Differentiation of *Frankia* strains by their electrophoretic patterns of intracellular esterases and aminopeptidases

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The closely related *Frankia* strains BR, S21 and Thr, isolated from the genus *Casuarina*, Allo2 and Dec from the genus *Allocasuarina*, and G80 from the genus *Gymnostoma*, could be distinguished by their esterase zymograms after ultra-low gelling point agarose–polyacrylamide electrophoresis. The kanamycin-resistant derivatives S21-kR and BR-kR could also be differentiated from their kanamycin-sensitive parental strains. Different patterns of intracellular esterases could be obtained by using β -naphthyl propionate or 3-indoxyl acetate as substrates. The substrate 5-bromo-4-chloro-3-indoxyl acetate proved particularly useful, revealing a variety of esterases from *Gymnostoma* isolates. Zymograms of aminopeptidases from all strains were found to be quite similar. Nevertheless, they allowed differentiation of *Frankia* strains BR and S21 from their kanamycin-resistant derivatives. Aminopeptidase and esterase zymograms obtained from *Gymnostoma* isolates were markedly different from all others. Zymograms of esterases and aminopeptidases may prove useful for the identification of some other *Frankia* strains.

Introduction

Trees of the family *Casuarinaceae* are widely used in agroforestry because of their association with the actinomycete *Frankia*, which can fix atmospheric nitrogen at rates comparable to the rhizobia of nodulated legumes (Torrey, 1978; Dommergues, 1987). The family *Casuarinaceae* is divided into four genera: *Casuarina* *sensu stricto* (18 species), *Allocasuarina* (58 species), *Gymnostoma* (18 species) and *Ceuthostoma* (2 species) (Johnson, 1988). All *Frankia*-type isolates are now classified within the genus *Frankia* (Becking, 1970). The general characteristics of all *Frankia* strains have been recently reviewed by Akkermans *et al.* (1992). Improved use of the *Frankia*–plant symbiosis implies not only

laboratory selection of optimally performing strains, but also tests in field conditions, for which consideration of properties such as persistence in the soil and good competitiveness of the released strains is essential. Studies to examine these properties require techniques to identify and follow individual strains after their release (Simonet *et al.*, 1990).

Various methods have been proposed to characterize and identify *Frankia* strains: the use of antisera, restriction analysis of the genome and specific DNA or RNA, determination of fatty acid composition and SDS-PAGE analysis of whole cell protein patterns all have their usefulness and limitations, and have been discussed and reviewed (Nazaret *et al.*, 1989; Mirza *et al.*, 1991; Akkermans *et al.*, 1992; Baker & Mullin, 1992). However, identification of *Frankia* strains by relatively simple, low-cost and reliable methods, which discriminate between closely related strains such as those isolated from trees of the family *Casuarinaceae*, has not been possible up to now.

The use of non-denaturing ultra-low gelling point agarose–polyacrylamide gel electrophoresis (ULGA-PAGE), as described by Benoist & Schwencke (1990), revealed diverse but reproducible patterns (zymograms)

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Abbreviations: BCA, bichinchoninic acid; BCIA, 5-bromo-4-chloro-3-indoxyl acetate; MNA, 4-methoxy β -naphthylamide; ULGA, ultra-low gelling point agarose; R_{fum} , ratio obtained by dividing the electrophoretic migration of a given esterase activity by the migration of prestained fumarase.

of aminopeptidases, dehydrogenases and esterases for *Frankia* strains. Previous studies have indicated that the electrophoretic analysis of enzymes patterns was a promising technique for analysis of the diversity of *Frankia* strains (Gardes & Lalonde, 1987; Prin *et al.*, 1991), but was limited by problems related to the heterogeneous nature of the *Frankia* cultures used (simultaneous presence of sporangia, lysing hyphae, growing hyphae and dead hyphae) resulting from non-balanced growth.

Here we show that by using cells grown in controlled agitated conditions in mineral medium (BAP) supplemented with egg yolk phosphatidylcholine (BAP-PCM), reproducible zymograms of esterases and aminopeptidases were obtained for every strain, allowing the strains to be differentiated.

Methods

Isolation of *Frankia* Allo2. *Frankia* Allo2 was isolated from young nodules formed on *Allocasuarina verticillata* plants after inoculation under greenhouse conditions with dried nodule samples collected in Uruguay. Nodule lobes were superficially sterilized in a 2% (w/v) aqueous solution of osmium tetroxide (Lalonde *et al.*, 1981) for 3 min. Nodule lobes were then thoroughly rinsed in sterile water, chopped into small pieces and transferred into a double-layer agar medium as recommended by Diem & Dommergues (1983). After 4 weeks, *Frankia* colonies grew out of nodule pieces and exhibited typical hyphae and sporangia. Nitrogen-fixing vesicles developed on nitrogen-free medium. Nodulation tests of the isolate were made on seedlings of the original host grown in tubes or sand culture. Infection tests showed that Allo2 was infective and effective in nitrogen fixation on species of genera *Allocasuarina* (*verticillata* and *decaisneana*) and *Casuarina* (*glauca* and *equisetifolia*).

Bacterial strains and growth conditions. The origin and designation of *Frankia* isolates are described in Table 1. Cultures of all *Frankia*

isolates were stored in complete Q_{mod} medium (Lalonde & Calvert, 1979) supplemented with 50 µg kanamycin ml⁻¹ for the kanamycin-resistant isolates BR-kR and S21-kR. Inocula of *Frankia* were prepared by washing the culture twice with sterile 0.85% NaCl, and passing through a sterile syringe needle (0.6 × 30 mm); this material was inoculated at 1 µg protein ml⁻¹ (Bradford, 1976) into BAP medium (Fontaine *et al.*, 1986) supplemented with egg yolk phosphatidylcholine mixture (P-4279 from Sigma; 1.7 µg l⁻¹) instead of soybean phosphatidylcholine mixture (P-3644 Sigma) and buffered with MES/Tris (10 mM, pH 6.8) [BAP-PCM medium as described by Schwencke (1991)]. This minimized post-exponential decrease in biomass during the early stationary phase. Isolates BR-kR and S21-kR were grown under the same conditions except that BAP-PCM medium was supplemented with 50 µg kanamycin ml⁻¹. Three pre-cultures were grown at 28 °C and magnetically stirred (200 r.p.m.). Mycelial cells were harvested and the cellular protein determined periodically by the Bradford (1976) method.

Preparation of bacterial extract and protein determination. After 5 d growth at 28 °C, cultures were centrifuged at 4000 g for 15 min in a swinging-bucket rotor. The pellets were washed twice with BAP-PCM medium and once with cold lysis buffer (20 mM-Tris/HCl, pH 7.5, 100 mM-KCl, 5%, w/v, glycerol). Then, 3 ml of cold lysis buffer was added to 1 gram (wet weight) of pelleted cells and the suspension (20 W; Branson Sonifier 250) sonicated eight times for 2 min at -20 °C (CaCl₂/ice). The homogenates were ultracentrifuged (186000 g, 60 min, 4 °C) and the supernates (cytosolic crude extracts) carefully collected. Glycerol was added to a final concentration of 10% (w/v). Protein concentration of cytosolic crude extracts was determined by the BCA (bicinchoninic acid) method according to the manufacturer's (Pierce) instructions, using bovine serum albumin as a standard. Cytosolic crude extracts were used immediately or after 2 d storage at -80 °C.

Electrophoretic conditions. Vertical, non-denaturing slab electrophoresis was performed in mixed agarose-polyacrylamide gels (Benoist & Schwencke, 1990). Resolving gels (135 × 130 × 2.0 mm) contained 7% acrylamide (Serva), 0.186% bisacrylamide, 0.4% ultra-low gelling point agarose (ULGA) from FMC Bioproducts, 0.375 M-Tris/HCl, pH 8.8, 0.075% ammonium persulphate, and 0.03% (v/v) TEMED (*N,N,N',N'*-tetramethylethylenediamine) from Serva. Stacking gels contained 4.5% acrylamide, 0.225% bisacrylamide, 0.2% ULGA, 0.125 M-Tris/HCl, pH 6.8, 0.075% ammonium persulphate and 0.07% (v/v) TEMED. Acrylamide polymerization was allowed to proceed at room temperature, then gelling was completed by cooling to 4 °C for 30 min. Gels were pre-electrophoresed twice with running buffer (0.050 M-Tris/0.384 M-glycine, pH 8.3) for 15 min at 40 mA (4 °C). The running buffer was supplemented with 50 µM-CoSO₄ or 50 µM-ZnSO₄ for aminopeptidase or 5 mM-MgCl₂ for esterase detection. Samples from crude cytosolic extracts containing 100–300 µg protein (determined by the BCA method) were electrophoresed at 25 mA constant current (4 °C) for 5 h. A prestained fumarase was used as a marker for migration.

Detection of esterases. After electrophoresis, the gels were equilibrated by gently shaking for 30 min with 100 ml 100 mM-HEPES/KOH, pH 7.5, 100 mM-NaCl, 5 mM-MgCl₂. The gels were then placed in plastic bags, substrates added from a 200 mM stock solution in DMSO, and Fast Garnet GBC added to 0.025% final concentration; the plastic bags were closed and shaken in the dark at room temperature for 30 min. Three different substrates (1 mM final concentration; all from Sigma) were used: β-naphthyl propionate, 3-indoxyl acetate and 5-bromo-4-chloro-3-indolyl acetate (BCIA). Esterases appeared as brown, red or blue-green bands with the three substrates respectively. A commercial (Sigma) acetylcholine esterase (AChE) (1 µg) was detectable after 5 min of incubation.

Table 1. Origin of *Frankia* isolates used in this study

Trivial designation	Original host	Geographical origin	Reference
BR	<i>Casuarina equisetifolia</i>	Brazil	Müller <i>et al.</i> (1991)
BR-kR	<i>Casuarina equisetifolia</i>	Brazil	*
S21	<i>Casuarina glauca</i>	Egypt	Girgis <i>et al.</i> (1990)
S21-kR	<i>Casuarina glauca</i>	Egypt	*
Thr	<i>Casuarina cunninghamiana</i>	Egypt	Girgis <i>et al.</i> (1990)
Allo2	<i>Allocasuarina verticillata</i>	Uruguay	This paper
Dec	<i>Allocasuarina decaisneana</i>	Senegal	Sougoufara (1990)
G80	<i>Gymnostoma nobilis</i>	Singapore	†

* S21-kR and BR-kR, kanamycin-resistant derivatives of isolates BR and S21 (M. Girgis, J. Schwencke, A. Akkermans & E. Duhoux, unpublished).

† A. Savouré & G. Lim, unpublished.

Detection of aminopeptidases. The assay was essentially as described by Benoist & Schwencke (1990) with the following modifications. After electrophoresis, gels were equilibrated by gently shaking for 30 min with 100 ml 100 mM-HEPES/KOH, pH 7.0, 200 μ M- CoSO_4 , 200 mM-NaCl. The gels were placed into plastic bags with 20 ml 50 mM-MES-KOH, 50 mM TES-KOH, pH 7.0, 1 mM substrate (from a 200 mM stock solution in DMSO), 200 μ M- CoSO_4 and 200 mM-NaCl. Two different substrates were used, L-leucine-4-methoxy- β -naphthylamide (L-Leu-MNA) or L-Pro-MNA from Bachem (Dübendorf, Switzerland). Freshly prepared Fast Garnet GBC coupling agent was added at 0.025% final concentration and the bags were then closed, and shaken for 10 min at room temperature. Red (L-Leu-MNA) or orange (L-Pro-MNA) bands corresponding to aminopeptidase activities usually appeared after 5 min of incubation. Incubations were done in the dark at room temperature and were stopped by immersing the gels in 10% (v/v) acetic acid for 30–60 min. The gels were then washed twice for 20 min with distilled water. In these conditions, a reasonably clear background was obtained without band destaining or gel cracking during drying at 70 °C. The relative mobility of bands was calculated as R_{fum} values, which were obtained by dividing the distance (mm) travelled by a given esterase by the distance (mm) travelled by prestained fumarase as a marker.

Results

Growth characteristics of Frankia isolates in supplemented BAP medium

For growth of all *Frankia* strains in BAP-PCM medium under stirring, the optimal concentration of the egg yolk phosphatidylcholine supplement was 1.7 $\mu\text{g l}^{-1}$ (Fig. 1a). The complete Q_{mod} medium (buffered with 10 mM-MES/Tris) allowed balanced growth until day 4 but maximal biomass yield was about one-sixth of that obtained in mineral medium BAP-PCM. All strains used in this study grew exponentially in BAP-PCM as shown in Fig. 1(b) for strains S21, Thr and Dec, indicating that nutritional conditions were optimal. As shown in Table 2, maximal mycelial growth was obtained at day 5 for all *Frankia* isolates tested. Except for BR-kR and

G80, a biomass yield of 17–22 $\mu\text{g protein ml}^{-1}$ was obtained for all strains.

Electrophoretic patterns of esterases

Zymograms of esterases revealed by 3-indoxyl acetate. Zymograms of esterases obtained using 3-indoxyl acetate are shown in Fig. 2(a). Isolates BR, Thr and Allo2 could not be clearly differentiated using this substrate, while all the other isolates showed a characteristic pattern of isoenzymes. Thus, strain Dec presented a unique band, R_{fum} 0.23. The *Gymnostoma* strain G80 showed three fast migrating bands, R_{fum} 0.58, 0.78 and 1.22, that were not detected in strains from *Casuarina* and *Allocasuarina*. Further, the esterase zymograms of the kanamycin-resistant derivatives BR-kR and S21-kR (Fig. 2d) were

Fig. 1. Growth kinetics of *Frankia* strains. (a) Effect of different phosphatidylcholines on growth of strain BR in buffered BAP-PCM, supplemented with soybean phosphatidylcholine (3.4 $\mu\text{g ml}^{-1}$, ○) or egg yolk phosphatidylcholine (3.4 $\mu\text{g ml}^{-1}$, □; 1.7 $\mu\text{g ml}^{-1}$, ●), compared with growth in the rich medium Q_{mod} buffered with MES/Tris (■). (b) Growth of *Frankia* strains in buffered BAP-PCM medium supplemented with egg yolk phosphatidylcholine (1.7 $\mu\text{g ml}^{-1}$). ○, S21; ●, Thr; □, Dec.

Table 2. Biomass yield of *Frankia* isolates growing in stirred, buffered BAP-PCM medium supplemented with egg yolk phosphatidylcholine

See Methods for details. Cultures were inoculated with 1 $\mu\text{g protein ml}^{-1}$. Each value is the mean of three replicates.

<i>Frankia</i> isolate...	Biomass obtained ($\mu\text{g protein ml}^{-1}$)*							
	S21	S21-kR†	BR	BR-kR†	Thr	Allo2	Dec	G80
Time (d)								
4	15.86	17.34	16.36	9.50	17.32	19.64	15.04	8.00
5	18.91	20.24	17.06	12.91	22.38	21.40	16.65	15.92
6	17.66	16.50	16.53	11.26	18.88	16.00	16.12	13.57

* Determined by the Bradford (1976) method.

† Kanamycin-resistant derivatives of BR and S21 respectively (M. Girgis, J. Schwencke, A. Akkermans & E. Duhoux, unpublished results).

Fig. 2. Electrophoretic patterns for esterases of some *Frankia* strains. For each *Frankia* strain 250 µg protein (BCA method) were loaded. Fum, prestained fumarase marker. R_{fum} , mobility calculated with reference to that of prestained fumarase. Gels were incubated with 1 mM-3-indoxyl acetate, 5 mM-MgCl₂ (a, d), 1 mM β -naphthyl propionate, 5 mM-MgCl₂ (b, c) or 1 mM-BCIA, 5 mM-MgCl₂ (e).

different from those of their original sensitive parental strains BR and S21. Thus, while two bands, R_{fum} 0.19 and 0.41, were characteristic for strain S21, they could not be detected in S21-kR. Also, a new very weak band, R_{fum} 0.08, was detected only in S21-kR. In addition, the parental strain BR showed two bands, R_{fum} 0.21 and 0.32, but neither was detected in its kanamycin-resistant derivative BR-kR.

Zymograms of esterases revealed by β -naphthyl propionate. Strain S21 showed six bands of esterase activity (Fig. 2b: two strong bands, R_{fum} 0.17 and 0.28, and five weak bands, R_{fum} 0.06, 0.33, 0.38 and 0.72. The esterase bands of R_{fum} 0.06, 0.17, 0.33 and 0.72 were present only in strain S21, allowing it to be differentiated from all other strains. Strains BR and Thr could not be differentiated using this substrate since they presented a

very similar isoenzyme pattern. Strain Dec showed a characteristic band, R_{fum} 0.23, which allowed it to be differentiated from all other strains. The *Gymnostoma* strain G80 was easily differentiated from all others by bands of R_{fum} 0.52 and 0.80.

Zymograms of esterases revealed by BCIA. This substrate, which has not been previously used for the detection of *Frankia* esterases, revealed a variety of esterases from the *Gymnostoma* strain G80. Seven characteristic main bands were clearly detected, R_{fum} 0.15, 0.22, 0.25, 0.29, 0.48, 0.63, 0.68, 0.79, 0.88, 1.10, 1.26, 1.38 (Fig. 2d, e), and a weak band, R_{fum} 0.48. Using 3-indoxyl acetate (Fig. 2a) or β -naphthyl propionate (Fig. 2b) revealed only three clearly defined bands for strain G80. All other strains from *Casuarina* or *Allocasuarina* showed rather similar esterase patterns with BCIA. However, strain S21

showed two bands, R_{fum} 0.15 and 0.22, which allowed it to be differentiated from all other strains. Also, strain Dec showed a characteristic band, R_{fum} 0.79. Clear differences were also found between the parental strains S21 and BR, and their kanamycin-resistant derivatives BR-kR and S21-kR. Thus, the band of R_{fum} 0.29 present in strain BR was absent in BR-kR. Similarly, the strong bands of R_{fum} 0.15 and 0.25 present in S21 were absent in S21-kR, which showed instead bands of R_{fum} 0.29 and 0.63; the latter was seen only faintly in strain S21.

Effect of storage on the pattern of esterases and aminopeptidases

For all experiments we used late exponentially growing cells (5 d old) and fresh extracts, or extracts stored for up to 2 d at -80°C . This is because extracts stored for more than 1 week showed an altered pattern of esterases, as shown in Fig. 2(e) for *Gymnostoma* extracts stored at -80°C for 2 d and 3 or 7 weeks. The maximum activity of esterases was obtained with the 2-d-old extract. Seven well-defined bands were detected, R_{fum} 0.15, 0.22, 0.68, 0.88, 1.10, 1.26 and 1.38. This pattern remained unaltered for 1 week (not shown). Analysis of extracts after a 3 week storage period at -80°C showed that the bands of R_{fum} 0.15 and 0.22 were no longer detectable, while after storage for 7 weeks, only four weak bands could be visualized, R_{fum} 0.68, 0.88, 1.10 and 1.38. Similarly, some aminopeptidase activities were also sensitive to storage, as seen in Fig. 3(a).

Aminopeptidase enzyme patterns

Similar aminopeptidase patterns for *Casuarina* and *Allocasuarina* strains (Fig. 3a) were obtained when extracts were electrophoresed in the presence of CoSO_4 and bands in the gel revealed using L-Leu-MNA as substrate in the presence of CoSO_4 and NaCl. Only strain S21 could be differentiated from other strains because it showed clearly separated bands, R_{fum} 0.79, 0.82 and 0.94, in a zone where all others showed diffuse bands. No other clear difference was revealed in these conditions for extracts of *Frankia* strains from the genera *Casuarina* and *Allocasuarina*. Strain G80 from the genus *Gymnostoma* gave a quite different aminopeptidase zymogram with three typical bands, R_{fum} 0.26, 0.32 and 0.99 (Fig. 3a; G80 2 d). Band R_{fum} 0.26 was undetectable in 3-week-old extracts.

As seen in Fig. 3(b), all strains from *Casuarina* and *Allocasuarina* showed a single, common aminopeptidase band, R_{fum} 0.86, when ZnSO_4 (50 μM) was present during electrophoresis and bands in the gel revealed using L-Leu-MNA as substrate in the presence of CoSO_4 and NaCl. The *Gymnostoma* strain G80 showed two different

bands: a strong band, R_{fum} 0.94, and a weak band, R_{fum} 0.31.

Finally, all the strains from the genera *Casuarina* and *Allocasuarina* showed similar patterns when CoSO_4 was present during electrophoresis and aminopeptidase activities were revealed using L-Pro-MNA as substrate in the presence of CoSO_4 and NaCl (Fig. 3c). However, strain S21 could be clearly differentiated from its kanamycin-resistant derivative S21-kR because it showed an AP band of R_{fum} 0.22 that was absent in S21. No clear differences could be observed between strain BR and its kanamycin-resistant derivative BR-kR. For strain G80, a characteristic well-defined aminopeptidase band, R_{fum} 1.01, was detected. As with esterase bands, storage of extracts for 7 d at -80°C resulted in a decrease in activity of all aminopeptidase bands from strain G80.

Reproducibility of band migration

Differentiation of strains on the basis of characteristic bands needs good band definition and reproducibility. This was obtained by growing *Frankia* strains under balanced conditions and by using ULGA-PAGE electrophoresis (Benoist & Schwencke, 1990). Further, we defined the relative mobility of each band by R_{fum} values. R_{fum} was calculated by dividing the migration distance of a band by the migration distance of a prestained fumarase marker. Standard deviations for R_{fum} values were within the range 0.01–0.04, calculated from three different electrophoretograms with bands revealed by 3-indoxyl acetate and β -naphthyl propionate. Similar small variations were observed for R_{fum} values when BCIA was used as substrate as well as for aminopeptidases (not shown).

Discussion

We report here that six *Frankia* strains isolated from the genera *Casuarina*, *Allocasuarina* and *Gymnostoma*, and two kanamycin-resistant derivatives, could be distinguished on the basis of the electrophoretic patterns of their esterases. With few exceptions (strains S21 and G80), aminopeptidase zymograms alone were less useful but helped to differentiate strains that showed identical or very similar esterase electrophoretic patterns.

Characterization of bacterial strains by the analysis of phenotypic characters presents the problem of reproducibility. This is especially pertinent for *Frankia* strains which grow slowly, non-exponentially and show morphological and physiological heterogeneity (hyphae, lysed hyphae and sporangia) under the usual static growth conditions (Benson & Schultz, 1990). This major problem has been solved here by growing *Frankia* strains under controlled stirring conditions in BAP medium

Fig. 3. Electrophoretic patterns for aminopeptidases of some *Frankia* strains. For each *Frankia* strain 250 μ g protein (BCA method) were loaded. Fum, R_{fum} , as Fig. 2. (a) Electrophoresis with 50 μ M-CoSO₄ and incubation with 1 mM-L-Leu-MNA, 200 μ M-CoSO₄ and 200 mM-NaCl. (b) Electrophoresis with 50 μ M-ZnSO₄ and incubation with 1 mM-L-Leu-MNA, 200 μ M-CoSO₄ and 200 mM-NaCl. (c) Electrophoresis with 50 μ M-ZnSO₄ and incubation with 1 mM-L-Pro-MNA, 200 μ M-CoSO₄ and 200 mM-NaCl.

supplemented with egg yolk phosphatidylcholine. The pattern of esterases showed good reproducibility and remained unaltered along the growth curve from day 3 up to day 8. Therefore, the zymograms obtained can be safely used to differentiate the closely related *Frankia* strains from the family *Casuarinaceae*.

A limitation to the method is that extracts cannot be stored for more than 6 to 7 d at -80°C because esterase activities diminish as a function of storage and some bands cannot be revealed with extracts older than 1 week. This is also true for some aminopeptidases. This may be due to proteolytic degradation in the extracts. Indeed, inhibitors of the proteasome-like particles described in *Frankia* (Benoist *et al.*, 1992) effectively protected esterase activities during storage (data not shown).

The use of the ULGA-PAGE method for electrophoretic separation of esterases under non-denaturing conditions (Benoist & Schwencke, 1990) was essential in order to obtain both optimal activity of esterases and good band definition after electrophoresis.

Frankia strains could be distinguished by the esterase zymograms obtained using (essentially) only two different substrates: β -naphthyl propionate and 3-indoxyl acetate. The use of BCIA as substrate is described here for the first time to visualize *Frankia* esterases. This substrate has been used to detect esterases in mammalian tissues (Holt & Withers, 1958). BCIA revealed a characteristic pattern of seven bands for strain G80 while only three bands were seen with either one of the other substrates. BCIA also revealed some new bands in strains from the genera *Casuarina* and *Allocasuarina*,

complementing the information obtained with β -naphthyl propionate and 3-indoxyl acetate.

The kanamycin-resistant strains S21-kR and BR-kR could also be differentiated from their parental sensitive strains by using β -naphthyl propionate as substrate. The observed band variation may be due to kanamycin-related alterations in gene expression or mRNA translation. However, positive selection of a naturally occurring resistant mutant cannot be excluded.

The results presented suggest that *Frankia* strains from the genera *Casuarina* and *Allocasuarina*, are phylogenetically very close, while strain G80 from the genus *Gymnostoma* is clearly separated from them. However, the general value of this preliminary conclusion should be confirmed by the analysis of esterase zymograms from other strains isolated from the genus *Gymnostoma*.

Until now, attempts to differentiate *Frankia* strains from the family *Casuarinaceae* have been unsuccessful. Thus, restriction analysis of DNA fragments from seven strains showed essentially identical hybridization patterns against *nifA*, *B*, *D*, *K* and *H* probes from *Alnus* (Nazaret *et al.*, 1989). The use of amplified rDNA sequences showed also that strains infective for *Casuarina* could be grouped in a single genomic species. Other molecular approaches, such as those based on the variable, non coding section of *Frankia* rRNA-encoding genes (Normand *et al.*, 1992) show promise. However, the appropriate probes are not available yet.

In conclusion, zymograms of esterase activities appear to be useful for differentiating among closely related *Frankia* strains by a moderately expensive and simple technique. It is clear that this method is limited to strains that can be isolated and grown under exponential growth conditions. If the method is to be applied to test strains isolated from *Casuarina* nodules after mixed inoculation experiments, stability of the esterase pattern for each strain after re-isolation is essential. Recent results from our laboratory indicate the patterns are stable for all strains assayed, i.e. S21, BR, Thr, S21-kR and BR-kR, opening the way for the use of esterase zymograms for identification of isolates in field studies.

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