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Localization of *nif* genes on a large plasmid in *Frankia* sp. strain ULQ0132105009

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Summary. We report that in DNA from one *Frankia* sp. strain at least some of the *nif* genes are located on a large indigenous plasmid of 190 kb. Using the cloned nitrogenase structural genes from *Klebsiella pneumoniae* as hybridization probes, homology was detected with a 5.6 kb *Eco*RI fragment from the *Frankia* sp. plasmid. This 5.6 kb fragment was cloned; used as a probe it hybridized to the *nif K, D* and *H* genes from *Rhizobium meliloti* and *Klebsiella pneumoniae*. The *nif* probes also hybridized on total DNA blots to a 10 kb *Eco*RI fragment that is not present on plasmid DNA, suggesting that the *nif* genes could be located on more than one replicon.

Key words: *Frankia* – *Nif* genes – Nitrogen fixation – Plasmid – Actinorhizal symbiosis

Introduction

Bacteria in the genus *Rhizobium* fix nitrogen in symbiotic association with leguminous plants. Many other genera of woody dicotyledonous plants have also been found to have established another type of nitrogen-fixing symbiosis with the actinomycete *Frankia* sp. (Akkermans and Houwers 1979). This makes *Frankia* a useful organism for the study of the expression of nodulation genes. Because of problems in the isolation in pure culture of *Frankia* sp. strains and in the definition of their growth requirements, the genetical and physiological studies of the symbiosis are not well developed (for review see Normand and Lalonde 1986). However, because large sequence homology has been found between the *nif K, D* and *H* genes from *Klebsiella pneumoniae* (Cannon et al. 1979) and those of other nitrogen-fixing species (Ruvkun and Ausubel 1980) one of the first steps in studying the symbiotic genes is to localize and isolate the *Frankia nif* genes. Recently, using the cloned nitrogenase structural genes from *Klebsiella pneumoniae* (Cannon et al. 1979) as hybridization probes, conclusive evidence has been presented to show that some of the *nif* genes in the *Rhizobiaceae* family are very often present on large plasmids (Robson et al. 1983). In some *Frankia* sp. strains, plasmids ranging in size from 8 to 190 kb (Normand et al. 1983; Simonet et al. 1984) have been detected. We tested for the presence of *nif* genes on the largest one, pFQ69, of about 190 kb (Simonet et al. 1984), detected in strain ULQ0132105009, isolated from *Alnus rugosa* (Du Roi) Spreng (Normand and Lalonde 1982). We report here that at least some of these sequences are located on a large indigenous plasmid in *Frankia* sp.

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Materials and methods

Bacterial strains and culture conditions. Frankia sp. strain ULQ0132105009 was obtained from Département des sciences forestières, Université Laval, Qué., Canada and was cultured at 28° C, for 2 months in 100 l of Tween liquid medium (Simonet et al. 1985) to obtain 30 ml of compact cells. *Rhizobium meliloti* strains were obtained from Thierry Huguet (Laboratoire de biologie moléculaire des relations plantes-microorganismes, INRA-CNRS, Castanet Tolosan, France) and were cultured in TY medium (Rosenberg et al. 1981). *Escherichia coli* strains were obtained from Francoise Laval and Thierry Huguet (unpublished results, Laboratoire de biologie moléculaire des relations plantes-microorganismes, INRA-CNRS, Castanet Tolosan, France) and were grown on LB medium (Miller 1972).

Preparation and analysis of total and plasmid DNA. Thirty millilitres of ULQ0132105009 strain cells were used in the plasmid extraction procedure described earlier (Simonet et al. 1985). Total DNA was obtained according to the same procedure but without the alkaline denaturation step, and purification was obtained with several phenol extractions and EtOH precipitations. Plasmid DNA was then purified on a caesium chloride-ethidium bromide (CsCl-EtBr) density gradient (35,000 rpm in Tl 50 Kontron rotor for 36 h). For restriction enzyme analysis and cloning purposes, *E. coli* plasmid DNA was isolated by the alkaline extraction procedure of Birnboim and Doly (1979). For rapid screening of plasmids small preparations were obtained by the method of Holmes and Quigley (1981).

Restriction enzyme cleavage. Digestions of DNA with restriction endonucleases (Boehringer, Mannheim, FRG) were done as specified by the manufacturer. Agarose gel electrophoresis was performed as described by Maniatis et al. 1982.

Nick translation. Purified plasmids or restriction fragments, isolated from agarose gels by a squeeze-freeze procedure

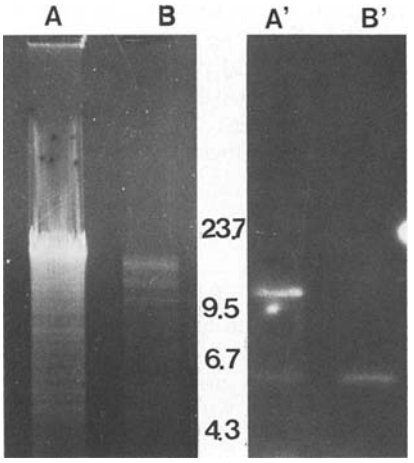

Fig.1. Agarose gel (*lanes A* and *B*) and autoradiogram (*A'*, *B'*) of *Eco*RI-digested total DNA from *Frankia* sp. strain ULQ0132105009 (*A*, *A'*) and its indigenous large (190 kb) plasmid (pFQ69) (*B*, *B'*). The digested DNA was electrophoresed for 3 h on 0.8% w/v agarose gels and transferred to a nitrocellulose filter. Hybridization was performed at 65° C for 15 h in the presence of 6× SSC, 1× Denhardt's solution, 0,1% SDS, 50 µg/ml of salmon testes DNA. The autoradiogram shows hybridization of ^{32}P-labelled *Klebsiella pneumoniae nif* DNA with a 5.6 kb *Eco*RI DNA fragment from plasmid pFQ69 (*B'*) and with total DNA (*A'*). A second hybridizing band (10 kb) was present on total DNA but not on plasmid DNA. The positions of lambda *Hin*dIII size standards are shown

(Tautz and Renz 1983), were labelled with α ^{32}P-dCTP (3,000 Ci/mmol, Amersham International, Amersham, England) by nick translation (Rigby et al. 1977). Specific activities of 50 µCi/µg DNA were obtained.

Southern transfer and hybridization. Transfer to nitrocellulose filters was done as described by Southern (1975) with a partial depurination step when non-digested plasmids were transferred (Whal et al. 1979; Singh et al. 1983). Prehybridization (65° C, 3 h) and hybridization (65° C, 15 h) were performed in 6× SSC, 1× Denhardt's solution (Denhardt 1966), 0.1% sodium dodecyl sulphate (SDS) and 50 µg/ml of salmon testes DNA. (SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 7.0.) Usually, the blots were washed with one change of 6× SSC, 0.1% SDS and 1× Denhardt's solution at 65° C for 15 min, then with six changes of 6× SSC, 0,1% SDS at 65° C for 30 min. Conditions of high stringency were realized with low concentrations (2, 0.4, 0.1 and 0.05×) of the SSC solution. Autoradiography was carried out at −80° C for 3 h to 7 days on Kodak Xomat AR film.

Cloning procedure. Using the restriction enzyme *Eco*RI, we constructed a bank of 500 *E. coli* colonies containing cloned fragments of the plasmid pFQ69. The cloning vector, pBR328, was dephosphorylated using calf intestinal phosphatase (Boehringer, Mannheim, FRG). Ligation was performed overnight at 15° C with one unit of T4 DNA ligase (Boehringer, Mannheim) as specified by the manufacturer and *E. coli* strain ED8767 (Murray et al. 1977) was transformed (Maniatis et al. 1982) with the recombinant plasmids. Because pBR328 has a single *Eco*RI site within the gene coding for chloramphenicol resistance, recombinant plasmids conferred resistance to ampicillin (50 µg/ml), and tetracycline (15 µg/ml) and sensitivity to chloramphenicol (30 µg/ml) on the transformants.

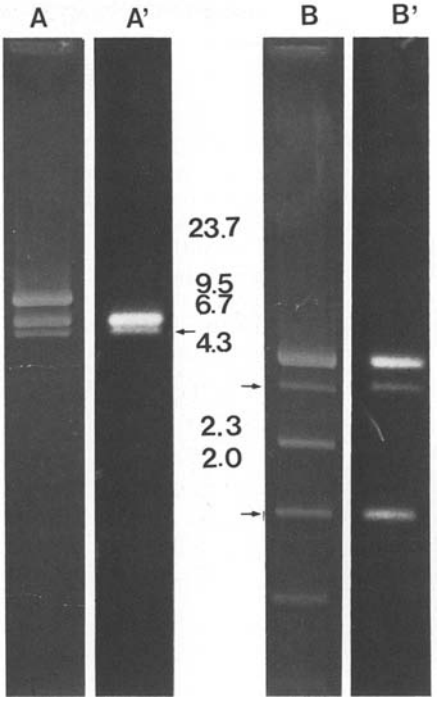

Fig. 2. Hybridization between ^{32}P-labelled cloned *nifD* and *H* DNA from *Rhizobium meliloti* (plasmid pRmR2) and pFQ307 plasmid DNA constructed with *Eco*RI fragments of plasmid pFQ69. Using the restriction enzyme *Eco*RI, we constructed a bank of *E. coli* colonies containing cloned fragments of plasmid pFQ69. Out of 100 colonies screened, only one contained DNA fragments that hybridized with the two *nif* probes used. pFQ307 possesses four *Eco*RI insert fragments (*lane A*): two identical 5.6 kb and two identical 8 kb fragments. Only the 5.6 kb fragments hybridized (*A'*) with the *nif* probe used. A negative control was given by the 8 kb fragments which showed no homology. The 5.6 kb *Eco*RI fragments possess one *Bam*HI site (*B*) generating two subfragments (1.6 and 4.0 kb) hybridizing (*B'*) with the *nif* probe. Three *Bam*HI sites are present on the 8 kb fragment generating four subfragments (3.9, 2.1, 1.5 and 0.5 kb). The *nif* DNA fragment from plasmid pRmR2 was purified from an agarose gel by the sqeeze-freeze procedure (Tautz and Renz 1983). Contamination with the pACYC184 vector occurred resulting in hybridization with the pBR328 vector from pFQ307. pACYC184 DNA used as a probe showed no homology with *Frankia* sp. DNA (results not shown). *Arrows* indicate the position of the pBR328 vector

Results

Hybridization of total DNA and pFQ69 DNA from Frankia *sp. strain ULQ0132105009 to* nif *genes*

Total DNA and pFQ69 plasmid DNA from *Frankia* sp. strain ULQ0132105009 was digested with *Eco*RI, separated by electrophoresis in agarose gels and transferred to nitrocellulose membranes. The *Klebsiella pneumoniae* DNA probe was a purified 6.6 kb *Eco*RI fragment of pSA30, containing the structural genes *nif K*, *D* and *H*. This ^{32}P-labelled *nif* DNA hybridized with a 5.6 kb *Eco*RI fragment of plasmid pFQ69 DNA. An additional 10 kb band as revealed when hybridizing the total DNA (chromosome plus plasmid) of strain ULQ0132105009. This indicates the presence of DNA homologous to the *nif* genes on both the *Frankia* sp. genome and the DNA of the large plasmid (pFQ69; Fig. 1). When total plasmid pFQ69 DNA was used as a probe, a strong hybridization signal was detected

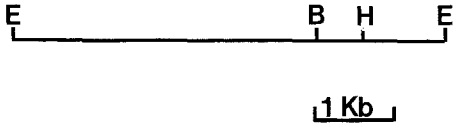

Fig. 3. Partial physical map of the cloned 5.6 kb *Eco*RI DNA fragment from the indigenous *Frankia* sp. plasmid pFQ69 which showed homology with the *nifD* and *H* genes from *Rhizobium meliloti* and the *nifK, D* and *H* genes from *Klebsiella pneumoniae*. Restriction sites: E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII. *Hin*dIII recognizes the sequence A/AGCTT. *Frankia* sp. strains DNA that have a high G+C content (around 70%; An et al. 1985) show no site for this restriction endonuclease. The *Hin*dIII site detected on the 5.6 kb *Eco*RI insert is the first detected in *Frankia* sp. DNA

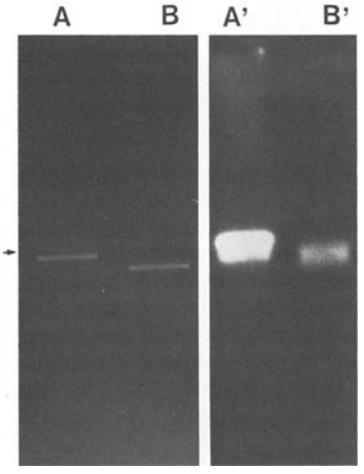

Fig. 4. Hybridization of *Rhizobium meliloti nifDH* (pRmR2) and *nifKD* (pLH3) with a ^{32}P-labelled 5.6 kb *Eco*RI insert cloned from the indigenous *Frankia* sp. plasmid pFQ69. Restriction fragments were generated by digestion with the enzyme *Eco*RI (pRmR2) or *Hin*dIII (pLH3) and separated on a 0.8% w/v agarose gel. Insert fragments (3.7 kb for pRmR2, *lane A*; 3.45 kb for pLH3; *lane B*) were purified by the squeeze-freeze procedure and again electrophoresed. DNAs were transferred to nitrocellulose and hybridized (*A'*, *B'*). Because contamination had occurred with the pACYC184 vector of pRmR2, a second hybridizing band (4.0 kb) was detected in *A'*. *Arrow* indicates position of pACYC184 vector

with the *nif Eco*RI fragment from pSA30 (results not shown), confirming that *Frankia* sp. *nif* sequences are located on the indigenous large plasmid pFQ69. Moreover, the same probe hybridized to the DNA of pSym megaplasmids from *Rhizobium meliloti* strains 2011, RM41 and L5 30 where *nif* sequences have been located.

Cloning of a Frankia *sp. plasmid DNA fragment homologous to* nif *probes*

We constructed a DNA library of *Eco*RI-digested plasmid pFQ69, using pBR328 as vector. One hundred individual clones from the library were assayed for the presence of *nif* genes using ^{32}P-labelled *Klebsiella pneumoniae nif* DNA and ^{32}P-labelled *Rhizobium meliloti nif D* and *H* DNA (plasmid pRmR2; Ruvkun et al. 1982) as hybridization probes on duplicate filters. Of the 100 colonies screened, only one contained DNA fragments (5.6 kb *Eco*RI inserts) that hybridized with the two *nif* probes used (results not shown). We digested the DNA of this plasmid, pFQ307, with *Eco*RI/*Bam*HI and hybridized again with the *Rhizobium meliloti nif* probe. We found two *Bam*HI subfragments of 4.0 and 1.6 kb that exhibited homology with *nif D* and *H* DNA (Fig. 2). A partial physical map of the 5.6 kb *Eco*RI insert is given in Fig. 3.

To confirm that some *Frankia nif* genes were located on this 5.6 kb fragment from the indigenous plasmid, we nick translated the *Eco*RI fragment and hybridized it with different fragments carrying *nif* genes from *Rhizobium meliloti*. Homology was detected (Fig. 4) with fragments from pRmR2 (*nif D* and *nif H* genes) and pLH3 (*nif K* and *nif D* genes). Identical Southern blots were washed in buffers of different ionic strength. The intensity of the heterologous *nif* hybridization band was unchanged in 2×, 0.4× and 0.1× SSC (results not shown) and decreased only in 0.05× SSC indicating that a large homology exists between the *nif K, D* or *H* genes from *Rhizobium meliloti* and the *Frankia* sp. strain ULQ0132105009 *nif* genes.

The same probe hybridized to the covalently closed circular (CCC) band of plasmid pFQ69, confirming that this cloned *Frankia* sp. *nif* fragment came from an indigenous plasmid (Fig. 5, lanes C and C'). With strain

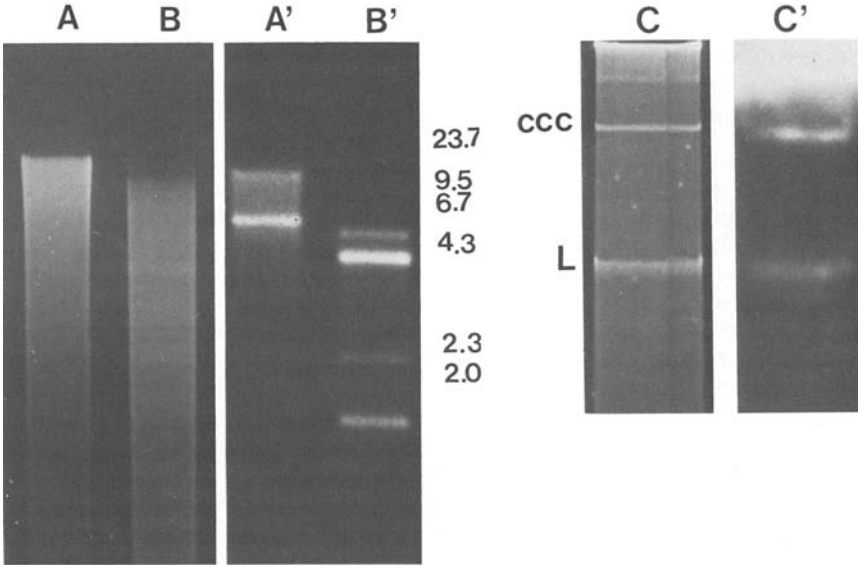

Fig. 5. Hybridization of *Frankia* sp. DNA with the ^{32}P-labelled 5.6 kb *Eco*RI insert of pFQ307, homologous to *nif* genes from *Rhizobium meliloti*. *A–C*, agarose gel electrophoresis; *A'–C'*, autoradiograms. *Frankia* sp. strain ULQ0132105009 DNA was digested by *Eco*RI (*A*) and *Eco*RI–*Bam*HI (*B*). Hybridization (*A'*) revealed the homologous 5.6 kb *Eco*RI fragment to be located on the plasmid, that fragment generated two *Eco*RI–*Bam*HI subfragments (4.0 and 1.6 kb; *B'*). The 10 kb *Eco*RI fragment (*A'*) located on the chromosome or on a larger plasmid (see text) generated three hybridizing *Eco*RI–*Bam*HI subfragments (4.5, 3.4 and 2.1 kb). Hybridization of the covalently closed circular (CCC) band (*C* and *C'*) confirms that the *nif* sequences detected on the 5.6 kb insert are located on pFQ69, the indigenous plasmid of *Frankia* sp. strain ULQ0132105009

ULQ0132105009 *Eco*RI-digested total DNA, hybridization occurred with the homologous sequences (5.6 kb *Eco*RI fragment and 4.0 and 1.6 kb *Eco*RI-*Bam*HI subfragments; Fig. 5) and also with a second band. This 10 kb *Eco*RI fragment corresponds to the band detected only in *Eco*RI-digested total DNA with the *Klebsiella pneumoniae nif* probe shown in Fig. 1. Thus with both the *Klebsiella pneumoniae* (pSA30 6.6 kb insert fragment; Fig. 1) and *Frankia* sp. (pFQ307 5.6 kb insert fragment; Fig. 5) *nif* probes, it has been shown that in this *Frankia* sp. strain, *nif* genes are located on plasmid DNA (pFQ69 plasmid) and on another replicon – the chromosome or another undetected larger plasmid. Such sequence reiterations have recently been described in *Rhizobium fredii* (Barbour et al. 1985) and in *Rhizobium phaseoli* (Quinto et al. 1982).

Discussion

In *Rhizobium* species *nif* and other symbiotic genes (required for the establishment of the symbiosis) have been shown very often to be located on plasmids (Robson et al. 1983). But in *Lignobacter* K17 (Derylo et al. 1981) and *Enterobacter agglomerans* (Singh et al. 1983) *nif* genes are also located on plasmids, showing that the incidence of plasmid-borne *nif* genes is not restricted to the *Rhizobiaceae*. Our results show that in the other type of nitrogen-fixing symbiosis, between woody dicotyledonous plants and the actinomycete *Frankia* sp., some of the *nif* genes are located on plasmids. For other smaller *Frankia* sp. plasmids tested for homology with pSA30, negative results were obtained (not shown). However, the isolation procedure used does not allow the routine detection of very large plasmids, pFQ69 being near the borderline. With a variation of Eckhardt's procedure (Simonet et al. 1984), a plasmid larger than 190 kb has been detected a few times in other strains (results not shown) and pFQ69 could be a deletion mutant of a symbiotic plasmid generally present in *Frankia* sp. With these results on *Frankia* sp. strain ULQ0132105009 DNA, plasmid-borne *nif* genes would appear more and more to be a common feature in the bacterial world.

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