular, can nodulate *Alnus* and *Myrica* species. Therefore, it is of great interest to know how host plants belonging to different families affect *Frankia* transcriptome in nodules. We harvested ACN14a-infected nodules formed on *Alnus nepalensis*, *Myrica gale*, and *Morella rubra* and used them for microarray analyses. Supplementary Figure 1 shows scatter plots of the fold expression changes (nodule vs. N-replete FL cells) for all pairwise comparisons of the trees. Most of the data plotted close to a diagonal line, and correlation coefficients between two trees were high (>0.9). There were 224 genes showing significant expression change in all the four trees (Table 1), and all of them, except for FRAAL0302, displayed similar up- or down-regulation (Supplementary Table 6). FRAAL0302, as mentioned above, displayed opposite regulation in the two data sets of *A. glutinosa*. Those results collectively indicate that nodule transcriptomes of *F. alni* are similar among phylogenetically distant plant families.

When genes without significant expression change were taken into account, we found several genes showing different expression patterns between the two plant families (Supplementary Table 7). Most of them showed similar up- or down-regulation, but fold change levels were different. Notably, genes encoding subunits of NADH dehydrogenase showed more obvious downregulation in *Myricaceae* nodules. This regulation may reflect differences in accessibility to oxygen,

probably caused by nodule architecture (Huss-Danell 1997). In addition, a *shc* gene (*shc2*, FRAAL2491) that did not show significant expression change in *Alnus* nodules was prominently up-regulated in *Myricaceae* nodules, suggesting a possible relationship with difference in vesicle structure between the two plant families (Huss-Danell 1997). Interestingly, the *shc2* gene is followed by a gene encoding a predicted redox sensor that could be implicated in regulating oxygen response in nodules (Alloisio et al. 2005).

Comparison of *Frankia* transcriptome with rhizobia.

We compared the nodule transcriptome of *F. alni* species with that of rhizobia. Although several microarray analyses were reported in rhizobia, we selected transcriptomic data from Capela and associates (2006) on *Sinorhizobium meliloti*, because their experimental conditions are similar to those used in the present study; they used FL cells at mid-log phase cultured in a defined minimal medium containing an organic source of nitrogen and symbiotic cells from young (8 days postinoculation) nitrogen-fixing nodules.

Frankia ACN and *S. meliloti* shared 1,179 orthologs (Supplementary Table 8). About 30% (345 genes) of them showed significant expression change in either ACN or *S. meliloti* between nodules and FL conditions (Table 7). In ACN, more genes were up-regulated in nodules (upregulated/downregulated genes [72:30] = 2.4:1), while more genes were down-regulated in *S. meliloti* nodule (upregulated/downregulated genes [72:223] = 1:3.1), indicating that transcriptomes in nodules were largely different between the two symbiotic bacteria. As a consequence, only 16 genes (4.6% of 345) showed consistent regulation (up- or downregulated in both bacteria). Dominant genes upregulated in both bacteria were *nif* as expected. Most of the upregulated genes in ACN were down-regulated or did not show significant expression change in *S. meliloti* (61 of 72 genes, 85%) (Table 7). Many of those genes are classified into COG classes C and J. They are related to 'energy production and conversion' (TCA cycle, respiration chain, ATP synthase) and 'translation, ribosomal structure and biogenesis' (ribosomal proteins, translation factors). Downregulation of such genes in nodules was also found in other rhizobial experiments (Barnett et al. 2004; Becker et al. 2004; Pessi et al. 2007). These results suggest that in nodules, *F. alni*, is metabolically more active than rhizobia, which is commensurate with the fact that *F. alni* is more autonomous than rhizobia; for instance, it must synthesize vesicles to deal with the lack of protection provided by the host.

Validation of microarray experiments.

To confirm the results obtained from microarray analysis, we performed quantitative reverse transcription (qRT)-PCR (Table 8). Fifteen genes showing various levels of expression change, belonging to different pathways, and spreading over the various positions of the genome were chosen. Comparison was made with data obtained from the same samples of RNA using the two distinct techniques. We found excellent agreement between the microarray and qRT-PCR data in both data sets, indicating the reliability of our microarray data, except only for a remarkable discordance in *hupL2* (FRAAL1829) in data set B, in which qRT-PCR detected a 207-fold upregulation, whereas the microarray detected only a threefold increase. This result was reproduced using a different pair of primers and RNA from other biological replicates (data not shown). This is probably due to the higher sensitivity of qRT-PCR for differential expression.

Conclusion.

In the present study, we conducted the transcriptional profiling of *F. alni* under N-replete FL and symbiotic conditions.

Table 7. Expression profiles of orthologous genes in *Frankia alni* ACN14a and *Sinorhizobium meliloti*

Strain	Expression	<i>Sinorhizobium meliloti</i>			Total
		Up ^a	Down ^b	N.S. ^c	
ACN	Up ^a	11	33	28	72
	Down ^b	3	5	22	30
	N.S. ^c	58	185	—	243
	Total	72	223	50	345

^a Number of genes upregulated in nodules.

^b Number of genes downregulated in nodules.

^c Number of genes that did not show significant expression change.

The data obtained showed that *F. alni* do not contain a symbiotic island consisting of numerous nodule-induced genes, as was found in some rhizobia. However they indicated that nodule-induced genes are distributed mostly over several regions with high synteny. In addition to the expected high upregulation of the genes related with nitrogen fixation (*nif*, *hup2*, *suf*, *ispG*, *shc1*, etc.), our genome-wide transcriptional analysis revealed some unexpected features concerning genes such as those involved in ammonium assimilation (*glnII*) or C₄-dicarboxylate uptake (*dctA*). A large number of induced genes involved, in particular, in transcriptional regulation, signaling processes, protein drug export, protein secretion, lipopolysaccharide, and peptidoglycan biosynthesis that may play a role in nodulation were also identified. Comparison of nodule and N-fixing FL *F. alni* transcriptomes allowed us to assign differential expression of some genes to nitrogen fixation and related functions. This *Frankia* nodule transcriptome of was highly similar among phylogenetically distant plant families. In contrast, the comparison of *Frankia* transcriptome with rhizobia indicates that the two bacterial transcriptomes were largely different in nodules and suggests that *F. alni* is metabolically more active, which could be related to vesicle biosynthesis. Globally, this transcriptomic pattern suggests that *F. alni* is a nitrogen-fixing machine in symbiosis.

Our *Frankia* microarray provides a useful tool to investigate gene expression pattern in various biological conditions, such as the early stages of infection, which are of particular interest in the absence of known signal molecules initiating symbiosis. However microarray analysis does not take into account the absolute transcript levels, so genes showing constitutive high expression, which must obviously play a role, are underestimated. Therefore other approaches such as proteomic or large scale cDNA sequencing could complete this first global gene expression dissection of *F. alni* in symbiosis.

MATERIALS AND METHODS

Bacterial strain and growth conditions.

In order to obtain rapid exponential growth, *F. alni* ACN14a (Normand and Lalonde 1982) was grown according to Schwencke (1991) at 28°C with stirring at 200 rpm in defined (BAP) containing 5 mM ammonium chloride (N-replete condition) or not (N-fixing condition) (Murry et al. 1984) with some minor modifications. Fe-citrate (10 mg/liter) was used instead

of FeNa-EDTA, CoSO₄·7H₂O (0.001 g/liter) was added in the micronutrient stock solution, and biotin was replaced by the vitamin mixture described by Tjepkema and associates (1981). Before starting cultures, *F. alni* cells were centrifuged and were washed twice with corresponding BAP media in order to discard ammonium chloride in N-fixing cultures. After 5 days of culture, stirring was stopped. Supernatants were carefully discarded, and cells were immediately stored at –80°C in the A experiments or treated with RNeasy protect bacteria reagent (Qiagen, Courtaboeuf, France) for stabilization of RNA before freezing at –80°C in the B experiments. Three biological replicates were performed.

Plants, growth conditions, and nodule isolation.

Seeds of *A. glutinosa*, *A. nepalensis*, *Myrica gale*, and *Morrellia rubra* were planted and grown for five to ten weeks in a soil/vermiculite substrate (1:1, vol/vol) in a glasshouse under fluorescent lighting with a 16-h light and 8-h nycthemeral regime at temperature 21 to 25°C. Seedlings were transferred to Broughton and Dilworth's solution (1971) in plastic pots. They were grown for four to nine weeks with 0.5 g of KNO₃ per liter and, then, for one week without KNO₃. *F. alni* ACN14a cells derived from 75 ml of log-phase culture were inoculated to a pot. Root nodules (≤3 lobes) were harvested into liquid nitrogen at 3 to 4 weeks postinoculation and were stored at –80°C. Nodules from about 24 plants per replicate were pooled for RNA extraction, and three biological replicates were performed.

RNA purification.

RNA from FL ACN and *A. glutinosa* nodules in A experiments and from nodules of *A. nepalensis*, *M. gale*, and *M. rubra* was purified following a procedure described by Kucho and associates (2009). In B experiments, RNA was purified from bacterial cells and nodules using RNeasy plant mini kit (Qiagen) and on-column DNA digestion with the RNase-free DNase set (Qiagen). In order to avoid any remaining DNA contaminations, a second DNase treatment was performed with RQ1 RNase-free DNase (Promega, Charbonnières-les-Bains, France), followed by RNA cleanup using RNeasy mini kit. Purity, concentration, and quality of RNA samples were checked using a NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific, Courtaboeuf, France) or Ultrospec 3300 pro photospectrometer (Amersham Biosciences, Buckinghamshire, U.K.) and agarose gel electrophoresis.

Table 8. Validation of microarray results by quantitative reverse transcription-polymerase chain reaction (qPCR)

Gene no. ^a	Gene Name	Product description	Fold change ^b			
			A		B	
			Array	qPCR ^c	Array	qPCR ^c
FRAAL0111	<i>glbA</i>	Citrate synthase	10.6	9.1	9.6	13.9
FRAAL0905	<i>amtB1</i>	Ammonium transport protein	6.7	6.4	4.7	17.3
FRAAL1239		Putative polysaccharide ABC transport subunit	1.5	0.8	3.2	2.8
FRAAL1248		Putative glycosyl transferase	1.2	1.3	3.9	5.1
FRAAL1829	<i>hupL2</i>	[NiFe] uptake hydrogenase large subunit	22.2	19.0	2.8	206.8
FRAAL2088	<i>dxs</i>	1-Deoxy-D-xylulose-5-phosphate synthase	7.5	14.1	5.2	6.7
FRAAL3103	<i>paaA</i>	Putative phenylacetic acid degradation protein	0.2	0.3	0.3	0.3
FRAAL3167	<i>katA</i>	Catalase	0.5	1.1	0.3	0.5
FRAAL3763		Putative sugar ABC transporter	0.19	0.08	0.02	0.04
FRAAL4147	<i>ctaD</i>	Cytochrome c oxidase polypeptide I	12.7	18.3	10.5	8.5
FRAAL4901	<i>pheA</i>	Prephenate dehydratase	1.8	0.3	1.6	3.2
FRAAL5116	<i>rr</i>	Rubrythrins	1.9	0.8	0.7	3.1
FRAAL5161	<i>glnA</i>	Glutamine synthetase I	0.05	0.04	0.2	0.5
FRAAL5772	<i>ispG</i>	1-Hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase	4.2	2.5	13.6	4.3
FRAAL6813	<i>nifH</i>	Nitrogenase reductase	396.4	1,023.3	111.1	7,003.7

^a Gene number, gene name, and product description according to the Genoscope database.

^b Ratio of nodules to N-replete free-living cells. Comparisons were made using a biological replicate of each condition.

^c qPCR = quantitative polymerase-chain reaction. Expression was normalized using the housekeeping *infC* gene (FRAAL5216).

Design of the *F. alni* whole-genome microarray.

Based on the sequence and annotation data for *F. alni* ACN14a genome publicly available (Normand et al. 2007b), a whole-genome high-density microarray was designed and manufactured by Roche NimbleGen, Inc. (Madison, WI, U.S.A.). Due to high GC content (72.8%) of the *F. alni* genome, a 60-mer oligo length was chosen for probe design. NimbleGen design rules and analysis algorithms found probes for 6,607 genes out of 6,786. In the microarray used for experiments B, 18 probes per gene were obtained for 6,340 genes and <18 probes per gene were obtained for 267 genes. In the microarrays used for experiments A and those for *A. nepalensis*, *M. gale*, and *M. rubra*, four-plex format was applied, in which 11 probes per gene were retained for 6,440 genes and <11 probes per gene were retained for 167 genes. Probes were duplicated on the array.

Microarray experiment and data analysis.

Quality analysis of RNA, synthesis, and labeling of double-stranded cDNA, hybridization, scanning, and data extraction were performed by the NimbleGen expression service laboratory (Tokyo and Reykjavik, Iceland) according to its standard protocol, except that three times more nodule cDNA than FL bacteria was used for hybridization in order to equalize amounts of bacterial cDNA in each condition, since the proportion of bacterial/plant RNA in nodules was roughly 1:3. Normalization of the signal intensity was performed using a quantile normalization method (Bolstad et al. 2003). Normalized gene expression values were generated using the robust multiplex average algorithm (Irizarry et al. 2003). We used GeneSpring gene expression analysis software 7.3 (Agilent Technologies, Massy, France) for statistical comparisons and filtering of the data. Genes showing significant differential expression between two biological conditions were selected based on the following three criteria: i) a *P* value ≤ 0.05 , using the Welch *t*-test with the multiple testing correction of Benjamini and Hochberg (1995), ii) a fold change ≥ 1.8 in all nine pairwise comparisons yielded by the three biological replicates of each conditions (3×3), and iii) an average fold change ≥ 2 . It is noteworthy that with such stringent criteria, putative differentially expressed genes with moderate fold change or standard deviation were lost.

qRT-PCR analysis.

qRT-PCR was performed using two procedures. In experiments A, reverse transcription was performed at 50°C for 15 min with a PrimeScript RT reagent kit (Perfect Real Time) (Takara Bio, Ohtsu, Japan) and real-time PCR was performed using 20 μ l of reaction mixture with SYBR Premix Ex Taq II (Perfect Real Time) (Takara Bio), using a 7300 real-time PCR system (Applied Biosystems, Foster City, CA, U.S.A.). The PCR conditions were as follows: 95°C for 10 s; 40 cycles of 95°C for 5 s, 60°C for 31 s. In experiments B, we performed reverse transcription at 55°C for 30 min with Transcriptor reverse transcriptase and random hexamers as primers (Roche, Mannheim, Germany) and real-time PCR using 20 μ l of reaction mixture with LightCycler 480 SYBR Green I Master (Roche), amplified on a LightCycler 480 (Roche). The PCR conditions were as follows: 95°C for 5 min; 45 cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 15 s. cDNA derived from 50 or 100 ng RNA was used for a PCR reaction in experiments B and A, respectively. Genes and primers used are listed in Supplementary Table 9. Expression values were normalized to the expression of *infC* (FRAAL5216), encoding protein chain initiation factor 3, whose expression showed little variability between nodule and FL conditions on arrays.

Comparison of *Frankia* transcriptome with rhizobium.

We obtained a list of *Sinorhizobium meliloti* genes showing the bidirectional best hit with each gene of *Frankia* ACN14a from the Kyoto Encyclopedia of Genes and Genomes sequence similarity database. We determined a *S. meliloti* gene to be an ortholog if it showed amino-acid sequence similarity with an ACN14a counterpart over 70% or more of its length. Microarray data of *S. meliloti* were obtained from supplementary data published by Capela and associates (2006). We determined an expression change was statistically significant if $\log_2$ FC (M) was >1 or <-1 and the *t*-test *P* value was <0.01 .

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Kyoto Encyclopedia of Genes and Genomes (KEGG) sequence similarity database (SSDB): www.genome.jp/kegg/ssdb
- Magnifying genomes microbial genome annotation system (MAGE): www.genoscope.cns.fr/age/mage
- NimbleGen Arrays User's Guide: Gene Expression Analysis (Version 3.2): www.nimblegen.com/products/lit/exp_userguide_v3p2.pdf