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Diversity of *Frankia* Stains Isolated From Actinorhizae of a Single *Alnus rubra* Cultivated in Nursery

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Abstract

Diversity of *Frankia* strains, isolated from root nodules of an *Alnus rubra* cultivated in nursery, has been estimated by comparing their protein and enzyme electrophoretic patterns. Protein patterns showed a large variation among the strains which was confirmed by the six enzymatic systems investigated. These electrophoretic analysis proved the diversity of these *Frankia* strains. The polymorphism observed with enzymes such as malate dehydrogenase, leucin aminopeptidase and phosphoglucomutase was lower than those recorded with diaphorase and esterases which gave multiple band patterns.

Keywords: *Alnus rubra*, electrophoresis, enzymes, DIA EST LAP MDH PGI PGM, *Frankia*, proteins

Abbreviations: DIA, diaphorase; EST, esterase; LAP, leucin aminopeptidase; MDH, malate dehydrogenase; PGI, phosphoglucoisomerase; PGM, phosphoglucomutase

1. Introduction

The first *Frankia* strain, a low growing actinomycete, able to form nitrogen fixing nodules on actinorhizal plants, was isolated by Callaham et al. (1978). Since, hundreds of strains have been isolated from various actinorhizal plant species (Baker and Torrey, 1979; Lalonde et al., 1981; Benson, 1982; Diem et al., 1982; Faure-Raynaud and Moiroud, 1983; Lechevalier, 1984).

Infectivity and effectivity studies have demonstrated heterogeneity among pure-culture *Frankia* isolates (Normand and Lalonde, 1982; Burggraaf and Valstar, 1984). Diversity of pure-culture *Frankia* strains has also been demonstrated in terms of morphology, physiology, biochemistry and cellular proteins (Baker et al., 1981; Benson and Hanna, 1983; Lechevalier et al., 1983; Lalonde et al., 1988). Analysis of protein patterns obtained by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Benson and Hanna, 1983) have been shown to be a useful tool to characterize *Frankia* strains (Benson et al., 1984; Gardes and Lalonde, 1987). In addition isozyme studies of *Frankia* isolates have presented evidence for diversity in some enzymes and their usefulness in the identification of *Frankia* strains (Puppo et al., 1985; Steele and Stowers, 1986; Gardes et al., 1987; Lalonde et al., 1988).

The nitrogen fixing ability of actinorhizal symbiosis and the potential for alder to colonize a large variety of soils and to increase soil nitrogen content explain their ecological importance. These actinorhizal plants are also economically important (Gordon and Dawson, 1979) by their utilisation in forestry programmes for forest preservation, biomass production or soil revegetation.

In the *Frankia*-actinorhizal symbiotic association, the concept of diversity affects microorganisms as well as host plants. The diversity, fundamental principle in ecology, occurs in the living world balance (Cauderon, 1987) and to preserve it, it is important to know characteristics of each member. *Frankia* symbiosis exhibits differences in plant growth, nodulation and nitrogen fixation with plant species and *Frankia* strains combination (Normand and Lalonde, 1982; Dawson and Sun, 1981; Redell and Bowen, 1985) and it is important to ascertain characteristics of each strain to realise the best association. Such studies need pure cultures of *Frankia* and cloned host plants. In identifying *Frankia* isolates it is necessary to recognize and select the most competitive, infective and effective strains to achieve an improved symbiosis. If natural *Frankia* population in soil exhibits diversity, different strains can inoculate the same root system and interact for many years. *Frankia* strain diversity in an alder stand has been described (Benson and Hanna, 1983; Simonet et al., 1989), but strain diversity on the same root system has not been studied. This paper reports a study of the diversity in *Frankia* strains isolated from a single *Alnus rubra* Bong. root system. Indeed for the past few years, *Alnus rubra* has been used in French programmes in improvement of forest trees for short term biomass production (Teissier du Cros, 1981) because of the high productivity of this species on poor sites.

2. Materials and Methods

Frankia *strains and culture conditions*

The *Frankia* strains analysed in this study have been isolated from a one years old *Alnus rubra* Bong. grown in the nursery of the INRA station of Ardon (France). The stains were obtained from 20 actinorhizae collected from this alder root system using the Lalonde et al. (1981) isolation nodules technique. Some of these isolations finally being unsuccessful, the 13 remaining isolates have been studied (Table 1).

All the strains were grown on F liquid medium added with Tween 80 (Faure-Raynaud et al., 1984) in flasks and incubated at 28°C. Each flask containing 400 ml of medium was inoculated with 10 ml of *Frankia* suspension from 18–20 day old colonies broken with a plastic syringe.

Electrophoresis

Preparation of extracts

For each *Frankia* strain, extracts were prepared from 3 to 4 weeks old cultures, according to the low *Frankia* development. The cultures were harvested,

Table 1. *Frankia* strains used, all isolated from the same *Alnus rubra* Bong.

Host plant	Strain designation	Laboratory code
A. rubra[1]	Ar 24 D_2	1
	Ar 24 D_7	2
	Ar 24 D_9	3
	Ar 24 D_{13}	4
	Ar 24 D_4	5
	Ar 24 D_{10}	6
	Ar 24 D_{11}	7
	Ar 24 D_{14}	8
	Ar 24 D_{20}	9
	Ar 24 D_8	10
	Ar 24 D_{12}	11
	Ar 24 D_{16}	12
	Ar 24 D_{19}	13

[1] Seedlot no. 81 041 3, Washington USA, 46°53 S, 128°38 E, 171 m.

centrifuged at 8300 g for 20 min at 4°C, and washed two times with distilled water. Cells were suspended in 62 mM Tris-HCl buffer, pH 6.8 and disrupted by sonication on ice for twice 3 min at 50 W (Labsonic 1510, B. Braun, Melsungen AG). The extracts were centrifugated at 20000 g for 5 min at 4°C and the supernatants centrifuged again for 30 min using the Ti rotor of a Beckman ultracentrifuge at 80000 g. The resulting clear supernatants were stored at −80°C until analysis.

Protein determination

The proteins present in the extracts were concentrated by incubation with acetone in 1/5 ratio (Hames, 1981). The mixture was centrifuged, the supernatant discarded and the remaining acetone was evaporated *in vacuo*. The resulting pellet was resuspended in 62 mM Tris-HCl buffer (pH 6.8) completed with 2.3% (w) SDS and 5% (v) β-mercaptoethanol. Samples were boiled for 3 min prior to electrophoresis.

SDS-PAGE

Thirteen samples per gel were run on a discontinuous slab gel as described by Laemmli (1970) and Gardes and Lalonde (1987). In each cell, was loaded approximately 25 μg of protein estimated by Bradford procedure (1976). Electrophoresis was run at 20°C and 25 mA until the tracking dye (Bromophenol Blue) had reached the bottom of the running gel, about 10 to 12 cm.

Straining of proteins

The gels were stained in 0.1% (w/v) Coomassie blue (Coomassie Blue R 250, Sigma) in a water:methanol:glacial acetic acid (5:5:5 v/v) mixture for 1 hr at 37°C. Then the gels were destained in the following mixture of water: methanol:glacial acetic acid (6:3:1 v/v) until the background was suitably clear. A reference protein mixture was loaded near the samples (Dalton Mark VII-L^{TM}, Sigma).

Enzyme systems determination

Frankia strains were tested for six enzyme systems described by Gardes et al. (1987): malate dehydrogenase (MDH; EC 1.1.1.37), diaphorase (DIA; EC 1.6.4.3), phosphoglucomutase (PGM; EC 2.7.5.1), esterases (EST; EC 3.1.1.1), leucin aminopeptidase (LAP; EC 3.4.11.1), phosphoglucoisomerse (PGI; EC 5.3.1.9).

PAGE

These enzyme systems were studied by discontinuous nondenaturing polyacrylamide gel electrophoresis which was carried out as described by Gardes et al. (1987). Thirteen samples were loaded per gel. The gels were run at 4°C under a constant current at 25 mA until the tracking dye reached the bottom of the gel.

Staining of enzyme systems

After electrophoresis, the gels were removed from the glass plates and placed directly into substrate solutions for enzyme staining. Histochemical reactions on the polyacrylamide gels were similar to those described in Pasteur et al. (1987). Extracts of each strain were subjected to electrophoresis at least three times to establish validity of data for each analysis of soluble protein or enzyme systems.

Numerical analysis

The term electromorph is used in this paper to designate the different patterns observed for one enzyme. Estimates of electromorph diversity (H) were calculated for each enzyme by the following formula: $H = 1 - Rx_i^2$, where x_i is the frequency of the ith electromorph (Gardes et al., 1987).

Jaccard similarity coefficients for all possible pairs of isolates were estimated as described by Gardes et al. (1987), involving the number of electromorphs shared divided by the total number of enzymes compared. These coefficients were deduced from data concerning combinations of electromorphs obtained for different enzymes analyzed (Table 2).

3. Results

Proteins

Identification of strains were performed by visual comparison of the electrophoregrams obtained for each strain. Only presence or absence of bands were compared and not their intensity of staining. Figure 1 shows the electrophoregrams of 12 of the strains isolated from *A. rubra*. A careful examination of the patterns revealed numerous protein band differences between the strains. Some bands with Rf values of 0.16–0.53 and 0.76 were present in all isolates whereas the band with Rf 0.33 was only present in some strains such as 1 – 4 – 9 and 10. A great homology is observed between strains 3 and 7 protein patterns, differences between them consisting of

Table 2. Electromorph types detected for 13 *Frankia* strains and electromorph diversity of six enzyme systems

Strains[1]	Electromorph rating for the following enzymes[2]					
	MDH	DIA	PGM	EST ind.	LAP	PGI
1	2	6	2	1	2	2
2	3	3	3	4	2	3
3	3	3	3	7	2	4
4	1	7	3	1	1	1
5	3	10	1	9	3	6
6	3	8	1	10	–	6
7	3	9	4	6	2	6
8	3	2	4	3	2	6
9	3	4	5	2	4	2
10	2	5	2	1	1	3
11	3	1	–	3	2	6
12	3	3	1	5	2	5
13	3	3	1	8	2	6
No. of strains with detectable activity	13	13	12	13	12	13
No. of electromorphs	3	10	5	10	4	6
Electromorph diversity (H)	0.400	0.853	0.764	0.876	0.515	0.605

[1] Laboratory code for strain designation
[2] Electromorphs were numbered in order of decreasing anodal mobility (Gardes et al., 1987)

9 bands among the 66 numbered. Strains showing greatest differences were strain 4 and strain 9. Strain 4 showed differences from the others by 3 bands (Rf 0.45 – 0.48 – 0.57) noticed on its pattern and by the absence of one band (Rf 0.30) present on the other electrophoregrams (Fig. 1). Strain 9 did not seem to have the Rf 0.25 and 0.62 bands while the Rf 0.82 and 0.88 bands were clearly marked on the pattern of this strain (Fig. 1). When observing carefully the protein patterns of these isolates, they all looked different.

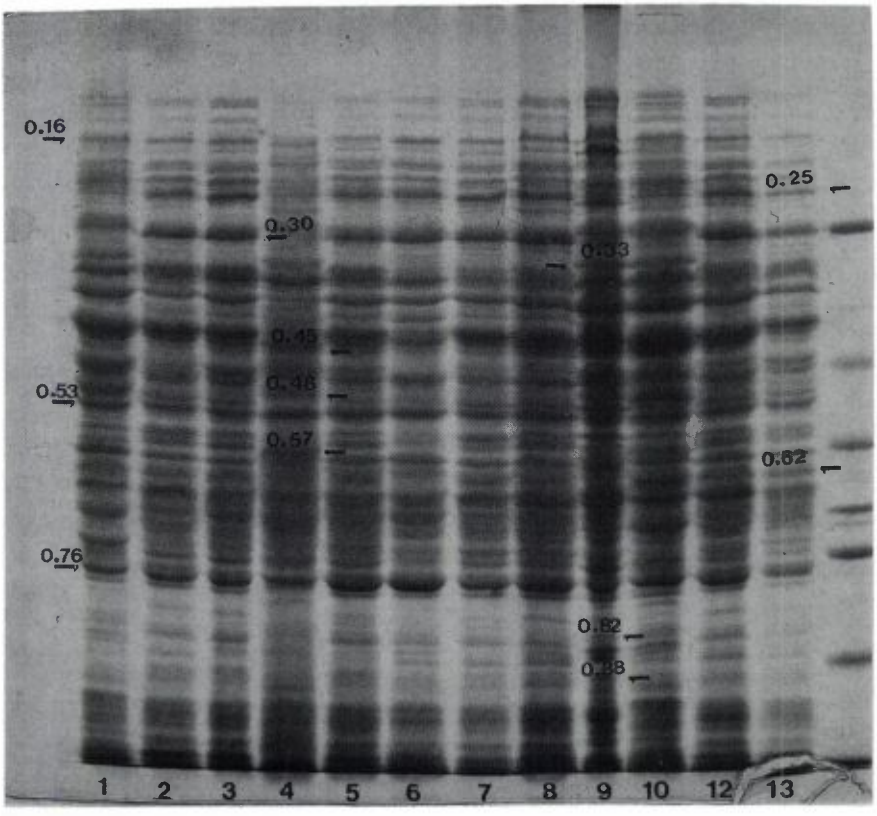

Figure 1. SDS-PAGE of soluble proteins from *Frankia* strains after 3 weeks of growth in F medium. Sample containing standard proteins was loaded in right well: from the top to the bottom, these proteins were: albumin bovine (66 kDa), albumin egg (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor soybean (20 kDa) and α-lactalbumin (14.2 kDa). *Frankia* strains were designated from 1 to 13. Migration was toward the anode at the bottom.

Enzyme system determination

Characterization of enzyme bands

Most of the strains exhibited activity for all the enzymes studied (Table 2), stains 6 and 11 were exceptions, for the respectively, LAP and PGM scored negatively.

MDH. Except for strain 4 which has another faster migrating band, each strain was characterized by a single band pattern of MDH (Fig. 2). Three different mobilities were observed. Three electromorphs were noted (Table 2).

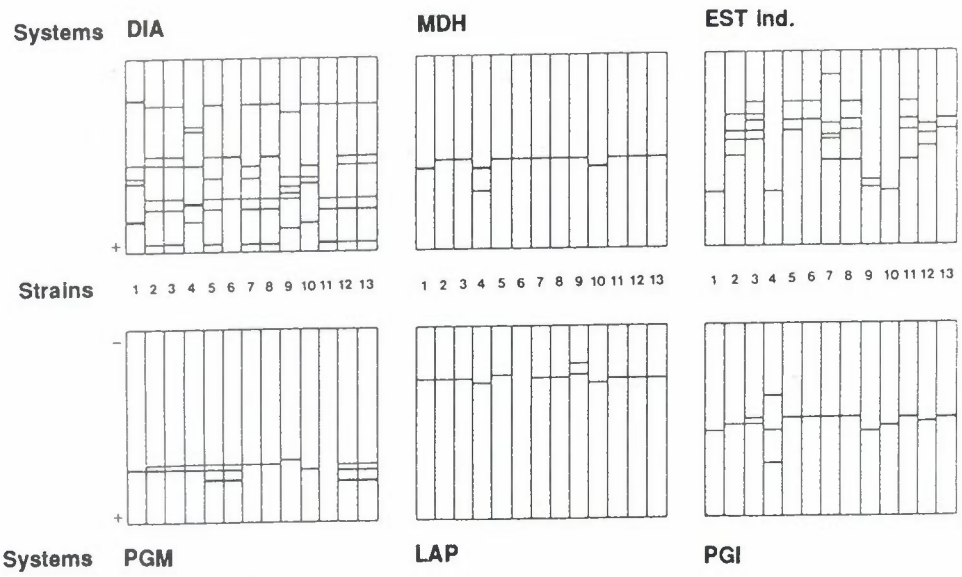

Figure 2. Schematic representation of the diaphorase (DIA), malate dehydrogenase (MDH), esterase (EST indoxyl), phosphoglucomutase (PGM), leucin aminopeptidase (LAP) and phosphoglucoisomerase (PGI) zymograms produced by electrophoresis of cell-free extracts from *Frankia* strains. Migration was toward the anode at the bottom.

DIA. For diaphorase activity, each of the strains had several isozymes, variable in number and mobilities (Fig. 2), 17 bands more or less intensely stained were numbered. A total of ten electromorphs were observed (Table 2).

PGM. No PGM activity was detectable for strain 11. For the others, one, two or three bands were observed (Fig. 2). Five electromorphs were noted (Table 2).

EST. Esterase enzymes were located by using indoxyl acetate as substrate. Striking variation was noted among strains (15 bands) (Fig. 2). A total of ten electromorphs was observed (Table 2).

LAP. No LAP activity was detectable for strain 6. The other strains, except the ninth which has also a slower cathodal band, were characterized by a single band with three different mobilities (Fig. 2). Four electromorphs were noted (Table 2).

PGI. Most of the strains had a single band for PGI activity for which three mobilities were noted (Fig. 2). For strain 4, two other bands were observed. A total of six electromorphs were detected (Table 2).

Diversity of strains

Frankia isolates diversity was shown by the analysis of the studied enzymatic system patterns. With some of them, as MDH and LAP, a single band was observed. For most of the strains, many isolated having the same patterns formed groups. With more polymorphic enzymatic systems, as DIA and EST-ind, for which several isozymes were detectable, dissociation of these groups was observed, each of the strains showing a particular pattern. Referring to the triangular matrix of pairwise Jaccard similarity coefficients (Table 3) strains 13, 11, 8, 7, 12 and 2 seem to be the less divergent, gathering the highest similarity coefficients. Likewise, the most divergent isolates are strains 9 and 4 which have their own patterns for respectively four (DIA – EST-ind – PGM and LAP) and three (DIA – MDH and PGI) enzymatic systems, followed by strains 10 and 1 which are very divergent too. Consequently, the lowest similarity coefficients are involved for these strains (Table 3). Strains 9 and 4 discrepancy has already been observed on protein patterns (Fig. 1).

The diversity of *Frankia* strains used in this study was high (Table 2), the average diversity per enzyme being 0.68. For PGM and PGI activities, moderate levels of diversity were found, the lowest estimates were noted for LAP

Table 3. Triangular matrix of pairwise Jaccard similarity coefficient between strains, as deduced from data in Table 2

Strain	7	8	11	12	13	6	5	2	3	9	1	10	4
7	1												
8	0.67	1											
11	0.60	0.80	1										
12	0.33	0.33	0.40	1									
13	0.50	0.50	0.60	0.67	1								
6	0.40	0.40	0.50	0.40	0.60	1							
5	0.33	0.33	0.40	0.33	0.50	0.60	1						
2	0.33	0.33	0.40	0.50	0.50	0.20	0.17	1					
3	0.33	0.33	0.40	0.50	0.50	0.20	0.17	0.67	1				
9	0.17	0.17	0.17	0.17	0.17	0.20	0.17	0.17	0.17	1			
1	0.17	0.17	0.20	0.17	0.17	0	0	0.17	0.17	0.17	1		
10	0	0	0	0	0	0	0	0	0	0	0.50	1	
4	0	0	0	0	0	0	0	0	0.17	0	0.17	0.33	1

and MDH activities. On the contrary, EST-ind and DIA, for which a large number of electromorphs was noted, had the highest estimates of diversity. In such studies, these enzymatic systems were the most discriminating.

4. Discussion

The determination of diversity among related microorganism strains by protein patterns, has been used in *Rhizobium* studies (Noel and Brill, 1980). It was extended by Benson and Hanna (1983) to actinorhizal endophytes for estimating the diversity of *Frankia* strains isolated from an alder stand and by Gardes and Lalonde (1987) for identification of *Frankia* strains from various host plants. We found this technique suitable to rate the strain diversity level of *Frankia* present on a single tree root system. The results obtained by gel electrophoresis of soluble proteins showed a large variety among the *Frankia* strains isolated from a single host. A careful examination of these protein patterns showed that each stain was distinguishable from each other. These results were different from those of Benson and Hanna (1983) who found among 43 isolates, 35 strains with the same protein patterns. Gardes et al. (1987) found some differences between strains belonging to the same alder species as *A. rugosa* or *A. crispa*, separating them into subgroups; although they had great homology in their protein patterns, some strains of these subgroups showed some specific bands.

Interpretation of protein patterns is tedious and delicate and some bands could be missed due to staining. One-dimensional electrophoresis is also limited in its resolution. Therefore, the strains were also evaluated by enzyme electrophoresis. The *Frankia* strains diversity, showed by whole cell protein electrophoresis, was confirmed by enzyme electrophoresis analysis, the average diversity per enzyme being 0.68. This strain heterogeneity was less visible for enzymes such as MDH, LAP and PGI, a single band pattern being observed for most of the strains. In agreement with Gardes et al. (1987), we observed the multiple electrophoretic band patterns given by DIA and EST. The diversity was directly demonstrated by a large isoenzymatic dissimilarity among *Frankia* strains. The analysis of six enzymatic systems has shown that each of these strains was different from the others.

The diversity ($H = 0.68$) among strains isolated from a single root system is as large as that noted by Gardes et al. (1987) in the study of strains belonging to various plant species ($H = 0.70$). Indeed, the number of electromorphs noted for the most discriminating enzyme systems, DIA and EST, for *Frankia* strains isolated from different *Alnus* species (Gardes et al., 1987) and from a single root system are quite similar. However, when less discriminating

enzyme systems, such as LAP, MDH or PGM are considered, *Frankia* strains isolated from a single root system seem to be more homogeneous than isolates from different alder species (Gardes et al., 1987).

High diversity among *Frankia* strains, isolated from young alders growing in several plots in the nursery of the same INRA Station, was demonstrated by Simonet et al. (1969). Plasmid analysis and hybridization data led Simonet et al. (1989) to delineate 7 groups among 12 isolates extensively studied: 3 groups including a single strain. Our results confirm the high diversity noted by Simonet et al. (1989).

In nursery soil natural selection among bacterial populations is generally weak. Indeed soil is regularly irrigated, ploughed and fertilized. There are also numerous rotations of alder species and non N-fixing trees. *Frankia* had been found in soils free from actinorhizal plants (Arveby and Huss-Danell, 1988) and in some soils, density of *Frankia* under non-host plants can be as high or even higher than under alders (Smolander*, 1988). Their importance in number and variety should be increased in soil by the presence of alders in the neighbourhood. This could explain the large diversity observed among strains isolated from the nodules formed by a single root system of a nursery-grown *A. rubra*.

The presence of highly divergent strains in nodules of alders grown in natural stands is questionable. Benson and Hanna (1982) separated the 43 isolates obtained from nodules of alders (*A. incana* s. sp. *rugosa*) growing in the field, into six groups only. The method used, protein pattern analysis, is not as resoluting as electrophoretic separation of isozymes to differentiate closely related strains (Young, 1985; Chun et al., 1985). For example, *Frankia* strains belonging to the same electrophoresis subgroup were easily differentiated by isozyme patterns (Gardes et al., 1987). Using DNA restriction pattern analysis and DNA hybridization, Bloom* (1988) demonstrated a considerable heterogeneity and a high degree of genetic diversity among isolates from a single plant of *Myrica pennsylvanica* growing in a natural site. Under natural conditions, colonization of the root system of an actinorhizal plant by highly divergent strains can be an ecological advantage. Indeed, as noticed by Prat (1989), growth of alder is better when the root system is bearing nodules induced by *Frankia* strains showing different isozyme phenotypes.

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