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

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Bacterial spores of *Frankia* produced in defined culture media were collected by filtration after washing in amounts approximating 10^6 spores/mL. *Frankia* strains UFGCeI5 from *Casuarina equisetifolia* and UFGCgI1 from *C. glauca* showed spontaneous release of spores in culture; strains HFPCcI3 from *C. cunninghamiana* and HFPAllI1 from *Allocasuarina lehmanniana* showed low spore release in culture unless homogenized. Spore germination was tested on plates of agar nutrient media under different physical and chemical environments. Strain CeI5 showed about 15% germination within 2 days in a defined (BAP) medium with an optimum pH of 6.0–6.8 at 28–35°C. Under these conditions, strain CcI3 germinated less than 0.5%. In a series of trials with increasingly complex media, strain CeI5 showed 75% spore germination in 3 days at 28°C and pH 6.7 in the most complex medium tested. Additions of specific single organic compounds to BAP medium caused either strong inhibition or slight stimulation of spore germination. *Frankia* strains that showed spontaneous spore release germinated better than strains that did not release. Spore germination in *Frankia* strains is markedly influenced by their strain origin and by the physical and chemical environment in which they are placed.

Key words: *Casuarina*, *Frankia*, life cycle, spore germination.

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Des spores bactériennes, produites dans des milieux de culture définis, ont été recouvrées par filtration après délavage en quantités approximatives de 10^6/mL. Les souches de *Frankia* UFGCeI5 provenant de *Casuarina equisetifolia* et UFGCgI1 provenant de *C. glauca* ont spontanément libéré leurs spores dans les cultures, alors que chez les souches HFPCcI3 issue de *C. cunninghamiana* et HFPAllI1 issue de *Allocasuarina lehmanniana* la libération des spores a été faible en culture, à moins d'une homogénéisation. La germination des spores a été vérifiée sur géloses nutritives dans différentes conditions ambiantes physiques et chimiques. La germination de la souche CeI5 a été d'environ 15% en dedans de 2 jours sur un milieu défini (BAP) avec un pH optimum de 6,0 à 6,8 et une température de 28 à 35°C. Dans ces mêmes conditions, la germination de la souche CcI3 a été inférieure à 0,5%. Dans un série d'essais sur des milieux de plus en plus complexes, la germination des spores de la souche CeI5 a été de 75% en 3 jours, à 28°C et à pH 6,7, sur le milieu le plus complexe. Des additions individuelles de composés organiques spécifiques au milieu BAP ont causé soit une forte inhibition de la germination des spores, soit une légère stimulation. Les souches de *Frankia* ayant fait une libération spontanée des spores ont mieux germé que les autres. La germination des spores chez *Frankia* est nettement influencée par l'origine de la souche et par les conditions physiques et chimiques environnementales dans lesquelles elles sont placées.

Mots clés : *Casuarina*, *Frankia*, cycle vital, germination des spores.

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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 UFGCeI5, kindly provided by H. Berg from an isolation from root nodules of *Casuarina equisetifolia* growing in south Florida, and *Frankia* strain HFPCcI3 (Catalog no. 020203),

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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 UFGCgI1, isolated from root nodules of *C. glauca* collected by H. Berg in South Florida, and HFPAlll1 (Catalog no. HFP022801), 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) NH_4Cl, 5; $MgSO_4$, 0.2; $CaCl_2$, 0.07; potassium phosphate buffer (pH 6.7), 10; sodium pyruvate, 10; and (in mg/L) FeNaEDTA, 10; H_3BO_3, 2.86; $MnCl_2 \cdot 4H_2O$, 1.81; $ZnSO_4 \cdot 7H_2O$, 0.22; $CuSO_4 \cdot 5H_2O$, 0.08; $Na_2MoO_4 \cdot 2H_2O$, 0.025; $CoSO_4 \cdot 7H_2O$, 0.001; thiamin-HCl, 0.1; pyridoxine-HCl, 0.5; nicotinic acid, 0.5; biotin, 0.225; folic 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 phosphate and pyruvate solutions were added to autoclaved (20 min at 15 psi) solutions.

The two strains of *Frankia*, CeI5 and CcI3, showed contrasting behavior in BAP medium. Both strains produced sporangia in abundance in this medium, but, after growth in liquid culture for 2–3 weeks, the medium of strain CeI5 was cloudy with spores spontaneously released, while in strain CcI3 spore release was sparse. The cultures were filtered through sterile Whatman no. 1 filter paper. Each spore suspension was washed twice with sterile distilled water and then was centrifuged at 3000–5000 × *g* for 30 min. The maturity of the spores as estimated from wall-staining properties was checked using a fuchsin – methylene blue staining method adapted from Baker (1967). The spore suspension was stored in sterile distilled water at 4°C for further experimental study. In experiments comparing germination of different *Frankia* strains, CcI3 and Alll1 behaved similarly to each other in showing low spontaneous spore release, and CgI1 was like CeI5 in showing high spontaneous spore release. Spore release by different strains grown in the same medium appears to be a strain character.

Spore germination tests

A standard method for testing a range of culture conditions was developed. The spore concentration of the *Frankia* spore suspensions was adjusted by dilution or concentration to approximately 10^6 spores/mL. A volume of 0.3 mL of spore suspension was released onto the surface of 15 mL of BAP medium solidified with 1.0% Bacto-agar (Difco) in a 10-cm Pyrex petri plate. The suspension was spread on the agar surface with a sterile glass rod. For each strain, two replicate plates were prepared for each treatment. Plates were covered and incubated in the dark, usually at 28°C, for periods of up to 2 weeks. For the longer term cultures, plates were sealed with Parafilm. Germination rates were assessed by removing the petri plate cover and examining the culture under phase-contrast light microscopy at 400×. The 10× eyepiece of the microscope was equipped with a Whipple disc etched with 100 small squares. For each plate, germinated and nongerminated spores within 100 squares in each field were counted and recorded. At least 30 such fields were scored per treatment and the average germination percentages were calculated. Statistical analyses employed Duncan's multiple range test. Spores with germ tubes that exceeded half their length or greater were rated as germinated.

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.

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).

Results

Time course of germination

Spores of *Frankia* strain CeI5 incubated 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 outgrowths from spores were 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. 5), 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 L-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 NH_4Cl 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

FIGS. 1–8. Germination and hyphal development of spores of *Frankia* strain CeI5 cultured on Nuclepore filters atop agar culture plates of BAP medium. Bars represent 5 μm. Fig. 1. Single germinated spore at day 2. Fig. 2. Dipolar spore germination at day 2. Fig. 3. Branching hyphae from germinated spore at day 4. Fig. 4. Hyphal colony, probably derived from two germinated spores. Fig. 5. Hyphae and swollen terminal branches at day 6 after germination. Fig. 6. A vesicle (V) formed on a short lateral stalk in a colony at day 6 after germination. Fig. 7. View of sporangium with evanescent sporangium wall leading to spontaneous release of spores. Fig. 8. A hyphal colony about 12 days after spore germination, showing extensive sporangium formation and spore release.

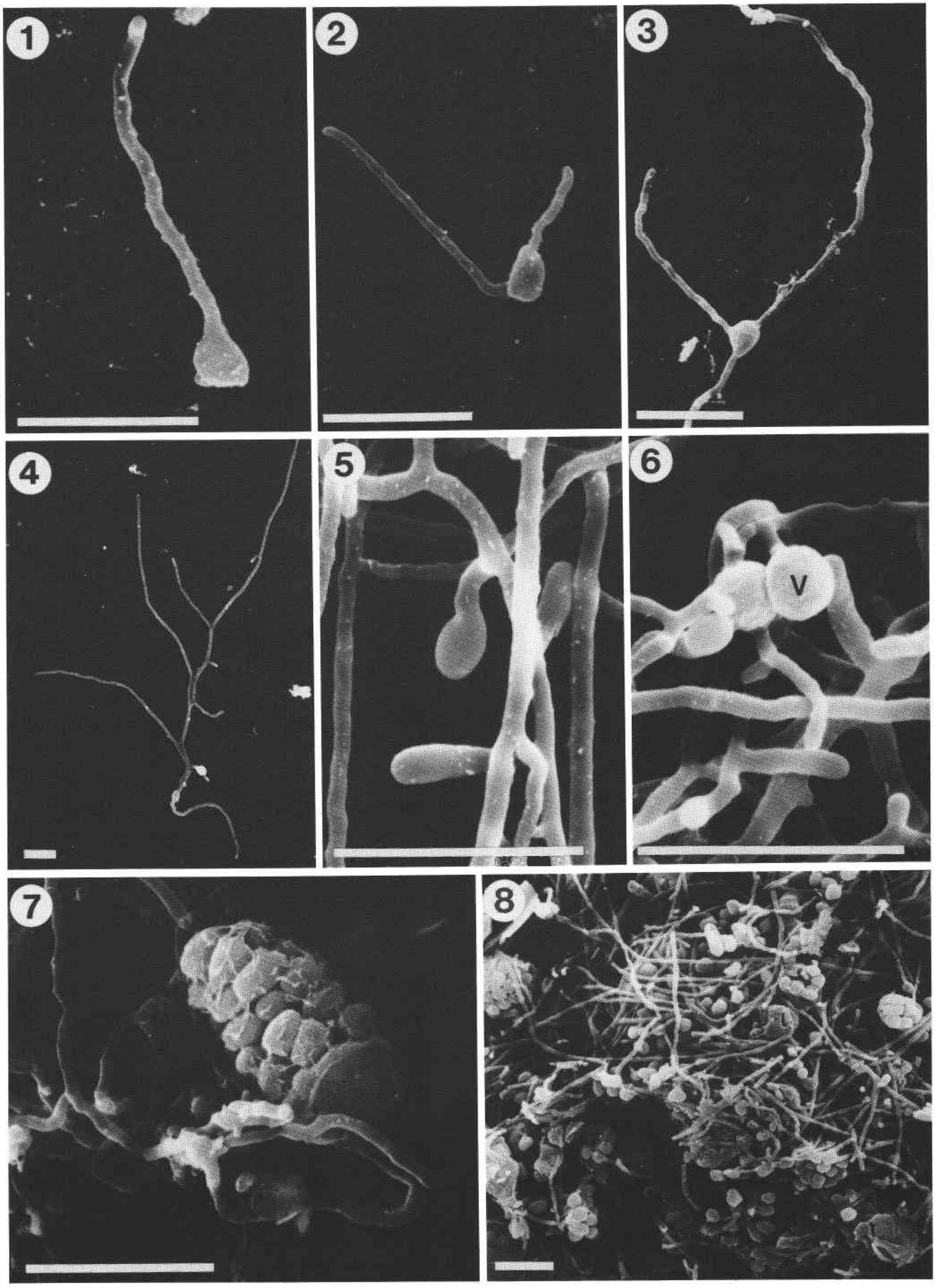

TABLE 1. Germination of spores of *Frankia* strain CeI5 on modified BAP agar medium under different temperature regimes during 2 days

Temperature (°C)	Germination (%)
0	0*d*
20	2.1*c*
28	12.5*b*
35	16.9*a*
40	0.1*d*

NOTE: The basic composition was as in BAP, pH 6.3, but pyruvate was replaced with 20 mM trehalose as carbon source, and NH_4Cl by 5 mM arginine as nitrogen source. Means with the same letter are not significantly different, according to Duncan's multiple range test ($p = 0.05$).

TABLE 2. Germination of *Frankia* strains CcI3 and CeI5 on modified BAP medium or BAP lacking carbon, nitrogen, micronutrients, or vitamins, respectively, after 2 days

	Germination (%)	
Deletion from BAP	CcI3	CeI5
None (control)	0.2*d*	14.3*a*
Carbon source	0.2*d*	7.9*c*
Nitrogen source	0.4*d*	7.0*c*
Micronutrients	0.4*d*	10.9*b*
Vitamins	0.5*d*	14.6*a*

NOTE: BAP was prepared with pyruvate as carbon source, except as noted. Final medium at pH 6.3. Means with the same letter are not significantly different, according to Duncan's multiple range test ($p = 0.05$).

reduced germination about 50% in strain CeI5, micronutrient omission reduced germination nearly 25%, while vitamin omission had no effect (Table 2). CcI3 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 CcI3 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 NH_4Cl 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.

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 mM in BAP medium resulted in germination of 21 and 47%, respectively. No significant stimulation in strain CcI3 was observed with any compound.

TABLE 3. Germination of *Frankia* strain CeI5 and CcI3 incubated on different culture media for 3 days at 28°C

Media		Germination (%)	
Designation	References	CcI3	CeI5
B	Murry *et al.* 1984	0.7*e*	26.2*b*
BAP	Murry *et al.* 1984	0*e*	13.5*c*
DPM	Baker and O'Keefe 1984	0.1*e*	14.4*c*
DPMNY	Baker and O'Keefe 1984	0.2*e*	8.0*d*
M6B Plus	Baker and Torrey 1979	0*e*	8.0*d*
QMOD	Lalonde and Calvert 1979	0.1*e*	75.4*a*
YCZ	Baker and O'Keefe 1984	0.1*e*	7.4*d*

NOTE: Means with the same letter are not significantly different, according to Duncan's multiple range test ($p = 0.05$).

TABLE 4. Germination of different *Frankia* strains on modified BAP and QMOD medium after 2 days

	Germination (%)	
Frankia strains	BAP	QMOD
CeI5	18.5*a*	76.5*a*
CcI3	0.1*c*	0*e*
CgI1	5.2*b*	33.0*b*
AllI1	0*d*	1.1*c*
Frankia spores from *M. gale* nodules*	0.2*c*	0.2*de*
Frankia spores from *Alnus incana* ssp. *rugosa* nodules*	0.1*c*	0.9*cd*

NOTE: BAP was prepared with sodium propionate at 10 mM; final medium at pH 6.7. Means with the same letter in vertical columns are not significantly different, according to Duncan's multiple range test ($p = 0.05$).

*Spore preparations were made of field-collected nodules from native populations of spore$^+$ nodules collected in early spring.

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 CcI3 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

were immature. A. J. P. Burggraaf (1984. Ph.D. thesis, University of Leiden, The Netherlands) studied several strains of *Frankia* from *Alnus* nodules that showed similar properties.

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 CpI1 (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 $EuI1_c$ 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 that 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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