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Electrophoretic comparison of enzymes from 22 single-spore cultures obtained from *Frankia* strain ORS 140102

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1. SUMMARY

Individual spores of *Frankia* strain ORS 140102 were allowed to germinate on Qmod medium. Germination percentages were approximately 30%. Colonies from germinated spores were individually picked off and subcultured on two media giving 22 single-spore cultures. Although colony morphology and pigmentation of the single-spore cultures differed, the study of the electrophoretic patterns of enzymes showed that malate dehydrogenase and esterase profiles of the 22 strains and the mother-strain were very similar.

2. INTRODUCTION

Actinomycetes of the genus *Frankia* are nitrogen-fixing microsymbionts living within root nodules of many non-leguminous shrubs and forest trees. Significant advances in defining the characteristics of *Frankia* by using biochemical, serological and molecular genetical methods have contributed to the improvement of the classification of the *Frankia* strains already isolated from various plant species [1–5]. However, due to considerable physiological and biochemical variations that occur in cultures of different *Frankia* strains, species definition in the *Frankia* group still remains unresolved [6]. Furthermore, cultural heterogeneity and spontaneous phenotypic changes have been already reported in cultures of the same strain [7,8], thus raising the question of the genetic stability of *Frankia* and indicating the need for obtaining strains from single cells such as spores and protoplasts.

Until now, subcultures of *Frankia* have been routinely obtained by inoculating fresh medium with a suspension of fragmented mycelia because germination rates of *Frankia* spores and protoplasts are very low. Thus single-cell *Frankia* strains are not available in laboratories throughout the world. Recently, high germination percentages were reported with *Frankia* strain Cel5 [9], thus suggesting that *Frankia* cultures might be obtained from single spores. In our laboratory, another *Frankia* strain, ORS 140102, isolated from

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Hippophae rhamnoïdes (sea buckthorn), was found to produce many sporangia and spores in liquid medium. When placed on solid Qmod medium, these spores exhibited germination rates of 30%.

This paper reports how single-spore cultures from the strain ORS 140102 were obtained, and focuses on the comparison of the enzyme profiles of single-spore cultures. Since analysis of enzyme profiles is considered to be one of the most discriminating techniques in bacterial taxonomy [10,11], this approach was used to answer the two following questions: (i) were all these cultures identical? and (ii) consequently, were the macroscopic variations between these strains, i.e. pigmentation, colony morphology and growth characteristics, reliable criteria to distinguish one strain from another?

3. MATERIALS AND METHODS

3.1. Spore purification

Spores were obtained from a 4-week-old culture of *Frankia* strain ORS 140102 on liquid BAP medium without nitrogen [12] at 28°C in the dark. The cultures were grown stationary in 250-ml flasks containing 100 ml of medium. The cell mass composed of hyphae, vesicles and numerous mature sporangia was homogenized by forced passages through a 0.8-mm diameter needle. During this step, spores were released into the liquid medium. The suspension was filtered on compacted cotton wool to separate spores from hyphae. Individual spores collected in the filtrate were examined microscopically to check for the absence of hyphal fragments. Spore concentration was evaluated by direct count using a microscope and a haemocytometer.

3.2. Spore germination

One drop of the spore suspension was spread onto the surface of agar Qmod medium [13] in a 55-mm diameter Petri dish in such a way that there were about 400 spores per dish. An overlay of Qmod medium was then added to cover the spores. Microscopic observations were performed with an inverted microscope (Nikon Diaphot) using Hoffman modulation contrast optics, ensuring that individual spores were separately distributed over the surface of the agar Qmod medium. After 2-day incubation at 28°C, the first germinating spores appeared. At day 7, microscopic observations clearly showed that germ tubes derived from individual spores had developed into microcolonies separated from each other.

3.3. Multiplication of Frankia obtained from microcolonies

Each young microcolony, originating from one spore was then axenically picked off the solid medium, homogenized in 100 μl distilled water in sterile 1.5-ml microtubes with an appropriate homogenizer (Polylabo Strasbourg, France). The suspension from each microcolony was then deposited onto the surface of solid Qmod medium in a 35-mm diameter Petri dish and covered with an overlay of the same medium. About 4 weeks later at 28°C, numerous *Frankia* colonies were visible in the Petri dish. The agar medium and the colonies of each dish were then gently crushed into granules using a 60-ml Potter homogeniser containing 10 ml of liquid BAP medium. The suspension was transferred into 60-ml sterile flasks and incubated stationary at 28°C for 2 weeks. As noticed by Diem et al. [14], *Frankia* grows profusely at the interface between the bottom layer of agar granules (solid phase) and the top liquid Qmod medium (liquid phase). At that time the colonies are ready to be classically subcultured in BAP or F medium [15].

3.4. Frankia biomass production for electrophoretic comparisons

The 22 single-spore strains of *Frankia* obtained, plus the mother strain ORS 140102, were cultivated in 250-ml flasks containing 100 ml of BAP or F medium at 28°C for 12 days. The biomass produced was estimated using the INT (Iodo-nitrophenyl tetrazolium chloride) Reduction Activity method [16]. The cultures were used to inoculate 1200-ml Roux bottles containing 400 ml of BAP and F medium, the inoculum being adjusted to 1 nmol INTF (Iodo-nitrophenyl tetrazolium formazan)/ml (v/v final dilution) [16].

The bottles were laid on their sides and incubated stationary at 28°C in the dark for 12 days.

3.5. Preparation of bacterial extracts

The cultures of *Frankia* were harvested by filtration through a GF/F Whatman filter. After several washings with sterile distilled water, the cells were harvested from the filter in Eppendorf tubes and the fresh weight of biological material was determined. The Tris-HCl buffer was added to each tube to a volume equivalent to 1.5 ml/g of fresh weight of cells. Lysis was performed on ice by intermittent exposure to ultrasonic oscillations (Branson Sonifier 250) at the lowest intensity and with a frequency of 60% of time for a total of 8 min. The protein content of the crude extracts supernatants obtained after centrifugation (Beckman J2 21 ME centrifuge, JA 18-1 rotor, 30 000 × *g*, for 1 h at 4°C) was measured by the Bradford procedure [17]. The extracts were stored at −80°C until use.

3.6. Electrophoresis

Electrophoretic analysis: horizontal slab acrylamide agarose gel electrophoresis (7% w/v, acrylamide, Tris/glycine buffer, pH 8.6) was performed as described by Uriel [18]. Characterization of esterases and malate dehydrogenase: esterases were stained using acetate, propionate and butyrate of α- and β-naphthyl and indoxyl acetate as substrates and tested for their sensitivity or resistance to diisopropylfluorophosphate (DFP) (10^{-3} M) as previously described [18,19]. Malate dehydrogenase was stained according to the method of Siciliano and Shaw [20].

In order to determine the optimal amount of protein per well necessary for a good electrophoresis banding, a preliminary assay was carried out using the following procedure: 30, 60, 120, 240 and 480 μg protein from one single-spore culture on BAP medium were deposited in each well of a polyacrylamide-agarose gel and allowed to migrate. The same experiment was repeated with another single-spore culture on F medium. Within the gel, α-naphthyl esterases were stained. This preliminary assay showed that a concentration of 100 μg protein was sufficient to allow a good electrophoresis banding.

4. RESULTS AND DISCUSSION

4.1. Spore purification and germination

Spore purification was easily obtained by filtration through compacted cotton, a technique previously used by Normand et al. [21] to purify a protoplast suspension from undigested hyphal fragments. Simonet [22] and Tzean and Torrey [9] also obtained good results in *Frankia* spores purification by filtration on Whatman filters.

The germination rate (germinated spores/total number of spores) of *Frankia* strain ORS 140102 was 30% on Qmod medium (3 replications, 10 counts per replication, standard deviation: 4%). The effect of other media i.e. BAP or TIS [23] on germination of spores of ORS 140102 was tested but none of them was superior to Qmod. Tzean and Torrey [9] reported similar observations with strain UFGCel5 whose germination rate on Qmod medium was very high (75.4%).

The germination percentages of some other strains of *Frankia* were estimated (data not shown), but the strains tested (i.e. HFP 070101, ORS 020602 and ORS 020608) failed to germinate on Qmod medium. Tzean and Torrey [9] did not obtain more than 0.7% spore germination with the strain Ccl3 which was isolated from *Casuarina cunninghamiana*. With two strains isolated from *Alnus glutinosa* (AgN1g and AGN10Al), Simonet [22] never observed more than 10% spore germination. It is therefore proposed that the germination ability of the spores of *Frankia* essentially depends on the intrinsic characteristics of the strain.

4.2. Single-spore cultures

From the present work, 22 single-spore cultures were obtained. They presented marked macroscopic differences in terms of colony morphology when grown on solid medium and pigmentation. On solid medium, colonies obtained from one germinated spore appeared either very compact and well delimited or more or less diffuse. These macroscopic variations in colony morphology could not be related to microscopic variations such as the presence or absence of sporangia. Similarly, some of the single-spore cultures exhibited a dark-red pigmentation both on solid and liquid media while others were orange or without

any pigmentation (white). However, these variations within a complete and well-standardized series of cultures were not always observed with other well-standardized series of cultures used as replication. Some authors have already described the emergence of 'variants' within the cultures of the same *Frankia* strain [7,8]. These variants also exhibited some macroscopic differences in terms of morphology and pigmentation compared to the original strain. According to Diem and Dommergues [24] the morphology of the *Frankia* colonies newly grown on solid medium may depend on the size of the *Frankia* clusters in the original inoculum and also on the mass, loose or dense, of the hyphae contained in these clusters.

4.3. Enzyme electrophoretic comparison of the single-spore cultures

The malate dehydrogenase and esterase activities were explored, both on F and BAP medium. Malate dehydrogenase profiles of the 22 strains on both media did not differ from each other and

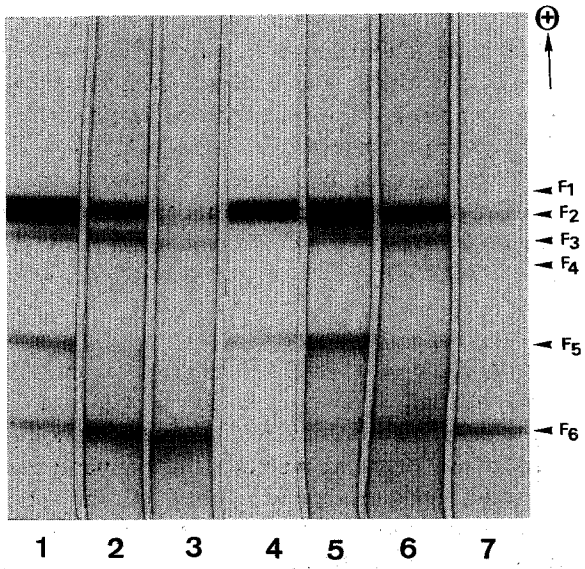

Fig. 1. Esterase bands F_1 to F_6 identified in *Frankia* strain ORS 140102 (mother strain, cultivated on F medium) after horizontal acrylamide-agarose gel electrophoresis, by their distinct hydrolytic profiles on: 1, α-naphthyl acetate; 2, α-naphthyl propionate; 3, α-naphthyl butyrate; 4, indoxyl acetate; 5, β-naphthyl acetate; 6, β-naphthyl propionate and 7, β-naphthyl butyrate.

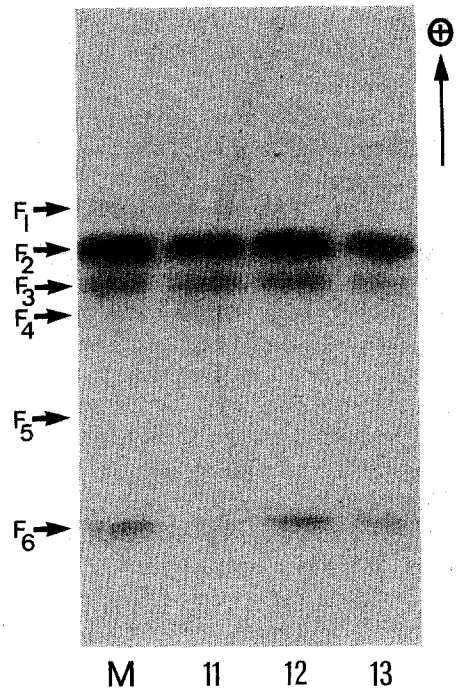

Fig. 2. Electrophoretic profiles (after horizontal acrylamide-agarose gel electrophoresis) of β-naphthyl propionate esterase from the mother strain (M) and three of the single-spore strains [11–13], after cultivation on BAP medium. No differences are detectable between the four profiles (esterase numeration similar to Fig. 1).

from the mother strain, with only one band and a migration front (M_f) value of 55. In the 22 strains, six types of esterase bands were detected, numbered in order of decreasing mobility towards the anode and identified by their distinct hydrolytic profiles on the seven substrates (Fig. 1) and by their sensitivity to DFP. Esterase F_1 was faint and active on β-naphthyl acetate and propionate. The major esterase F_2 hydrolysed the 7 substrates but was more active on acetyl and propionyl esters than on butyryl esters whereas band F_3 had a comparable activity on all substrates. Band F_4 was faint and slightly hydrolysed β-naphthyl propionate and butyrate. Esterase F_5 hydrolysed mainly α- and β-naphthyl acetate and indoxyl acetate and α- and β-naphthyl propionate slightly. Band F_6 was active on all substrates except indoxyl acetate. Esterases F_1, F_3, F_4, F_5 and F_6 were sensitive, to DFP whereas esterase F_2 was resistant

to this agent. The six enzymes were detected in all strains and shared the same M_f values from one strain to another at M_f 54 for esterase F_1; M_f 49 for esterase F_2; M_f 45 for esterase F_3; M_f 41 for esterase F_4; M_f 30 for esterase F_5 and M_f 16 for esterase F_6. Consequently, the 23 strains tested had the same zymotype for malate dehydrogenase and esterases (Fig. 2). Since the latter enzymes have been described as among the most discriminating enzymes in various taxonomic studies [11,25], this analysis suggests that there were little or no differences between the 22 single-spore strains and the mother strain ORS 140102 which can be therefore considered as a pure strain if the pigmentation and colony morphology variations can be ascribed to physiological variations (such as inoculum effects, even though the inoculation techniques were as standardized as possible) but not genetic factors.

However, for specific biochemical and genetical studies of *Frankia*, it would probably be safer to use single-spore cultures whose purity would be indisputable.

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