The Determinants of the Actinorhizal Symbiosis

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The actinorhizal symbiosis is a major contributor to the global nitrogen budget, playing a dominant role in ecological successions following disturbances. The mechanisms involved are still poorly known but there emerges the vision that on the plant side, the kinases that transmit the symbiotic signal are conserved with those involved in the transmission of the Rhizobium Nod signal in legumes. However, on the microbial side, complementation with Frankia DNA of Rhizobium nod mutants failed to permit identification of symbiotic genes. Furthermore, analysis of three Frankia genomes failed to permit identification of canonical nod genes and revealed symbiosis-associated genes such as nif, hup, suf and she to be spread around the genomes. The present review explores some recently published approaches aimed at identifying bacterial symbiotic determinants.

Key words: actinorhizal plant, Frankia, nitrogen fixation, root nodule, symbiosis

Introduction

Actinorhizal plants are key pioneer species that colonize nitrogen-depleted biotopes such as glacial moraines, burned forests, landslides, or volcanic lava flows (9), mostly in temperate and polar latitudes. The best studied situation is that of Glacier Bay, Alaska where glacier retreat has been monitored for decades, and the growth of green alders (Alnus crispa) and yellow avens (Dryas drummondii) shown to precede that of more nitrogen-demanding species such as willows and poplars and eventually of climatic species such as fir (53, 54). Another emblematic event is that which occurred on the Krakatoa volcanic island in Indonesia that was sterilized in the catastrophic eruption of 1883, was monitored by naturalists who noted less than 20 years afterwards the presence, among others, of filaos (Casuarina equisetifolia) (15). A similar situation is found in Japan, on the Sakurajima volcano slopes where major eruptions, the most recent ones having occurred in 1914 and 1946, have been covered by lava flows that contain mainly Si, Al, and Fe oxides with little nitrogen are colonized (Fig. 1) among others by Japanese green alder (Alnus firma) (105).

In parallel, actinorhizal plants have gained use as man-made improvements of anthropically degraded sites such as mine spoils, dam dykes, clear-cut forests or over-exploited agricultural lands. In Canada, the giant Hydro-Quebec dams comprise dykes that have been stabilized and revegetated through the plantation of thousands of green alders (Alnus crispa) (www.hydroquebec.com/eastmain1/en/proteger/deboisement.html), a similar approach is being implemented for treating Alberta’s tar sands residues (90) (www.ptac.org/env/dl/envf0701p04.pdf). The largest present-day project is the initiative of the UNEP “Plant for the planet: the billion tree campaign” that aims to establish a belt of several species, among which filaos, across the Sahel region in Africa, in particular in Senegal (http://www.unep.org/billiontreecampaign/CampaignNews/sahel.asp).

Actinorhizal plants can invade nitrogen-depleted sites because of their root nodules, modified secondary roots that are colonized by nitrogen-fixing soil actinobacteria collectively called Frankia spp. These bacteria, through a molecular dialog, penetrate the root tissue, colonize the cortical cells and fix nitrogen using plant photosynthates to fuel the energy-demanding nitrogenase. The symbiosis is used empirically but the bacterial determinants permitting it are so far unknown, a prerequisite for eventual extension to non-symbiotic plant species. The purpose of the present review is to recapitulate

Fig. 1. Vegetation of Sakurajima in 1962 as described by Tagawah (105). Are indicated in shades of grey the three latest lava flows (1779, 1914 and 1946), by capital letters the three summits (Kita-dake at 1118 m (K), Naka-dake at 1060 m (N), and the still active Minami-dake at 1070 m (M)) and by stars the Alnus firma-dominated circum-summit community.

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what has been done to identify the bacterial symbiotic determinants and describe what is known in the case of the Rhizobium-legume symbiosis.

**Frankia phylogeny**

The closest phylogenetic neighbours on the basis of the 16S rRNA gene are the thermophilic Acidothermus cellulolyticus (60, 75), the radiation tolerant Geodermatophilaceae, the compost dweller Sporichthyaecae, and finally the soil microbes Cryptosporangiaceae and Nakamurellaceae, forming the suborder Frankineae (76) (Fig. 2). The Frankineae thus represent one of the most contrasted groups of bacterial taxa, having differentiated and specialized to colonize markedly contrasted biotopes such as plant root tissues, hydrothermal springs, gamma-irradiated substrates, calcareous stones, soils and composts.

A recent pangenomic phylogeny, done in the framework of the GEBA project (Genome Encyclopedia of Bacteria and Archaea) using 31 proteins shows a somewhat different topology (119) with the members of the Frankineae spread out among the actinobacterial line of descent, not forming a clade contrary to what is seen with the 16S rRNA marker. Such multigene approaches may eventually modify our vision on the phylogeny of Frankineae.

**Frankia** in pure culture produces vegetative hyphae, reproductive multicellular sporangia as well as nitrogen-fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3). Vesicles contain a hopanoid derivative, the phenyl-acetate ester of hopanetetrol (11) that is fixing vesicles (Fig. 3).

**Determinants of Rhizobium symbiosis**

Rhizobium is another group of bacteria that establishes nitrogen-fixing root nodule symbiosis with plants. Rhizobia include diverse bacterial species classified into 13 genera such as Rhizobium (Allorhizobium), Azorhizobium, Bradyrhizobium, Burkholderia, Cupriavidus (Ralstonia), Devosia, Herbaspirillum, Mesorhizobium, Methylobacterium, Octobactrum, Phyllobacterium, Shinella and Sinorhizobium (Ensifer) (42, 68, 104). Free-living nitrogen-fixing ability is limited to several exceptional bacteria. Lineage of those rhizobial genera is intertwined with that of non-symbiotic bacteria such as pathogens. All rhizobia are Gram-negative Proteobacteria, which are phylogenetically distant from the Gram-positive actinobacterium Frankia. Host plants of rhizobia are limited to family Leguminosae with only one exception, Parasponia, that belongs to the Ulmaceae.

In most cases, rhizobia enter the host plant cells through

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**Fig. 2.** Phylogenetic tree of the Frankineae based on a 16S rRNA gene analysis. Done with recognized species according to the approach given in Normand and Benson (75).
root hair (42). When rhizobia establish contact with root hair, they elicit root hair deformation that entraps microbial cells. Then, a tubular structure called infection thread is developed through the root hair to guide bacteria into plant cells. In parallel, plant cortical cells begin to divide to form nodule primordia that then develop into mature nodules containing nitrogen-fixing bacteroids. Legume nodules have stem-like structures peripherally surrounded with vascular bundles, which is different from actinorhizal nodules with lateral root-like structure (Fig. 5).

**Nod factor.** In general, host specificity of rhizobial symbiosis is more stringent than that of *Frankia*. Collectively, they establish symbiosis with the plants belonging to only a single plant family *Leguminosae* (26). In extreme cases, a rhizobial strain distinguishes difference in cultivar of the host plants although such processes are often controlled by Nod factor-independent mechanisms (106). Host specificity of rhizobia shows less correlation with bacterial phylogeny based on 16S rRNA. Molecular bases of the host specificity determination were well characterized in rhizobia with genetic approaches. Rhizobial genes involved in nodule formation are denoted as *nod* genes that are clustered in a particular region of genome, symbiosis island (47) or symbiosis plasmid (36). Some *nod* genes are conserved in all rhizobia (common *nod* gene) but others are not (specific *nod* genes) (26). Common *nod* genes are involved in production and secretion of a lipo-chito-oligosaccharide called Nod factor which determines host range (Fig. 6). Although local structures of Nod factors are different depending on rhizobial strains, they share a common backbone structure which is a chitin oligomer (β1,4 linked N-acetylglucosamine [GluNAc]) with an N-acyl chain at its nonreducing end. The common structure is synthesized by the common *nodABC* genes. *NodC*, which shows homology with chitin synthases, catalyzes oligomerization of GluNAc and *NodB* and *NodA* proteins subsequently deacetylate and add the N-acyl chain, respectively (86). Nod factors from different rhizobial strains are subjected with different modifications and host plants recognize only the Nod factor produced by its compatible rhizobial strains. Such modifications were added by strains specific *nod* genes mostly at reducing- and nonreducing-terminal GluNAc residues of chitin oligomer (86). Genetic modifications of the specific *nod* genes can alter the host range (30, 97). Nod factor itself elicits not only root hair deformation but also cortical cell division and in some cases, formation of nodule structure (99, 107). This fact indicates that development of the nodule structure relies on plant ability with the rhizobia just turning on the developmental program.

Expression of *nod* genes is induced in response to flavonoids released from plant roots (Fig. 6). Structure of
flavonoids is different depending on plant species and flavonoids secreted by the host plant show the highest inducing activity of nod genes of its compatible rhizobial strain (26). Host plant specific flavonoids are perceived by a transcription factor NodD and direct binding of the two molecules, the plant-derived luteolin and the transcriptional regulator NodD, has been strongly suggested (82). The nodD genes are found in all rhizobia but their C terminals have diverged, which enables the recognition of different flavonoids in a strain specific manner. Indeed, modification of the C terminal region resulted in alteration of host range (45, 64, 87). Conversely, the N terminal portion of NodD contains a helix-turn-helix DNA binding domain to interact with DNA as a transcription factor. In the presence of specific flavonoid, NodD activates expression of the nod genes by binding to a conserved sequence motif called nod box in the promoter regions of nod genes, and then production of Nod factor is initiated.

Most probable candidates of plant receptors for Nod factor are NFR1 and NFR5 proteins in Lotus japonicus (59). They are receptor kinases consisting of LysM-related extracellular domains and intracellular serine/threonine kinase domains linked by a transmembrane domain and mutation of those genes resulted in loss or attenuation of all Nod factor induced plant responses. LysM domains are found in bacterial peptidoglycan-binding protein and chitinases, suggesting their role for Nod factor perception. Transfer of Lotus japonicus LjNfr1 and LjNfr5 genes to Medicago truncatula or Lotus andiculis extended their host range to incompatible rhizobial strains which normally infect L. japonicus (88). In addition, domain swapping and site-directed mutagenesis experiments showed that LysM domains were responsible for the host range alteration. These genetic evidences strongly support that NFR proteins are the Nod factor receptor although biochemical data showing specific interaction between the two molecules are awaited.

Thus, correct recognition of symbiotic partners in legume-rhizobia interaction is realized by mutual exchange of signaling compounds released from both bacteria and plants. It should be noted, however, that some Bradyrhizobium strains use Nod factor-independent recognition system (40).

Surface polysaccharides. In addition to the Nod factor, surface polysaccharides of rhizobia are crucial for establishment of successful symbiosis (Fig. 6). Outer surface of Gram-negative bacteria contains several different types of polysaccharides such as extracellular polysaccharide (EPS), capsular polysaccharide (CPS, K-antigen or KPS), lipopolysaccharides (LPS) and cyclic β-glucans. Unlike nod genes, most of the genes involved in polysaccharide synthesis are located outside of symbiosis island/plasmid. In Sinorhizobium meliloti, those genes are not induced by compatible plant flavonoid (7) whereas some EPS-related genes are responsive to seed extracts of its host plant soybean in Bradyrhizobium japonicum (116). Symbiotic phenotypes caused by defects in polysaccharide synthesis vary depending on rhizobial species, individual genes mutagenized and host plant species, which makes difficult to draw a definitive conclusion for the functions of those molecules. Nevertheless, a rough sketch of how these polysaccharides contribute to successful establishment of symbiosis is presented. Unlike Nod factor, surface polysaccharides themselves do not actively evoke plant symbiotic program but they are important to make the host plants allow intracellular inversion of rhizobia presumably through suppression of defense responses.

**Fig. 6.** Molecular dialogs between plant and soil microorganisms to achieve specific symbiotic interactions. Root hair deforming factor, RHDF; lipopolysaccharide, LPS; polysaccharide, PS.
EPS is not attached to the cell surface but secreted to environment. EPSs are heteropolymers with repeating units ranging from 7 to 9 hexose residues (35). Their structure is largely diverse among rhizobia in sugar composition, glycoside linkage, degree of polymerization and noncarbohydrate substitutions (96), suggesting its potential as a determinant of host specificity. Disruption of genes involved in EPS synthesis usually resulted in more severe impairments of symbiosis than in the cases of other surface polysaccharides. Although numerous works on EPS mutants are reported (22, 25, 27, 51, 52, 57, 63, 83, 96, 117, 120), their phenotypes can be summarized into abortion of the infection threads followed by formation of non-nitrogen-fixing nodules with no or less bacteroids. The defective nodules exhibited features characteristic of plant defense responses such as thickened cell wall and accumulation of callose, phenolic compounds, phytoalexin and chitinase (71, 80) suggesting their role in suppression of defense responses. Defects of nodulation in EPS mutants are complemented by addition of purified EPS fraction (41, 115) and the complementing ability requires the proper structure (8, 27), suggesting a role as a signaling molecule for host specificity determination. However, significance of EPS in host range determination does not seem to be dominant because in some cases only a change in Nod factor structure can alter the host specificity without changing EPS structure (52, 110).

Unlike EPS, KPS adhere to the bacterial cell surface but are not anchored to the outer membrane like EPS because they lack lipid chains. Most of our knowledge about structure and symbiotic activity of KPS has been obtained from works on Sinorhizobium. Precise structures of KPS are different from strain to strain even within Sinorhizobium species but there is a conserved motif consisting of a disaccharide repeat containing a hexose and 3-deoxy-D-manno-2-octulosonic acid (Kdo) or its derivatives (35). A mutation in the rkpZ gene in S. meliloti strain Rm41 affected the size distribution of KPS and resulted in a fix− phenotype (118). Interestingly, symbiotic defect in an EPS mutant (exoB) of strain Rm1021 was suppressed by introducing the rkpZ gene (89), suggesting functional redundancy between EPS and KPS.

LPS is anchored in the bacterial outer membrane with hydrophobic lipid chains. LPS shares a common backbone structure consisting of 3 parts with an O-antigen polysaccharide, a core oligosaccharide and lipid A (35). The O-chain is positioned on the outside of the cell and the core oligosaccharide connects it with the lipid A which is embedded in the outer plasma membrane. The O-antigen is the most variable domain of the LPS, showing strain by strain diversity (35). LPS mutants of rhizobia exhibited extensive levels of symbiotic impairments ranging from complete lack of nodule formation (98), formation of non-nitrogen-fixing nodules devoid of bacteroids (73, 74), nodules with less bacteroids and nitrogen fixation (17), abnormally developed nodules devoid of bacteroids (73, 74), nodules with less levels of symbiotic impairments ranging from complete lack of nodule formation (81, 112) to increased competitiveness (31, 50, 93). Nevertheless, the majority of the LPS mutants can invade plant tissue more or less, suggesting that LPS is particularly important in later stage of symbiosis. Up to now, the most severe impairments in nodulation were reported in mutants deficient in O-chain (73, 98). Significance of lipid A in symbiosis remains unclear because most of lipid A mutants show weak phenotypes such as delay and reduced competitiveness for nodulation (93, 112) or sometimes no apparent symbiotic lesion (46, 111). LPS may contribute to the establishment of symbiosis as a suppressor of plant defense responses because LPS from S. meliloti have been found to suppress defense responses in a host plant Medicago sativa cell culture induced by a yeast elicitor (1).

If lipopolysaccharides are considered ubiquitous among Gram-negative bacteria, Gram-positive bacteria have membrane-anchored polymers such as lipoteichoic acids (LTA) as for Bacillus, Streptococcus, Clostridium or lipoglycans like in the Mollicutes and Actinobacteria lineages (39). Usually, LTA is a linear polymer of 16 to 40 phosphodiester-linked glycerophosphate residues covalently linked to a membrane anchor which is generally analogous to a glycolipid or glycosphospholipid found among the free lipids of the cell membrane. The structures, functions, and biosynthesis pathways of these conventional LTAs have been extensively reviewed, comprising a few Gram-positive bacteria. Conversely, the number of Gram-positive bacteria known to lack classical LTA is steadily increasing. The first Gram-positive bacterium found to lack LTA was Micrococcus luteus (103). Lipoglycans like lipaorabinomannans from Mycobacterium (LAM) are the most common components of actinobacteria cell envelopes (102). Among them, it seems that N-glycolycuramic acid could be only observed in the peptido-glycan of bacteria from genera Rhodococcus, Tsukamurella, Gordonia, Nocardia and Micromonospora that are phylogenetically close to Mycobacterium (37). Mycobacterial LAM are lipoglycans composed of three domains, a polysaccharide backbone (mannan and arabian cores), the MPI anchor (fatty acids) and the capping motifs (mannooligosaccharide units or phosphoinositide units). Diverse lipoglycans have been identified in a number of actinobacterial strains, including Corynebacterium matruchotii (101), Dietzema maris (102), Gordonia rubropertincta (33), Rhodococcus rhodni (32), Turicella ottidis, Rhodococcus ruber and Amycolatopsis sulphurea (72). It could even be remarked that lipoglycan distribution may be of chemotaxonomic value (100, 102). However, the main interest in lipoglycans is that they appear to play a role, for instance in Mycobacterium.

Cyclic β-glucans are circular molecules consisting of several tens of glucose residues linked by β-(1,2) or β-(1,3) and β-(1,6) glycosidic bonds which are unique to Rhizobiales bacteria (16). Cyclic β-glucan is a predominant constituent of rhizobial cell (5 to 20% of the total cellular dry weight), most of which are localized in periplasmic space (16). Part of the glucose residues are substituted by phosphoglycerol, phosphocholine or succinyls. Cyclic β-glucans are required for successful symbiotic interaction because ndv mutants which are defective in the glucan biosynthesis resulted in abortion of infection thread and pseudonodule formation in S. meliloti (28). Although the mode of action of cyclic β-glucan remains elusive, several results suggest its role as a suppressor of host defense responses (13, 66).

Little is known about the surface lipopolysaccharides of Frankia despite the role such compounds play in virulence of the actinobacteria Mycobacterium spp. (92) and Corynebacterium (55, 56). Beside the analysis of cellular fatty acids,
menaquinones and diamino acids (78) done for taxonomic purposes, a study of whole-cell sugars has permitted to show that all *Frankia* strains tested harbored a unique mono-saccharide, 2-O-methyl-D-mannose that may play a role in the specific recognition by the plant of the microbial symbiont (67). The genomic region at coordinates FRAAL6189-6265 in particular contains several *Frankia* specific genes involved in mannose metabolism, LPS and transferases that could be involved in its synthesis (Fig. 7).

**Known infection steps**

**Entry routes, root hair deformation.** Root hair deformation, a well described phenomenon in several legumes such as *Medicago*, is also known to occur in *Alnus, Myrica* and *Casuarina* (10). This deformation on *Alnus*, as in *Medicago*, is known to start a few hours after the application of compatible bacteria onto the root hair. It also occurs following the application of supernatant of *Frankia* cells grown in pure culture. This physiological reaction initiates a series of molecular events that involves kinases that perceive the Nod factor and transmit a signal to the nucleus for the synthesis of a series of nodule specific proteins collectively called nodulins (38). One major and immediate consequence of this root hair deformation is the entrapment of bacterial cells in the folds, from which is initiated the acropetal growth of bacterial cells towards the basis of the root hair cell, in the thread.

There, in *Alnus* and *Casuarina*, the *Frankia* cells contained in the infection thread multiply and induce limited plant cell divisions in the cortex to form a small protuberance called prenodule. Prenodules contain infected *Frankia* cells but they do not develop into genuine nodules. Accompanying the prenodule induction, formation of nodules, which are modified adventitious secondary roots with a thickened cortex, is initiated from the pericycle and they get colonized by hyphae (Fig. 5). In *Alnus*, hyphae later differentiate into nitrogen-fixing diazo-vesicles. In *Casuarina*, on the contrary, oxygen diffusion is limited by the existence of a suberin layer and the synthesis by the host plant of leghae-moglobin, making the synthesis of vesicles unnecessary, even though CcI3 in pure culture under nitrogen-limited conditions, is able to form vesicles and fix nitrogen (81). Another compound synthesized by *in vitro* grown *Frankia alni* is phenylacetate (PAA), an aromatic compound that is an auxin, and that can induce the emergence of adventitious secondary roots (44).

Another compound synthesized by *in vitro* grown *Frankia alni* is phenylacetate (PAA), an aromatic compound that is an auxin, and that can induce the emergence of adventitious secondary roots. Other auxins have been described in the supernatant of *Frankia*, in particular IAA (12), which together with the fact that an inhibitor of auxin influx was shown to perturb nodule formation and that a functional auxin influx carrier was found to be produced specifically in *Frankia*-infected cells (84), point to a central role for auxin synthesis and transport in nodule formation. Application of pure PAA at $10^{-3}$ to $10^{-5}$ M resulted in a marked number of adventitious roots with a stunted oversized morphology reminiscent of that of nodules (44).

**Intercellular penetration.** Some actinorhizal host plants such as *Elaeagnus* or *Datisca*, do not deform root hairs but...
penetrate the host root tissues through intercellular spaces, also called cracks (12). This intercellular process does not involve the formation of a prenodule. Some Frankia strains are able to infect both Alnus and Elaeagnus forming in the first case root hair deformation and prenodules and in the second case no deformation nor prenodule (65), thus implying that the mode of root penetration is host-dependent (5).

**Plant symbiotic response to microbial effectors**

The plant symbiotic genes involved in symbiosis are so far still poorly known beyond the recent demonstration of a role for a SymRK kinase (38), similar to the one existing in legumes that governs the nodulation process as well as the establishment of mycorrhizae. The targeted knock-out of various symbiotic genes known in legumes should progress rapidly in Casuarina where a RNAi approach is now possible (34). Another approach that should help identify candidate genes is an EST approach of root and nodules tissues in both Alnus and Casuarina that is underway at present (www.genoscope.cns.fr/spip/Alnus-glutinosa-Casuarina-glauca,660.html).

**Root hair deformation assay**

**Actinorhizal plant.** Root hair deformation occurs in Alnus, Myrica and Casuarina (12) while the other actinorhizal plants get nodulated through intercellular spaces. Root hair deformation in Alnus starts a few hours after application of compatible bacteria onto the root hair or following application of cells supernatants. The compound synthesized by Frankia and responsible for this reaction has not been identified so far however it has been characterized as small (<3,000 Da), hydrophilic, thermoresentant (20 min at 120°C), concentrated (10⁶ dilution still active) (21). It was also assessed for sensitivity to enzymes and found to be attacked by pronase and somewhat sensitive to chitinase (20), however, a search for N-acetyl-D-glucosamine using labeled precursors was unsuccessful (21).

**Leguminous plant.** Rhizobial Nod factor is the active molecule that induces root hair deformation on leguminous plants. Root hair deformation by rhizobia is different from those by Frankia in some aspects. Production of Nod factor by rhizobia always requires induction with specific flavonoid contained in host plant’s root exudates. A particular Nod factor induces root hair deformation only on the host plant (29). Nod factor can induce cortical cell division and formation of genuine nodule structure (99, 107).

**Genetics**

**Complementation of Rhizobium.** Many nodule mutants of Rhizobium have been obtained by various approaches, mostly by transposon insertion and cosmid complementation. Given the similarities existing between Frankia and Rhizobium, it was hypothesized that the genes determining the ability to synthesize the Nod factor could be sufficiently conserved to make possible a cross-genera complementation. To that end a whole-genome bank was constructed in E. coli wide spectrum cosmid pLAFR3, conjugated into Rhizobium leguminosarum biovar viciae and Sinorhizobium meliloti nodB and nodC mutants and inoculated onto pea or alfalfa, respectively (20). However, no complementation was achieved except for a few nodules obtained following excision of the Tn5 from nodC. Chemical complementation was also a failure since no root hair deformation was obtained with Rhizobium supernatant on Alnus root hair, nor with Frankia alni supernatant on legumes root hairs (20). In retrospect, complementation may not have been expected to happen since the study of the genome has permitted to note that most Frankia genes have poor sigma recognition promoters and poor Shine-Dalgarno ribosome binding sites for them to work in a Proteobacterium like Rhizobium, there is so far only one instance of a Frankia gene, sodF, expressed in Proteobacteria to complement a mutant from its own promoter (61) and in this particular case, only minimal transcription was deemed sufficient to permit complementation.

**Genomes.** The recent determination of three genomes (79) from Frankia strains of contrasted host specificity has shown a surprising polymorphism in size (5.3–9.0 Mb) and gene set, and permitted to initiate various evolutionary works on hup (58) or kat (91) genes. The region of the genome with the highest sequence conservation was the replication origin (Fig. 7). The most surprising conclusion of this work though was that there were no canonical nod genes (79) in Frankia, despite the fact that its supernatant contains a root hair deforming factor with characteristics comparable to those of Rhizobium (21). This confirms the fact that bacterial nodulation in Rhizobium and Frankia would represent a case of convergent evolution.

Numerous other Frankia genomes are in the process of being sequenced, from all clusters described above, from the various genomic species identified (77). Also, the genomes of phylogenetic neighbors Acidothermus (6) and Geodermatophilaceae (JGI and Genoscope) are being determined. These genomes should help reduce the Frankia-specific core genome expected to contain the symbiosis genes, as well as help identify genes related to specific niches occupied by the diverse Frankia.

A phyloprofile made on the Mage platform (109) using the presently available genomes of three Frankia strains as well as Acidothermus (6), Geodermatophilus (119) and other more remote actinobacteria at a threshold level of 30% in amino acids similarity permits to show there are 335 conserved genes (Table S1), of which 199 are simply “hypothetical”, “secreted hypothetical” or “membrane hypothetical”, 14 are transporters, 12 are kinases, 10 are regulators, 6 are glycosyl transferases, besides the nif and hup clusters. These genes are mostly situated in the “upper” part, that close to the origin of replication.

**Transformation.** Molecular bases of rhizobial nodule have been elucidated using genetic approaches in which first nodule mutants were isolated and then genes responsible for the impaired phenotypes are identified (forward genetics) or a target gene of interest was knocked out and then symbiotic phenotypes of the mutant are observed (reverse genetics). Transformation system is essential for both strategies. In spite of numerous trials over time (9, 24, 49, 69, 70, 95), nobody has succeeded in transforming Frankia.
so far, which remains a major hurdle to identify nodulation genes in *Frankia*.

Cournoyer and Normand introduced plasmid DNA into *Frankia* strains by electroporation (24). They used plasmid DNA derived from a *Frankia* strain, those stably maintainable in *Streptomyces*, or composites of those. They confirmed by Southern hybridization that DNA was successfully delivered inside *Frankia* cells after electric pulse. Introduced DNA was maintained over 5 days without conformational changes. Nevertheless, they could not obtain antibiotic resistant colonies on selective media. Myers and Tisa, using electroporation, introduced chromosomal DNA of a *Frankia* strain EAN1pec, which is resistant to lincomycin and kasugamycin, into a *Frankia* strain EuI1c, which is sensitive to those antibiotics, and obtained antibiotic resistant EuI1c colonies (70). Frequency for appearance of the resistant colonies was about 10⁻⁵, which was higher than those for spontaneous resistant. However, it is not clear whether they were true transformants because the resistant strains were not characterized at the molecular level. Recently, Kucho et al. employed several new technical attempts to transform *Frankia* strain CcI3 (49). They assumed that antibiotic resistance genes generally used in bacterial transformation are not expressed efficiently in *Frankia* cells because of differences in promoter sequences and/or codon usage frequency since *Frankia* genomes are extremely rich in GC (79). They generated fusion marker genes by combining promoters of the strain’s translation initiation factor 3 gene and tetracycline resistance gene with a high codon usage similarity to *Frankia*’s (49). The marker genes were designated to integrate into chromosome by homologous recombination. *Frankia* cells transformed with the construct DNA by electroporation grew in selective liquid media. PCR and Southern blot analysis of the genomic DNA showed that at least a part of resistant cells contained the fusion marker gene at the targeted position but majority of them did not have marker gene insertion in the chromosome. In addition, the marker genes declined in the transformant population during maintenance in selective liquid media, showing that the transformants were unstable. It was probable that spontaneous mutants which acquired antibiotic resistance by natural mutations occurred during prolonged selective culture.

Isolation of single-cell derived colony on solid media is apparently necessary to obtain genuine transformants. It is difficult to get such a colony with *Frankia* hyphae because they are multicellular like other actinobacteria. *Frankia* differentiates single-cell spores under nutrients-limiting conditions (9). So controlling sporulation and spore germination are important issues for successful transformation. Burleigh and Dawson optimized sporulation condition of *Frankia* strain CcI3 (18). The best sporulation efficiency was achieved in media lacking nitrogen and phosphorus, yielded more than 10⁷ spores per 1 mL culture. Addition of aliphatic L-amino acids stimulated sporulation. However, the same condition did not increase sporulation of other two *Frankia* strains (18). Germination rates of *Frankia* spore were largely different depending on strains from less than 1% to 75% (48, 108). Sporulation was stimulated by a gentle heat shock, low concentration of phenol, root extracts of a host plant (48), and some secondary plant products (108). Culture medium also affects germination efficiency (108). Effects of those modifications on germination efficiency varied among strains tested. Unfortunately, however, none of the model *Frankia* strains whose genome sequences were determined (ACN14a, CcI3 and EAN1pec) are optimized for both sporulation and germination so far.

**Expression**

**Proteome.** *Proteome* is the whole of the protein set present at a given time under a given set of circumstances. This supposes the possibility to characterize all proteins present which constitutes an ideal goal in the case of *Frankia*. Nevertheless this has been attempted using two-dimensional protein electrophoresis of *in vitro* grown cells exposed to plant exudates followed by end-sequencing (43), which showed mainly stress proteins such as Sod. Later, nitrogen-fixing cells were studied comparing nitrogen-replete cells of *Frankia alni* ACN14a, permitting to characterize 126 proteins individually harvested by Maldi-TOF (2) as well as those proteins induced by *Myrica* exudates (4). Technical progress is rapid in this field and now the whole proteome can be characterized without tedious 2D separation by liquid chromatography coupled to tandem mass spectrometry. This approach permitted to identify 38 proteins in the culture supernatant of *Frankia* sp. strain CcI3, only three of which had predicted export signal peptides raising the question of whether these proteins originate from leaking or broken cells or the way these proteins were secreted into the medium. Symbiotic cells can also be studied with this approach, which permitted to identify 42 signal peptide containing proteins in *Frankia* strain CcI3 in *Casuarina cunninghamiana* and *Casuarina glauca* root nodules, while 73 and 53 putative secreted proteins containing signal peptides were identified from *Frankia* strains in field-collected root nodules of *Alnus incana* and *Elaeagnus angustifolia*, respectively. Solute-binding proteins were the most commonly identified secreted proteins in symbiosis, in particular putative branched-chain amino acids and peptides transporters (62). Few of the proteins recovered under these symbiotic condition were present in the three genomes.

**Microarray.** Follow-up of transcription has been undertaken to compare symbiotic cells with *in vitro* grown cells (3), using Nimblegen commercial arrays. This approach has permitted to show that functions known to be related to symbiosis such as *nif* (nitrogenase), *hop* (uptake hydrogenase), *suf* (sulfur-iron cluster synthesis) and *hop* (hopanoid lipids synthesis) were indeed up-regulated. Also, stress-related determinants were in general down-regulated. However, no evident symbiosis island emerged from this work.

**Lectins**

Plant lectins have been shown to be determinants of specificity, since cloning of a soybean lectin into *Lotus corniculatus*, which is normally nodulated by *R. loti*, was instead nodulated in response to *Bradyrhizobium japonicum* (113). An even more distant transfer has seemingly been obtained by transforming *Hippophae rhamnoides*, normally nodulated by *Frankia*, with a pea lectin gene, resulting in
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nodulation by *Rhizobium* (113). No lectins have been described in actinorhizal plants, however several lectin genes have been found in one of the three genomes studied so far, a phenotype remains to be ascribed to it (79).

**Perspectives**

The identification of *Frankia* symbiosis determinants will be attempted in the coming years using genomic, transcriptomic, proteomic, metabolomic and genetic approaches, alone or in combination.

The availability of high throughput sequencing makes it feasible to envision that all *Frankia* isolates as well as all strains of related *Frankinaceae* will have their genomes sequenced in the coming years. This in turn will permit to reduce the specific core *Frankia* genome. Hopefully, phenotyping will be intensified in parallel to correlate the presence of proteins in the genome and metabolic capacities, prominent among which of course will be those related to symbiosis.

Transcriptomics that has started with the use of DNA arrays (3). However, this approach is tedious and has many biases, related to the high G+C of *Frankia* and the resulting important background noise. Given the existing increased high throughput sequencing capacities, and the possibility to study RNA after retro-transformation into DNA, it is likely that symbiosis will be followed, using this approach. Proteins interacting with DNA, such as transcriptional regulators, can be analyzed through approaches such as chromatin-immunoprecipitation or ChIP-on-chip (114), where an antibody can pull down DNA adhering to a given protein.

Proteomics has also interesting perspectives, given the increased proteins identification capacities with ever decreasing thresholds for size, solubility and complexity. Both partners can be analyzed in this fashion, as well as sub-fractions (secreted, membrane or those that interact with a given metabolite, another protein).

Metabolomics, the global study of metabolites has an important potential for analyzing the phenotype of mutants because the demonstration of a link between a gene and a function still remains the most convincing approach in analytical biology. Therefore, efforts underway are expected to continue.

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**References**


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