Spore germination and the life cycle of *Frankia in vitro*

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Introduction

The infection of actinorhizal plants by *Frankia* usually can be achieved by inoculating seedling roots with crushed or ground actinorhizal nodular tissues, soil from the actinorhizal plant rhizosphere, or washed suspensions of pure *Frankia* isolates grown in liquid culture (Torrey 1987). The nature of the infective particles from these various inocula remains unclear although we presume that infective inocula must consist of fragments of mature hyphal filaments or germinated spores or both. The infection process itself, whether it occurs by root hair penetration (Berry et al. 1986) or by direct epidermal penetration (Miller and Baker 1985), involves *Frankia* hyphal entry.

In studies of *Alnus glutinosa*, Van Dijk (1978) reported that inoculum derived from crushed nodules of field-grown plants in which *Frankia* sporulation occurred in the nodule (spore\(^+\) plants) had far greater infectivity (300- to 2000-fold higher) than inoculum from nodules lacking spores (spore\(^-\) plants). The presumption was (Akkermans and Van Dijk 1976; Van Dijk 1978; Houwers and Akkermans 1981) that the superiority of inoculum from spore\(^+\) nodules was due to the presence of “granulae” or spores as well as hyphal filaments. No direct demonstration of the involvement of spores in the infection process was made.

Although spore germination in *Frankia* has been mentioned by some authors (Lalonde and Calvert 1978; Quispel et al. 1983; Burggraaf et al. 1981; Normand and Lalonde 1986; M. J. McBride and J. C. Ensign. 1983. Annu. Meet. Amer. Soc. Microbiol., Abstr.; A. J. P. Burggraaf. 1984. Ph.D. thesis, University of Leiden, The Netherlands), no description of the process has been published. In the following account, we report a study of spore germination in a *Frankia* strain grown in pure culture under a range of culture conditions, and we describe the life cycle of *Frankia*, which involves spore germination, hyphal filamentous growth, sporulation, and spore release. For comparison, the behavior of other *Frankia* strains tested under the same conditions is reported.

Materials and methods

Preparation of *Frankia* spore suspensions

*Frankia* strain UFGC15, kindly provided by H. Berg from an isolation from root nodules of *Casuarina equisetifolia* growing in south Florida, and *Frankia* strain HFPCC13 (Catalog no. 020203),...
isolated by Zhang (Zhang et al. 1984) from C. cunninghamiana, were cultivated in a modified BAP medium (Murry et al. 1984) with pyruvate as the carbon source. Other Frankia strains tested included UFGCg1, isolated from root nodules of C. glauca collected by H. Berg in South Florida, and HFPAAl (Catalog no. HFPO22801), isolated by Zhang and Torrey (1985) from nodules of Allocasuarina lehmanniana and grown on modified BAP medium. Culture conditions were as described earlier (Fontaine et al. 1986). Frankia strains are referred to hereafter by trivial designations.

The modified BAP medium contained (in mM) NH4Cl, 5; MgSO4 · 7H2O, 0.2; CaCl2, 0.07; potassium phosphate buffer (pH 6.7), 10; sodium pyruvate, 10; and (in mg/L) FeNaEDTA, 10; H3BO3, 2.86; MnCl2 · 4H2O, 1.81; ZnSO4 · 7H2O, 0.22; CuSO4 · 5H2O, 0.08; Na2MoO4 · 2H2O, 0.025; CoSO4 · 7H2O, 0.001; thiamin-HCl, 0.1; pyridoxine-HCl, 0.5; nicotinic acid, 0.5; biotin, 0.225; folie acid, 0.1; calcium pantothenate, 0.1; and riboflavin, 0.1. The pH of the medium was adjusted to pH 6.3 with dilute KOH. The spore concentration of the spore suspensions of Frankia strains CeI5 and CcI3, which showed good germination, was compared with that of strain CcI5 incubated on the BAP medium.

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**Results**

**Time course of germination**

Spores of Frankia strain CeI5 germinated on the BAP medium in the dark at 28°C showed no germination at 12 h. By 24 h, some spores became phase dark, indicative of activation, and showed outgrowth of the first hyphal filament. Hyphal outgrowth from spores was observed at 2 days (Figs. 1 and 2). By the 4th day, mono-, di-, and tri-polar germination had occurred (Figs. 1-4), and branching hyphae had formed small colonies. The original spore size was unchanged.

By the 6th day, terminal branch endings showed enlargement (Fig. 3), suggesting early sporangial formation. An occasional spherical vesicle on a short stalk was observed by day 4 on complex (QMOD), and by day 6, on BAP medium (Fig. 6). By the 12th day, colonies derived from single spores had formed enlarged sporangia filled with numerous spores (Figs. 7 and 8). The sporangial wall in this strain is thin and readily breaks down, releasing spores.

Spore suspensions of Frankia strain CeI5 were cultured on a modified BAP medium with added organic nitrogen in the form of 1-tyrosine at 5 mM and trehalose at 20 mM in place of pyruvate. Spore germination, scored every 12 h, was as follows: 12 h, 0%; 24 h, 8%; 36 h, 9.5%; 48 h, 14.5%; 60 h, 14.5%; 72 h, 14.0%; and 84 h, 17.5%. This time course of germination was fairly typical, allowing one to score germination after 2-3 days.

**Effects of the physical environment**

Spores of Frankia strain CeI5 were germinated on modified BAP medium at a range of temperatures. Maximum germination after 2 days occurred at 35°C (Table 1). Germination was suppressed at both high (40°C) and low (0°C) incubation temperatures.

**Effects of the chemical medium on germination**

Using BAP medium with pyruvate and NH4Cl as carbon and nitrogen sources, respectively, we determined the effects of different buffers on spore germination used to adjust the pH of the medium (pH 5.2 to 9.0). Germination was highest with potassium phosphate buffer at pH 6.0-6.8. In further experiments, modifications of the nutrient components in BAP medium were studied. For these studies the Frankia strain CeI5, which showed good germination, was compared with strain CcI3, which showed poor germination in preliminary trials. Omission of either the carbon or nitrogen source and physical conditions on germination were studied. Stable values could be achieved by sampling after 48 h.

Scanning electron microscopy of germinated Frankia spores

Sterile polycarbonate Nuclepore membrane filters, pore size 0.4 μm, were placed on top of the agar surface of the BAP medium and spore suspensions of Frankia strain CeI5 were spread on the membrane and allowed to germinate. After incubation periods of 1 to 14 days, the membranes supporting germinating spores of different developmental stages were fixed and processed for scanning electron microscopy (Berry and Torrey 1979).

**Culture conditions tested for maximum germination rate**

Using the standard spore germination test, the effects of chemical and physical conditions on germination were studied. Stable values could be achieved by sampling after 48 h.
reduced germination about 50\% in strain CeI5, micronutrient omission reduced germination nearly 25\%, while vitamin omission had no effect (Table 2). CeI3 almost totally failed to germinate under all conditions.

Various media regularly used in our research program, including BAP, were tested without modification in accordance with published formulations (Table 3). Strain CeI3 always showed low germination percentages. In contrast, strain CeI5 showed remarkable variation in germination. The greatest germination (75\%) was in QMOD, the most enriched medium. BAP is a completely defined medium. B medium is related to BAP, but lacks NH4Cl and is highly buffered with MOPS buffer. DPMNY, M6B Plus, and YCZ are media containing yeast extract and caused reduced germination. QMOD, in contrast, contains several complex components, including yeast extract, Bacto-peptone, and lecithin.

Germination of spores from several different strains of Frankia on modified BAP medium and on QMOD medium were compared (Table 4). In addition, spores collected aseptically from spore\(^+\) nodules of field-grown plants of Myrica gale and Alnus incana ssp. rugosa were tested. Frankia strains CeI5 and CgI1, both of which spontaneously release spores in culture, showed the highest germination. QMOD was the most effective medium. Strains that showed sparse spore release in culture exhibited poor germination as did the spores taken from field-collected spore\(^+\) nodules.

\section*{Effects of possible activators of spore germination in Frankia strain CeI5}

Since specific substances in root exudates might serve to stimulate spore germination in Frankia, concentrations of known secondary plant products were added to the BAP medium. In nonamended BAP medium, spore germination of Frankia strain CeI5 was about 15\% (Table 3). The addition to BAP medium of single nitrogen- containing compounds at 5 mM resulted in marked inhibition or slight stimulation in germination. Compounds that caused complete inhibition of germination in CeI5 were L-tryptophan, DL-dihydroxyphenylalanine, DL-ethionine, adenine sulfate, and 5-amino uracil. Germination varied from 21 -33\% with 5 mM additions of L-lysine, L-tyrosine, L-asparagine, and DL-phenylalanine. Among phenolic compounds tested, p-hydroxybenzoic acid or p-coumaric acid at 1 \(\mu\)M in BAP medium resulted in germination of 21 and 47\%, respectively. No significant stimulation in strain CeI3 was observed with any compound.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
Temperature (\(^\circ\)C) & Germination (\%) \\
\hline
0 & 0d \\
20 & 2.1c \\
28 & 12.5b \\
35 & 16.9a \\
40 & 0.1d \\
\hline
\end{tabular}
\caption{Germination of spores of Frankia strain CeI5 on modified BAP agar medium under different temperature regimes during 2 days}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Deletion from BAP & Germination (\%) \\
\hline
None (control) & 0.2d & 14.3a \\
Carbon source & 0.2d & 7.9c \\
Nitrogen source & 0.4d & 7.0c \\
Micronutrients & 0.4d & 10.9b \\
Vitamins & 0.5d & 14.6a \\
\hline
\end{tabular}
\caption{Germination of Frankia strains CeI5 and CeI3 on modified BAP medium or BAP lacking carbon, nitrogen, micronutrients, or vitamins, respectively, after 2 days}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Designation & References & Germination (\%) \\
\hline
B & Murry et al. 1984 & 0.7e 26.2b \\
BAP & Murry et al. 1984 & 0e 13.5c \\
DPM & Baker and O'Keeffe 1984 & 0.1e 14.4c \\
DPMNY & Baker and O'Keeffe 1984 & 0.2e 8.0d \\
M6B Plus & Baker and Torrey 1979 & 0e 8.0d \\
QMOD & Lalonde and Calvert 1979 & 0.1e 75.4a \\
YCZ & Baker and O'Keeffe 1984 & 0.1e 7.4d \\
\hline
\end{tabular}
\caption{Germination of Frankia strain CeI5 and CeI3 incubated on different culture media for 3 days at 28\^\circ}C}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Frankia strains & BAP & QMOD \\
\hline
CeI5 & 18.5a & 76.5a \\
CeI3 & 0.1c & 0e \\
CgI1 & 5.2b & 33.0b \\
AllI & 0d & 1.1c \\
\hline
Frankia spores from M. gale nodules* & 0.2c & 0.2de \\
Frankia spores from Alnus incana ssp. rugosa nodules* & 0.1c & 0.9cd \\
\hline
\end{tabular}
\caption{Germination of different Frankia strains on modified BAP and QMOD medium after 2 days}
\end{table}

\section*{Discussion}

Spores from cultures of isolates of Frankia grown in sterile nutrient culture are notoriously difficult to germinate. In our experiments, Frankia strain CeI5 proved to be especially favorable for two reasons that may be related. Cultures of CeI5 formed sporangia abundantly, and then spontaneously released mature spores into the medium. Cultures of CeI3 of the same age in the same medium produced abundant sporangia, but did not release spores unless the cultures were homogenized or sonicated, and then many released spores...

Our earlier studies of the process of spore germination in Frankia met with limited success. We observed germination of random isolated spores in cultured Frankia strain Cp11 (unpublished observations of D. Callaham, D. Baker, and W. Ormerod), but did not observe predictable and abundant germination of free spores. M. J. McBride and J. C. Ensign (1983. Annu. Meet. Amer. Soc. Microbiol., Abstr.) reported 25% germination of Frankia spores of strain Eu11, on complex medium after a gentle heat shock. Other strains they studied... "were constitutively dormant and difficult to germinate."

Diem and Dommergues (1985) found no germination of spores released from sporangia in Frankia cultures isolated from root nodules of Casuarina junghuhniana. They reported what may have been anomalous structures referred to as "torulose hyphae" that underwent a type of branching they equated to sporelike behavior. No such structures were ever observed in our study.

In the present experiments, bona fide sporangiospores were released spontaneously by Frankia strains CeI5 and CgI1. Spores from CeI5 germinated in high percentages if provided with favorable conditions, while CcI3 showed almost no germination. The hyphae produced at germination branched and rebranched, forming terminal vesicles and abundant sporangia in some media. The complete cycle from germinated spore to mycelial hyphal mat to sporangium formation and spore release required 12 days. Frankia strain CgI1, although studied less extensively, behaved like CeI5 with respect to both sporangial release and spore germination, but with lower germination percentages in the media tested.

Inoculation of seedling roots of actinorhizal plants with appropriate cultured strains of Frankia has been based on the assumption that infective inoculum should consist of hyphal pieces, sporangia, and spores (Lalonde and Calvert 1979; Berry and Torrey 1979, 1985; Perinet et al. 1985; Stowers and Smith 1985). The assumption has been that spores represent resistant structures that survive in the soil and could serve as sources of infection. Yet, the evidence is clear that both in root hair infection (Berry et al. 1986) and in direct epidermal penetration (Miller and Baker 1985) infection involves hyphal penetration and entry. The role for germinating spores in these infection processes remains to be demonstrated.

Germination of spores in response to secondary products produced by the appropriate host root may well be an early event in the plant-microbe recognition systems, analogous to what is described in Rhizobium-legume symbiosis (e.g., Peters et al. 1986). The availability of a spore germination test system such as described here should make a study of root-spore interactions among actinorhizal plants open to direct experimentation.

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