Plant and Soil 90, 429–453 (1986). Ms. NF 34

The genetics of actinorhizal *Frankia*: A review

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Key words Actinorhizae *Frankia* Genetics Nitrogen fixation Nodulation Symbiosis

Introduction

In 1978, nothing could have been said about the genetics of a micro-organism which had never been isolated except by Pommer[70] who, in an unnoticed report, described an actinomycete now recognized as being a true *Frankia*. But in the six years that have elapsed since Callaham *et al.*[19] isolated CpI1 in pure culture the question of the genetics of *Frankia* has arisen for many reasons. The economic importance of biological nitrogen fixation is well established especially with regard to leguminous plants for the agriculture of less developed countries which cannot afford the intensive use of fertilizers required for the sustenance of their rapidly growing populations. Similarly, forestry in many areas faces the problem of clear-cut or burned land on which it takes as long as fifty years for the natural succession to replenish the timber to an economically profitable stage, due in large part to nutrient removal from the sites[4]. Non-legumes or actinorhizal plants associated with the appropriate Frankiae provide the main input of biologically-fixed nitrogen in temperate regions and they are already being used for the re-afforestation of mine spoils in Pensylvania[22], to establish rapid plant covers on dam dykes in New Québec[32] to serve as nurse trees for the production of highly valuable black walnut in Illinois[33] or Douglas fir on the West Coast[4], and in general, as windbreaks, highway landscaping, for land reclamation and as ornamental species[30]. In Africa and the coastal regions of China, *Casuarina* or filao is being planted on an enormous scale to stabilize the coastal sand dunes and protect the agricultural fields lying behind it from being buried under metres of sand (Dommergues and Zhao, pers. comm.). But other tree species are more important economically than alder or filao, for pulp and paper production or furniture and house building.

The actinorhizal symbiosis is quite different in its interactions from that of *Rhizobium* with legumes[37]. For instance, *Frankia* has a much more varied host spectrum, being in symbiosis with 19 genera of dicotyledonous plants belonging to seven families[1]. Fundamental

research into the genetics of *Frankia,* a nitrogen-fixing actinomycete, is in itself interesting. But a broadening of its already large host spectrum to other more economically important plants would yield tangible benefits. Such an extension of the symbiosis to genera related to actinorhizal ones such as *Betula,* for instance, may be made possible by a study of the molecular biology of *Frankia* and of its host plant. For that purpose, an understanding of the genome organization, including its plasmids, is important. Furthermore, gene transfer and cloning systems will have to be developed. These aspects will be reviewed here, as well as the symbiotic properties of *Frankia* whose genetic study and eventual cloning are our long term aims. The following review, therefore, sets out to describe the state of research on the genetics of *Frankia* and suggests directions for future work.

It must be pointed out that less is known at present about the genetics *per se,* than about the phenotype, of *Frankia.* But it has already been noted that an important heterogeneity exists in populations of natural strains for a number of characters, some related to the symbiosis[13,17,63]. Such genetic diversity constitutes a reservoir from which desirable genetic traits might eventually be drawn.

Note. A list of the strains mentioned in the text is given in Table 1 with their new standardized acronyms[52]. It must also be mentioned at this point that a number of results cited below were presented at symposia held in Madison, Wisconsin, USA (International Conference on the biology of *Frankia,* August 1982) and in Wageningen, The Netherlands (International Workshop on *Frankia* Symbioses, September 1983), the proceedings of which have been published. These results, and others presented at Laval University, Canada (International Symposium on Current Research on *Frankia* and Actinorhizal Plants, August 1984) will be discussed here because they afford valuable insights into the workings of the actinorhizal symbiosis even though some have not been, or will not be, published. If not designated as 'in press' or 'submitted for publication' they will be cited as 'oral presentation, Laval 1984', for example.

'Unpublished' data are those of the authors of this review unless otherwise stated.

Genome characteristics

Genome size

The mean genome size of an organism gives an idea of the complexity of its metabolism, since as was expressed by Watson[91], for each protein, there is at least one gene. The large size of the human genome (Table 2) may reflect the enormous complexity of its nervous sytem while that of algae is understandable considering their many biosynthetic capabilities[16]. The genome size is by no means an absolute guide to the complexity of an organism since redundancies, introns and splicing all tend to skew the correlation.

Frankia is a pleomorphic actinomycete (Fig. 1) both *in vivo*[37] and

Table 1. List of *Frankia* strains with their standardized catalogue numbers*

Group A strains (belonging to *Elaeagnus* host-specificity group)			
EUN1f	ULQ132500106	EUN1wb	ULQ132500155
TX30$^{\underline{SA}}$ *b*	ULQ00230026	SCN10a	ULQ190201001
EUI1b	DDB130120	EAN1pec	ULQ130100144
HrN18a	ULF1401	EUN1fS20	ULQ1325001061
EAN1b	ULQ130100102		
Group B strains (belonging to *Alnus* host-specificity group)			
CpI1	HFP0701	ARgX17c	ULQ013221703
ARgN22d	ULQ013202204	AvcI1	DDB010110
ArI3	HFP0131	ArI4	DDB010210
ACN1$^{\underline{AG}}$	ULQ0102001007	TN18b$^{\underline{AC}}$	ULQ00018024
TX38b$^{\underline{AC}}$	ULQ00238024	TX41b$^{\underline{AC}}$	ULQ00241024
ANN1	ULQ0102001997	AgN11a	ULF010701101
AcoN24d	ULF01010244	AgN24h	ULF0107
ARgP5$^{\underline{AG}}$	ULQ0132105009	ARbN4b	ULQ01310042
ATP1d	ULQ013710107	AGN1g	ULQ010700107
CPX34g	ULQ070123407		
Other strains			
CcI3	HFP0202	Cj isolate	ORS021001

* According to Lechevalier[52].

Table 2. Genome size of selected species

Species	Genome size (kb)	Reference
Phage MS2 (RNA)	4	24
Phage lambda (DNA)	47	24
Phage T2	200	24
Mycoplasma sp.	300	26
E. coli	4000	24
Klebsiella spp.	5000	26
Frankia EuI1	7000	2
Frankia ArI4	10000	2
Streptomyces	10000	12
Drosophila	200000	24
Human	3000000	24

in vitro[19], producing branched septate hyphae, sporangia containing non-motile spores[54] and pedunculate thick-walled specialized structures called vesicles[47] that have been associated with nitrogen fixation[35,84] as well as secondary metabolites such as pigments[53] and volatile actinomycete-like compounds (unpublished).

The mean genome size of two *Frankia* strains was measured by An[2] using a technique based on the kinetics of reassociation of denatured total DNA. He found values of 7.10^6 for *Frankia* strain EuI1 isolated from *Elaeagnus umbellata* and of 10^7 for strain ArI4 isolated from

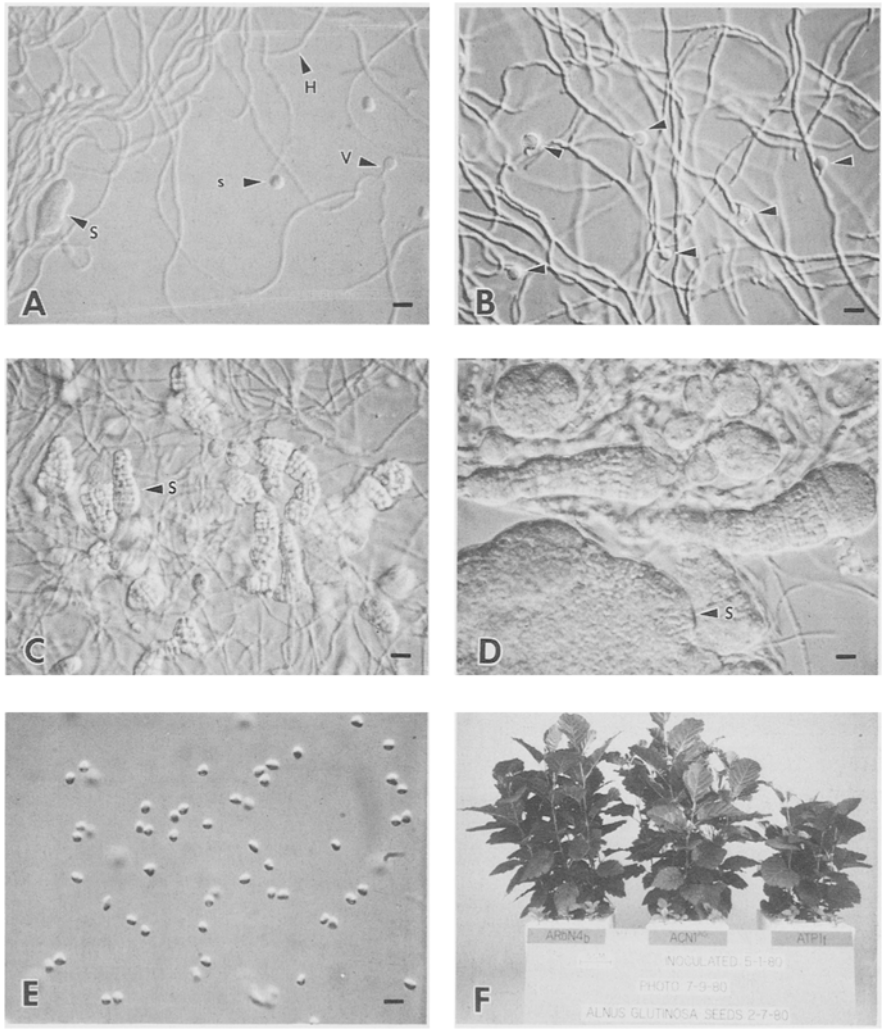

Fig. 1. *In vitro* morphology of *Frankia* and influence of sporulation phenotype on nitrogen fixing efficiency.

A) *Frankia* strain ACN1^{AG}, typical of group B strains, (belonging to the *Alnus* host-compatibility group) grown on Qmod B medium. Pleomorphism of this microorganism is illustrated by the presence of branched and septate hyphae (H), a pedunculate thick-walled vesicle (V), a sporangium (S) and free spores (s). Bar is 4 μm.

B) *Frankia* strain EAN1b, typical of group A strains, (belonging to the *Elaeagnus* host-compatibility group) grown on Qmod B medium. Group A strains produce numerous vesicles even on a nitrogen-rich medium such as Qmod B but fewer sporangia than group B strains. Bar is 4 μm.

C) Group B strain AGN1g grown in Qmod B medium, with the small *in vitro* produced sporangia (S) that are typical of Type N strains (strains whose sporulation in the nodule is inhibited by the host plant). Bar is 4 μm.

D) Group B strain ATP1f grown under identical conditions as in 'C' with the large (up to 150 μm in diameter) *in vitro* produced sporangia (S) that are typical of Type P strains (those

Alnus rubra. This represents a genome about twice that of *E. coli* but is similar to that of *Streptomyces* (Table 2). From a practical point of view, these values also proved useful in the measurement of plasmid copy numbers, as was done by Simonet *et al.*[81].

DNA base composition (G + C%)

One of the most easily determined parameters of a given genome is its overall DNA base composition, in other words, the proportion of guanine and cytosine residues. An *et al.*[3] determined the G + C% of 12 *Frankia* strains isolated from widely different host plants. They found their G + C% to lie between 68 and 72% depending on which method was used, either thermal denaturation kinetics, buoyant density in CsCl gradients or direct nucleoside analysis by HPLC, the three methods yielding slightly different values for the same DNA. All strains tested yielded similar values indicating that the G + C% could not be used to identify classes within the genus[3]. This was to be expected since organisms with G + C% values differing by more than about 20%[26] are not considered as belonging to the same genus. Similar G + C% values do not necessarily, however, indicate a close relatedness between strains.

These results establish *Frankia* as a typical actinomycete since these microorganisms generally have a high G + C% (Table 3). Taking advantage of the high G + C% of *Frankia* relative to that of plants in general, Mullin *et al.*[60] were able to isolate pure endophytic DNA directly from nodule tissues. As expected for DNA with such a high G + C%, restriction endonucleases such as Sst I, Sph I and Bam HI that have a high G + C% recognition site, cut total DNA or plasmid DNA from all strains tested much more often than enzymes such as Eco RI, or Bgl II with a low G + C% recognition site (unpublished).

DNA homology

It is now well recognized[91], that not only do microorganisms transfer

Fig. 1. Contd.

strains that sporulate in the nodule). The mass of spores relative to that of hyphae is much more important in *in vitro* grown Type P strains than in Type N strains. Bar is 4 μm.

E) Spores isolated on a Percoll density gradient from a pure culture of strain EUN1f. Purified spores germinate but asynchronously and at a rate of only 5–10% (unpublished). Such purified spore suspensions nevertheless provide single cell colonies necessary for a classical genetic study of *Frankia*. Bar is 4 μm.

F) Evaluation of the efficiency of Type N and of Type P strains in symbiosis with *A. glutinosa* seedlings grown under nitrogen-free conditions. Those seedlings inoculated with Type N strains ARbN4b and $ACN1^{AG}$ had a significantly more important biomass after 9 weeks than the Type P *Frankia* strain ATP1f.

their genetic information to daughter cells together with slight mutations, but that 'lateral' transfer also occurs. Three main mechanisms have been studied: transformation with naked DNA, conjugation with sex-plasmids and transduction through phage infection. This laterally transferred DNA, in turn, also undergoes mutations. The long term effect of all these mutations is to decrease the overall relatedness of the DNA of donor and recipient strain[5]. Brenner[15], who has used one of the many hybridization procedures developed to quantify homology between enteric bacteria, estimates that two microbes with less than 70% homology cannot be considered as belonging to the same species. An[2] has shown that an homology of 67 to 94% existed between strain ArI4 and *Frankia* strains belonging to the *Alnus*-host specificity group (group B strains as described by Lechevalier[53]). On the other hand, the degree of homology between EuI1 and strains belonging to the *Elaeagnus* host specificity group (group A of Lechevalier[53]) was less than 39%. EuI1 may not have been a good choice for reference since that strain is not typical of group A strains by its non-effectivity[9] and the presence of large amounts of fucose (D. Baker, pers. comm.). One could argue that if another strain had been chosen as reference, more homogeneity would have been detected. However, similar results were obtained by Simonet[79] who tested all combinations of three isolates from group A (strains EUN1f, EAN1pec and HrN18a) and one from group B (strain AgN11a). In all cases, he found that each isolate could be considered as forming a separate species on the basis of Brenner's[15] assumption, a conclusion similar to that of An[2]. This situation is similar to that found in the genus *Streptomyces* where the homology lies between 27 and 100%[26], reflecting, that for *Frankia*, a wide evolutionary variation must have taken place in the genus.

Genetic stability in pure culture

Many of the parameters that can be measured to evaluate the genetic stability of *Frankia* in pure culture are qualitative. The question of genetic stability is of obvious interest since large scale cultivation of inoculum for commercial purposes has begun in earnest[67].

The technique routinely used in all laboratories for subculturing *Frankia* is to break the mycelial mats and subdivide the obtained slurry without attempting to obtain single-cell colonies as is considered standard practice for *E. coli* or for *Streptomyces*. The reason for that is that, routinely, spore germination rates much below 10% are obtained (McBride and Ensing, Oral presentation, Madison 1982), spores being one of the structures used with mycelial organisms to obtain single-cell colonies, the other being protoplasts. One *Casuarina*

junghuniana isolate, though, produces spore-like structures that germinate readily and rapidly[28] and these could be used for genetic studies. These germinating 'spore-like' structures have not been described for other strains. However, spores may be purified (Fig. 1) and used to obtain single cell colonies though their use is very time consuming considering *Frankia* growth rate[61,82]. The obvious consequence of not using single cell colonies is the heterogeneity maintained in the culture.

Some specific instances of heterogeneity in liquid cultures have already been observed. In an *A. rubra* isolate (WEY0131391), a variant with a much increased *in vitro* growth rate, a more aerophilic growth and a modified protein pattern was found[66]. An C.S. (pers. comm. 1984) found, between strain AvcI1 subcultures, differences concerning an 11 kb DNA sequence, specifically in its copy number and in the restriction pattern of bands that hybridized with it. Two morphologically distinct types of colonies have been found in one tube containing a strain, ARbN4b, isolated from *A. rubra* (unpublished). Spontaneous phenotypic changes have also been observed in other strains, for example, ATP1d started synthesising a black pigment, $ACN1^{AG}$ colonies stopped sporulating and EUN1f subcultures became resistant to high levels of streptomycine (unpublished). In EUN1f, plasmids were also shown not to be stable (section II A, Fig. 2).

According to M. P. Lechevalier (pers. comm.), a complex medium reduces the chances of mutations in actinomycetes. But results such as those obtained by Burrgraaf and Valstar[17] on the phenotypic heterogeneity (*e.g.* in pigment production, sporulation, nodulation and efficiency) of single cell colonies and reisolates from a 'single strain', show that a rich medium is not enough to prevent mutations, selection, or both, from taking place.

Plasmids

Plasmids have been recognized as forming an important part of bacterial cells and of some eukaryotic organisms, coding for certain functions which are generally not essential to the subsistence of the host. They can also be used to characterize strains in ecological studies. Examples of functions carried by plasmids are: antibiotic resistance and synthesis, heavy metal resistance, bacteriocin production, conjugation, pathogenic functions, symbiotic functions, enterotoxin production, degradation of toluene and of other generally toxic carbon compounds[38]. Plasmids have also been widely used as vectors in the development of cloning systems[23,58].

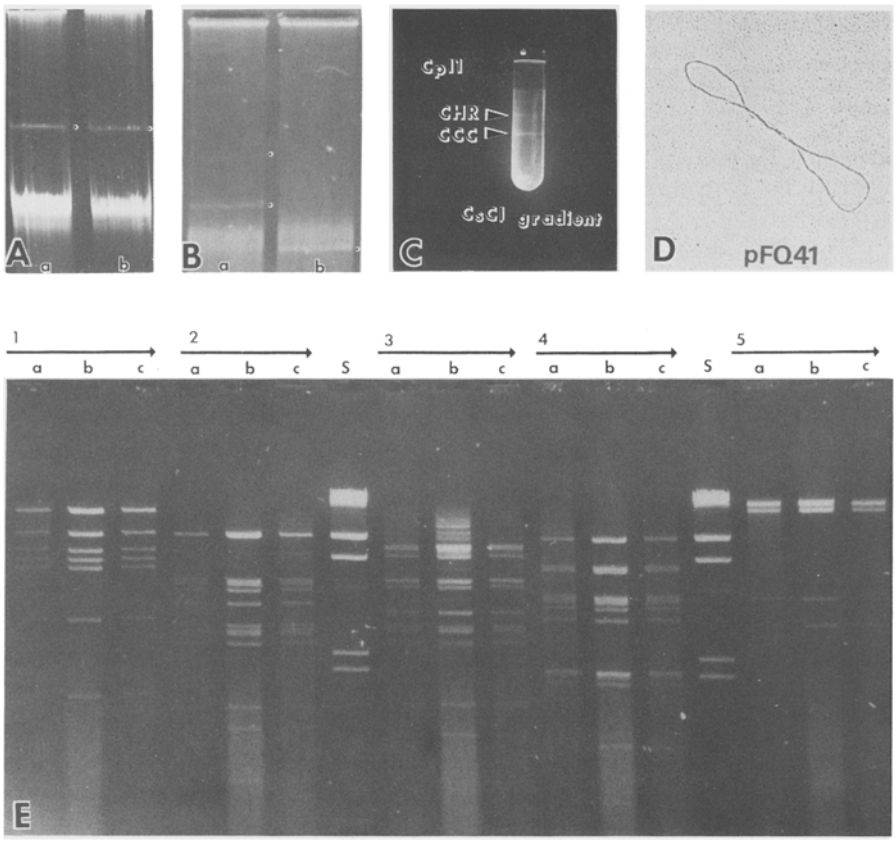

Fig. 2. Plasmids from pure cultured *Frankia*.

A) Plasmids from *Frankia* strain ACN1^{AG} (a) and TX38b$^{\underline{AC}}$ (b). Lysis was done on top of the gel according to Simonet *et al.*[80], and electrophoresis was left to proceed at 5Vcm^{-1} for 3 h in Tris (89 mmol/l), EDTA (2.5 mmol/l) and borate (89 mmol/l) buffer. Migration rates of these two plasmids (dots above the linear chromosomal DNA) were similar, and when compared with standards yielded size values of 50 kb. (Courtesy of P. Simonet, Université Lyon I, Villeurbanne, France).

B) Plasmids from two subcultures of *Frankia* strain EUN1f. Plasmid extraction was done according to Normand *et al.*[64] and electrophoresis with the same conditions as in (A). Culture 'a' yielded a major band of 40 kb and a faint band of 80 kb (dots). Under the electrophoresis conditions used, these two bands cannot be linear or open-circular forms of a 14 kb plasmid, nor can the higher band (80 kb) be a linear or an OC form of the smaller 40 kb plasmid. Culture 'b' yielded only a 14 kb band (dot) that migrated below linear DNA. Other cultures did not yield any visible plasmid DNA. *Frankia* strain EUN1f thus appears to be unstable as far as plasmids are concerned.

C) Plasmid isolation on a CsCl density gradient. Twenty litres of culture, yielding 3 ml of packed-cell volume were necessary to obtain such a ccc-DNA band. Such CsCl purified plasmids preparations were to measure the plasmids molecular sizes with the electron microscope[64] and to digest with restriction endonucleases for cloning (unpublished) and for 'fingerprinting'[81].

D) Electron micrograph of pFQ41, the smaller plasmid of strain EUN1f spread on a monomolecular layer of cytochrome C and platinum-shadowed[64] (Courtesy of J L Butour, Centre

Plasmids found in pure cultures

For the first time in 1982, Marvel found plasmids in two *Frankia* strains, ArI3 and CpI1 (pers. comm.). Benson. An and Kirby (pers. comm.) also mentioned that they had found plasmids in some *Frankia* strains without giving many details. Normand *et al.*[64], screening a large number of *Frankia* strains using a rather drastic method (SDS 10%, at 90°C and vortexing), showed that plasmids (Fig. 2B) were present in 4 strains with different backgrounds (Table 4). Simonet *et al.*[80], using a new, gentler method (making use of achromopeptidase), found larger plasmids in strains ACN1^{AG} (Fig. 2A) and ARgP5$^{\underline{AG}}$ (Table 4) in which no plasmid had been detected using the hot SDS method of Normand *et al.*[64].

These plasmids have already been used in ecological studies to identify strains (Fig. 2). Simonet *et al.*[81] studied some of these plasmids by restriction analysis and found that strains isolated from locations geographically distant by as much as 3000 km apart had identical plasmids. One of the plasmids listed below, the 8 kb plasmid from strain ArI3, has been cloned in pBR322 and in pKT230 and a restriction map of it made with seven restriction endonucleases, a task that would have been much more difficult with native plasmid DNA (unpublished). The replication origin of subclones of this recombinant plasmid are currently being tested in *Streptomyces*.

It was found that a marked difference in plasmid stability existed between strains belonging to group A and those of group B. Strains of group B such as CpI1, ArI3 or ACN1^{AG} have been subcultured fifty times or more and few, if any, changes have been noted in copy number or restriction patterns. In contrast, in strains EUN1f, TX30$^{\underline{SA}}$ or SCN10a of group A, the plasmids described in Table 4 appeared and disappeared with different subcultures (Fig. 2B). This might be related to the presence of more active exonucleases in strains of group A, that would result in the intense 'streaking' noted in agarose gel electrophoresis of plasmid DNA from those strains[64]. It could also be related to an excision and integration mechanism whereby plasmids

Fig. 2. Contd.
de Pharmacologie et Toxicologie, CNRS Toulouse, France).
E) Gel of plasmid preparations from strains TX41$^{\underline{AC}}$ (a) ACN1^{AG} (b) and TX38b$^{\underline{AC}}$ (c) isolated from Sacré-Coeur PQ; Tadoussac, PQ and from Manitounuk Island, PQ, respectively. The first two locations are 5 km apart, while Manitounuk is about 800 km away from the others, yet the plasmid restriction patterns of the three strains were found to be identical with all enzyme tested. Plasmid DNA was digested with restrictions endonucleases Sph I ('1'), Sst I ('2'), Bcl I ('3'), Pst I ('4') and Cla I ('5') and electrophoresed on a 0.6% agarose gel with a voltage of 5V/cm for 3 h[81]. Size standard ('S') is phage lambda digested with Hind III.

would interact with the chromosome, as has been found to be the case in mitochondria[94] or in *Streptomyces*[41]. Such a mechanism of DNA sequence amplification has been found to occur in group B strain AvcI1 (An *et al.*, oral presentation, Laval 1984).

A number of small, and so far cryptic, plasmids from strains CpI1, ArI3, ARgN22d and EUN1f (Fig. 2D) have been used in hybridization studies with pSA30 with negative results (unpublished), as was to be expected considering the size of these plasmids (Table 4) relative to that of the *Nif* cluster of *Klebsiella pneumoniae* (about 25 kb)[6] and of the *Sym* plasmids of *Rhizobium* strain (100–500 kb)[5,71].

Plasmids isolated from actinorhizal nodules

Plasmids isolated directly from root nodules have been described by Dobritsa[29] who separated *Frankia* vesicles from *Alnus glutinosa* nodules and isolated ccc-DNA on CsCl-ethidium bromide gradients. She described plasmids of 14 different size classes, from 1.3 kb to 85 kb as measured with the electron microscope. These sizes range from that of the smallest described plasmids ever[29], to large symbiotic or pathogenic plasmids. The main objection to that work concerns the origin of the ccc molecules, since they may be from the host plant cells, its organelles, pathogenic or commensal microbes.

An attempt to isolate the endophyte of these nodules in pure culture should be done to define whether or not these nodule plasmids are to be found in the *Frankia* endophyte.

Genetic transformation systems

Phages

Phages have been found to be useful for strain typing with *Rhizobium*[25,55]. Cloning vectors have also been built using phage PhiC31 for *Streptomyces*[23] and phage Lambda for *E. coli*[58]. They have been used for genetic studies since they can penetrate cell walls, are stable replicons, allow the cloning of large DNA fragments, facilitate the detection of desired clones by direct plaque hybridization and also permit the establishment of chromosomal gene linkage maps.

No *Frankia* phage has yet been detected after many attempts in our laboratory. On the other hand, M. P. Lechevalier (pers. comm.) has mentioned that she had found one. She pointed out that to detect phages, plaques are the most used tool; unfortunately, *Frankia* does not form lawns on which plaques could be seen. Liquid cultures and electron microscopy could detect phages but so far, not much success has been obtained in that area.

One of the ways in which microbes defend themselves against phages

Table 3. Comparison of the G + C% of various organisms

Species	G + C%	Reference
Calf (thymus)	43	91
Virus (phage T2)	35	91
E. coli	50	91
Azotobacter vinelandii	66	16
Rhizobium spp.	59–65	16
Thermoactinomyces spp.	54–55	77
Nocardia asteroides	67–77	26
Streptomyces coelicolor	72	26
Actinoplanes spp.	71–76	26
Chainia spp.	71–72	26
Frankia sp.	68–72	3

Table 4. List of strains carrying plasmids

Strain	Host*	Geographic origin	Number of plasmids	Sizes of plasmids (mol . wt $\times 10^3$)	Reference
CpI1	CP	MA, USA	2	8, 18	64
ARgN22d	ARg	PQ, CAN	6	8, 16, 20 23, 27, 32	64
ArI3	ARu	OR, USA	2	8, 18	64
EUN1f	EU	IL, USA	3	14, 39, 80	64
ACN1$^{\underline{AG}}$	AC	PQ, CAN	1	55	80
TX38b$^{\underline{AC}}$	AC	PQ, CAN	1	55	81
TX41b$^{\underline{AC}}$	AC	PQ, CAN	1	55	81
TN18b$^{\underline{AC}}$	AC	PQ, CAN	1	30	unpublished
TX30$^{\underline{SA}}$	SA	PQ, CAN	1	25	unpublished
SCN10a	SC	PQ, CAN	1	14	unpublished
ACoN24d	ACo	France	2	8, 40	80
AGN24h	AG	France	2	8, 40	80
ARgP5$^{\underline{AG}}$	ARg	PQ, CAN	1	190	80
ARgX17c	ARg	PQ, CAN	2	26,28	81
CPX34g	CP	PQ, CAN	1	60	81

* The hosts are *C. peregrina, A. rugosa, A. rubra, E. umbellata, A. crispa, S. argentea, S. canadensis, A. cordata* and *A. glutinosa.*

is through the synthesis of restriction enzymes, endonucleases which cut DNA at specific sites. The way in which microorganisms protect their own DNA against these enzymes present in the cytoplasm is by modifying their DNA, adding a methyl group, for instance, to all cytosine residues, thereby preventing the recognition and cutting of the site by their own restriction enzyme. No such modified base has been detected by An[2] who used chromatography of total DNA of strain EUN1wb on a Deaminex A6 column. This, of course, does not mean that no *Frankia* phage exists in that strain.

Conjugation

Conjugative or sex plasmids have been extensively used in classical genetic studies, for instance, F′ plasmids that cointegrate with the chromosome and transfer it to the female cell have provided the main input for the construction of chromosomal linkage maps such as that of *E. coli* K-12[7]. They also accelerate the introduction into different hosts of DNA sequences too large to be manipulated *in vitro*. It was by using such sex plasmids that Johnston *et al.*[43] showed that symbiotic *R. leguminosarum* genes were plasmid-borne and that the host range of a given strain could be altered by introducing into it the appropriate plasmid from another *Rhizobium* species.

So far, there has been no indication that a conjugation system exists in any *Frankia* strain. The fact that known plasmids (Table 4) are all cryptic does not help in the search for conjugation. Similarly, no conjugation pili have ever been described in the admittedly mixed cultures that have been studied by electron microscopy[42,51,62].

Protoplasts

Protoplasts of *Frankia* have been worked upon for two reasons. First, they would provide the means of producing single cell colonies since abundant and synchronous spore germination has not been obtained. Second, they could be used to transform strains with naked DNA, such as has been done for *Streptomyces*[23]. They could also help in the search for the functions coded for by cryptic plasmids (Table 4) since plasmid curing has been done by protoplast formation and regeneration in *Streptomyces*[40].

Simonet *et al.*[80] showed that $10\,mg.ml^{-1}$ of lysozyme decreased the O.D. of a suspension of *Frankia* but that it could not succeed alone in forming protoplasts. Although hyphae are rapidly attacked by lysozyme, it would appear that vesicles and spores are more resistant as can be deduced by the persistance of refraction of these specialized structures after lysozyme and hot SDS treatment (unpublished observation). Furthermore, it has been shown by Simonet *et al.*[80] that achromopeptidase, an enzyme described by Ogawa *et al.*[65] (and produced by Wako Chemicals USA, Dallas, Texas) to be active in the formation of protoplasts from *Streptomyces*, was also quite active on *Frankia* cell wall, resulting, in conjunction with lysozyme, in the rapid and efficient formation of protoplasts.

However, Tisa and Ensign (Oral presentation, Laval 1984) found that low concentrations ($250\,\mu g.ml^{-1}$) of lysozyme alone resulted in the formation of viable protoplasts when using 4–6 day-old colonies of different strains from groups A and B grown in the presence of 0.1%

w/v glycine in a medium described earlier[82].

Protoplast regeneration of various *Frankia* strains have been obtained (Tisa and Ensign, Oral presentation, Laval 1984) under conditions comparable to those described for *Streptomyces*[23] with slight modifications. A soft agar overlay was essential, optimal pH was 6.5, optimal buffer was MOPS, optimal N-source was NH_4, optimal yeast extract concentration was 0.1% w/v, and the optimal regeneration temperature (25°C) somewhat lower than that optimal for growth (Tisa and Ensign, oral presentation, Laval, 1984).

The phenotype of these regenerated protoplasts has not yet been characterized with respect to symbiotic properties or genetic stability in pure culture; the results presented by Tisa and Ensign (Oral presentation, Laval 1984) were only preliminary but quite promising.

Selection markers

Resistance to antibiotics is most useful since it is not only a stable marker of strains, it also enables one to use positive selection in genetics work and more specifically in cloning.

The background sensitivity of different *Frankia* strains to various antibiotics was determined by Luc Simon in our laboratory (Table 5). Background resistance to *D*-cycloserine, ampicillin, penicillin G, rifamycin and tetracycline was found to be very high, thereby making their use as markers in mutant selection less interesting. On the other hand, gentamycin, kanamycin, neomycin, streptomycin, vancomycin and thiostrepton were deemed potentially useful since background resistance to them was minimal except for EUN1fS20, a mutant or a variant of EUN1f with a thousandfold increased resistance to streptomycin over that of its parent strain.

Such intrinsic antibiotic resistance results were used when pFQ31 was cloned into *E. coli* vectors (unpublished), *i.e.* the vectors used contained genes coding for resistance to antibiotics to which *Frankia* was sensitive.

Pigment synthesis has also been used in the genetic study of *Streptomyces*, for instance in the construction of cloning vectors with a tyrosinase gene from *S. antibioticus*[44]. Different pigments have been found to be synthesized by various *Frankia* strains and the genes coding for them may be useful in cloning.

Genes related to the symbiosis

The steps involved in the establishment of an effective symbiosis are numerous[27,90]. Some of the postulated genes and functions are described below.

Table 5. Background resistance to selected antibiotics of different *Frankia* strains*

Antibiotic	*Frankia* strain					
	ACN1ag	AGN1g	EAN1pec	EUN1fS20	EUN1f	ARgN22d
Ampicillin	100	10	10	10	nd	nd
D-Cycloserine	1000	1000	100	1000	nd	nd
Gentamycin	0.01	0.01	0.01	0.1	nd	nd
Kanamycin	0.1	0.01	0.01	0.01	nd	nd
Neomycin	0.1	0.1	0.1	0.1	nd	nd
Penicillin G	10	1	1	1	nd	nd
Rifamycin	100	10	1	10	nd	nd
Streptomycin	0.1	0.1	0.01	100	nd	nd
Tetracycline	100	100	1	10	nd	nd
Vancomycin	0.1	0.1	0.1	1	nd	nd
Thiostrepton#	nd	nd	nd	nd	0.05	0.5

* Normal growth after 60 days in liquid Qmod B medium containing X $\mu g \cdot ml^{-1}$.
# Tested on solid medium containing X ppm (normal growth after 30 days). nd, not done.

Early nodulation steps

Root hair deformation has been associated for a long time with the application of a *Frankia* inoculum to *Alnus* roots[11,45] but it was not clear whether the deformation was caused by *Frankia*[14] or by the concomitant application of plant compounds present in the crushed-nodule inoculum[69]. This deformation, however, appeared to be more limited in other actinorhizal genera such as *Comptonia*[19]. An *Alnus crispa* clone, AC4, in which very few root hairs were produced, was found to be non-nodulating except with one *Frankia* strain[85]. Callaham *et al.*[20] showed that a root hair deformation reaction was obtained with a *Frankia* pure culture and other authors showed that this deformation of *Alnus* root hairs could be induced at a distance from the *Frankia* cells growing on the root surface[18,89]. It has been shown that in the *Rhizobium* symbiosis, a similar reaction was coded for by microbial genes located close to other *nod* genes, and that the function was necessary for the establishment of nodules[73]. It has also been shown that in *Rhizobium*, that function was non-specific[74]. It would appear to be a straightforward experiment to see if the same is true for *Frankia* and this cloned *Rhizobium* gene might provide a good marker to screen *Frankia* nodulation genes.

Delays in nodule formation have been noted with some less compatible combinations of host plant and *Frankia* strains using crushed-nodule inocula[46]. In such cases, only the formation of the prenodule, or swelling on the parent root, was found to be delayed, the succeeding sequence of events (leading to the formation of a true effective nodule) occurring in the normal time scale with no increase in delay. Using pure cultures and pure spore suspensions, Nesme (unpublished) could

classify a large number of host plant-*Frankia* strain combinations with respect to nodulation delays. Such a phenomenon was shown to be genetically controlled in part by the microbial partner and not to be correlated with the growth conditions nor by the host plant from which the strains had been isolated. Such a trait must be considered in strain selection. Its use as a genetic marker, however, will be seriously hampered by the complexity and the time involved to measure this parameter.

Another set of functions that may be necessary for *Frankia* to establish an effective symbiosis is that related to phytohormone synthesis. It has been known for some time that the metabolism of auxins, gibberellins[93] and cytokinins[59] becomes modified in actinorhizal root nodules. Miguel *et al.*[59] also showed that root outgrowths looking externally very much like nodules were produced by the topical application of phytohormones. But the demonstration of *in vitro* synthesis of IAA in the presence of the precursor, tryptophan, shows that the genes coding for that symbiotic function are present in *Frankia* The amounts detected by Wheeler *et al.*[92] were very low, though, making it a not very convenient marker of symbiotic functions.

Host specificity

The question of host specificity has been thoroughly studied with the *Rhizobium*-legume symbiosis where it was used as the basis for species definition: *i.e. R. leguminosarum* is defined as nodulating peas; *R. trifolii,* clover; *R. melilotii* alfalfa, *etc.*[90]. However, it was found later that the *nod* genes were plasmid-borne and transferable, reducing the value of that species definition. In *Frankia,* no species are recognized in the genus at present because, among other reasons, of the confused situation regarding host specificity.

Crushed nodules were used prior to the isolation in pure culture of CpI1[19] to define cross-inoculation or host specificity groups. For instance, it was thought that *Myrica* spp. and *Alnus* spp. endophytes were different enough to be defined as species[10] on the basis of the very different shape of the vesicles and on the lack of cross-nodulation. That situation was modified with the use of pure cultures when it was shown that the two endophytes were cross-inoculable[46], and that soluble nodular compounds both reduced[8] and slowed down nodulation (Nesme, unpublished).

The host-specificity groups defined with crushed nodules are quite different from those determined with pure culture[86]. The current host-specificity results are summarized in Table 6. It can be seen that strains isolated from *Alnus, Comptonia* and *Myrica* on the one hand

Table 6. Host-specificity of pure-cultured *Frankia* strains

Host plant on which *Frankia* strain was tested	*Original* host plant from which Frankia strain was isolated																	
	AC	ARu	AG	AT	ASi	AS	ARb	CP	MG	MP	S	HR	EA	EU	CiS	CaE	CaJ	CaC
AC	e	e	e	e	i	e	e	e	e	e				n				
ARu	e	e	e		n		e	e		e								
AG	e	e	e	e		e	e	e	ei					i				
ASi			e	e		e												
AS						e		e										
ARb	e		e	e	i	e	e	e		e				n				
CP	e		e	e	i	e	e	e		e				n				
MG	e		e	e	e	e	e	e	ei	e				n		i		
MP								e		e								
S			n	n		n	n		e									
HR	n	n	n	n		n	n		e			e		e	e	e	n	en
EA			n	n		n	n				e	e	e	ei	e	e	n	
EU			n	n		n	n				e		e	ei				
CiS	n							n	n			e			e	e	n	
CaE												n			n	en	e	en
CaJ												n			n	n	n	
CaC																		en
	AC	ARu	AG	AT	ASi	AS	ARb	CP	MG	MP	S	HR	EA	EU	CiS	CaE	CaJ	CaC

Host plant abbreviations are *Alnus crispa* (AC), *A. rugosa* (ARu), *A. glutinosa* (AG), *A. tenuifolia* (AT), *A. sinuata* (ASi), *A. serrulata* (AS), *A. rubra* (ARb), *Comptonia peregrina* (CP), *Myrica gale* (MG), *M. pensylvanica* (MP), *Shepherdia* spp. (S), *Hippophae rhamnoids* (HR), *Elaeagnus angustifolia* (EA), *E. umbellata* (UE), *Colletia spinosissima* (CiS), *Casuarina equisetifolia* (CaE), *C. junghuniana* (CaJ) and *C. cunninghamiana* (CaC). . . . . .

e: effective nodulation; i: ineffective nodulation and n: no nodulation.

The source of the results summarized here is the catalogue of *Frankia* strains being compiled by M. P. Lechevalier (unpublished), and unpublished observations from our laboratory and other sources[9,19,28,36,46,48,63,66,95].

and those from *Shepherdia, Hippophae, Elaeagnus* and *Colletia* on the other, form two coherent cross-inoculation groups. The tests made with the one strain isolated from *Purshia* and with the one *Ceanothus* strain have been limited in scope to a few genera, while the situation with *Casuarina* strains is quite confused. About half of the *Casuarina* strains are not infective on that host while they are infective and effective on *Hippophae* and on *Elaeagnus.* However, the other half of the strains that were isolated, were infective and effective on *Casuarina* and non-infective on *Hippophae* and *Elaeagnus*[36,95]. This may indicate that more than one strain is present in actinorhizal nodules, especially those of *Casuarina,* and that the growth conditions are not adequate for some *Frankia* strains, for instance those from *Casuarina* and *Myrica* and also for *Alnus* Type P strains.

From the large number of actinorhizal plant genera described so far[1] only ten, or about half, have had their endophyte isolated (M.P. Lechevalier, unpublished). It is therefore impossible to have a comprehensive opinion about the genetic functions involved. Nevertheless, it would appear that at least three cross-inoculation groups exist in the genus, and that these host-specificity groups are based on *Frankia* genes or gene clusters. A small number of strains were found to cross these cross-inoculation barriers and these would be interesting to study but their description is beyond the scope of this review.

Such host specificity may prove useful in a cloning strategy, providing a strong selection pressure for recombinant clones.

Nitrogen fixation

The presence of nitrogen fixation genes in *Frankia* is the reason for its economic importance and for the interest in the genetic study of the symbiosis. At the moment, little is known about the *nif* genes of *Frankia,* except that there is homology between a *nif* K, D and H probe from *Klebsiella pneumoniae* (the probe pSA30 contains the genes coding for nitrogenase structural proteins) and two EcoRI fragments of 7.4 and 5.2 kb from CpI1 total DNA[72]. This homology has been used to identify a *nif* clone from a *Frankia* total DNA gene bank made in *Escherichia coli* (Ligan, Poster presented at the Fourteenth Steenbock Symposium on Nitrogen Fixation and CO_2 metabolism, June 1984, Madison, WI). Using this clone, a study of the organization of the *nif* cluster has been started.

The phenotype of reduced symbiotic nitrogen fixation noted with type P strains[63] is not, in all likelihood, related only to nitrogen fixation *per se* but also to host-endophyte compatibility and energy availability[78].

Late nodulation steps

Haemoglobin has the function of binding to gaseous O_2 and to carry it toward a sink in the organism. It has been most studied in animals but a very similar compound, with a haem ring and a protein moiety, has been detected in all leguminous nitrogen-fixing plants tested so far[83]. Its function is to supply the essential O_2 to the cells, but without the usual concomitant high pO_2 that would rapidly and irreversibly denature nitrogenase. Up till recently, it was generally thought that *Frankia* protected its nitrogenase in a manner similar to that of heterocystic cyanobacteria, that is, through the synthesis of thick walls in vesicles (Fig. 1) that establish a steep pO_2 gradient. The vesicle cell walls are thick enough to do that but haemoglobin has recently been detected in nodules from *Casuarina* and *Myrica* and as traces in nodules of *Elaeagnus* and *Alnus*[83]. Lalonde[46] showed that the same strain, CpI1, formed club-shaped vesicles in *Comptonia* nodules and spherical vesicles in *Alnus* nodules presumably responding to a host plant stimulus. Such a vesiculation reaction may be induced *in vitro*[68] using plant phenolic compounds. Vesiculation would therefore appear as a genetic function of *Frankia,* finely modulated by the plant.

It has been known for some time that *Frankia* strains could be induced to synthesize nitrogenase *in vitro* by reducing the amount of fixed nitrogen in the medium, resulting in the synthesis of provesicles[31] that differentiate into mature specialized vesicles[35,83]. Tisa and Ensign (Oral presentation, Laval 1984) also showed that isolated, *in vitro*-induced, vesicles could account for most, if not all, nitrogenase activity. It has also been shown recently (J. G. Torrey, pers. comm.) that the vesiculation function of strain CcI3 was not induced concomitantly with nitrogenase in the absence of oxygen in the medium. What does appear from the preceding facts is that vesiculation is a function necessary for the expression of nitrogenase genes only under certain conditions of high pO_2. But vesiculation also occurs in nitrogen-rich media such as Qmod B, and especially so with group A strains (Fig. 1B).

In a study of nitrogen fixing root nodules, Schubert and Evans[75] found that while a large proportion of *Rhizobium*-induced nodules released considerable amounts of hydrogen into the atmosphere, *Frankia*-induced nodules did not. Hydrogen formation is an unavoidable byproduct of dinitrogen reduction, but a recycling system based on a hydrogenase uptake protein (*hup* gene product) is synthesized by many nitrogen-fixing microorganisms to avoid the waste of energy involved. Using *in vitro* induced vesicles, it should also be possible to determine if the very efficient hydrogenase uptake system of

actinorhizal nodules[76] is synthesized by the host plant or by *Frankia*. *In vitro* hydrogenase uptake assays have been developed for *Rhizobium*[57]. These should be adaptable to *Frankia* making *hup* an interesting marker of genetic functions.

Most *Frankia* strains isolated so far have been found to sporulate (Fig. 1) in pure culture[19,48,63], and in actinorhizal nodules of most plants studied so far, spores have been seen, for instance in *Alnus*[88], *Myrica, Elaeagnus*[34], *Casuarina, Hippophae* and *Comptonia* (J. G. Torrey, pers. comm.). In actinorhizal nodules, however, the proportion of strains that sporulate is lower; even strains that sporulate *in vitro* do not do so *in vivo*[63]. Inhibition of that function was obtained *in vitro,* again by using plant phenolic compounds[68]. It would thus appear that most if not all *Frankia* strains have the necessary genes for sporulation, genes normally expressed *in vitro,* but that the host plant, maybe through the synthesis of the appropriate molecular signal (*e.g.* phenolics), inhibit the function. Furthermore, such *in vivo* sporulating strains (Type P) would respond differently to a given stimulus than the others. With the sporulation (Type P/Type N) phenotype have been correlated other traits: delayed nodulation (Nesme, unpublished), different *in vitro* morphology (Fig. 1C–D) and reduced nitrogen fixation[63]. These traits are rather inappropriate for commercial purposes. From an ecological point of view, however, Type P strains have been found to stay alive longer in soils without host plants[31] and were suggested to be more osmotolerant[39].

The transfer of nutrients from the host to the endophyte is a crucial step in the symbiosis. It would appear that the pectic capsule that surrounds hyphae and vesicles in the nodule[50] could serve to feed the endophyte which then transforms it into glucose and phospholipids and trehalose for storage[56]. It has been shown that *Frankia* could grow on pectin in pure culture[48]. Berry *et al.* (Oral presentation, Laval 1984) showed electron micrographs demonstrating that *Frankia* penetrated *Alnus* root hairs in the folds produced by the deformation. It had previously been shown that the penetration of host cell wall involve no mechanical disruption[49] and the existence of genes coding for pectinase and cellulase are thus good candidates as symbiotic genes.

Conclusion

Frankia has a large economic importance, matched only by the difficulties involved in working on it, especially when one wants to study its genetics. Despite the drawbacks that affect research on this organism, such as: its slow growth rate (average doubling time of 12–48 h[61,82]); its inability to form typical lawns; its low and asynchronous

spore germination rate, and its marked tendency to yield mutants or variants in culture, much work has been done on it recently. Many of the tools necessary for a genetic analysis have already been developed and others will be soon.

For instance, the broad outlines of the genetic organization of *Frankia* and of its symbiosis with actinorhizal plants are beginning to be understood. It is now well established that *Frankia* is a typical actinomycete in many respects[3]. Its genome size and DNA base composition, for instance, are normal by actinomycetal standards. The same can be said for the large evolutionary diversity in the genus reflected by DNA homology studies between strains[2] and its instability in pure culture[17].

Also, small *Frankia* plasmids have been isolated[64], cloned with various resistance markers added onto it (Normand, unpublished) and protoplast formation and regeneration has been achieved (Tisa and Ensign, Oral presentation, Laval 1984) making cloning into *Frankia* a near possibility. A replication origin, promoter sequences compatible with *Frankia* RNA polymerase, and a transformation procedure are, of course, still needed.

In many respects, the behaviour of *Frankia* parallels that of *Rhizobium*. The same kind of multi-step nodule establishment has been noted, with root-hair deformations[20], phytohormone involvement[93], synthesis of haemoglobin in the nodule[83], differentiation of the specialized structures that are vesicles and bacteroids, synthesis of nitrogenase and of hydrogenase uptake. Nevertheless, one must not be carried away by such analogies. From a genetic point of view, while it is true that pSA30 will hybridize with both *Rhizobium* and *Frankia* DNAs, a probe made with the nodulation genes of *R. japonicum* was found not to hybridize with *Frankia* total DNA (An *et al.*, Oral presentation, Laval 1984), and many steps in the establishment of both types of nodules are different[37]. Therefore, even though it is true that it would facilitate the study of the symbiosis if the *nod* and *nif* genes were situated on plasmids[71] as in most *Rhizobium* strains and species, there is no indication as yet that they are so situated in *Frankia*.

Species definition has not been made possible by the genetic work done on *Frankia* so far. Therefore, the debated question of the taxonomy of the genus[10,53] remains unresolved although some guidelines have been proposed[2].

The important goal to be achieved appears, at this point, to be the development of a genetic cloning system for use with *Frankia*, and then to use this sytem to clone and study the symbiotic genes. There exists a large natural reservoir of genetic diversity that has only begun to be

investigated. The exploitation of this wealth may result in more adapted, improved strains, and maybe, eventually to the extension of the host range of *Frankia.*

Acknowledgements PN received an NSERC of Canada scholarship and ML an NSERC of Canada grant (#U-0209). Thanks are also expressed to Luc Simon for communication of his unpublished results.

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