Characterization of an effective actinorhizal microsymbiont, *Frankia* sp. AvcI1 (Actinomycetales)

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The actinomycete, *Frankia* sp. AvcI1, isolated from root nodules of *Alnus viridis* ssp. *crispa* was grown in axenic culture and used to inoculate host seedlings. This bacterium has been shown to be an infective and effective nitrogen-fixing microsymbiont which can be distinguished from other frankiae, *in vitro*, on the basis of size, distinctive morphology, and growth characteristics. Cross-inoculation studies indicated that the host range of this symbiont encompasses all of the members of the genera *Alnus*, *Myrica*, and *Comptonia* tested. In all cases, the symbioses developed were effective in fixing atmospheric dinitrogen.

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L'actinomycète, *Frankia* sp. AvcI1, isolée des nodules racinaires de *Alnus viridis* ssp. *crispa* a été cultivée en condition axénique et utilisée pour l'inoculation de plantules-hôtes. Cette bactérie, que l'on peut distinguer des autres *Frankia*, *in vitro*, par ses dimensions, sa morphologie propre et ses caractères distinctifs de croissance, s'est avérée un microsymbiote infectieux efficace pour la fixation d'azote. Des études d'inoculations croisées indiquent que la gamme de ses hôtes couvre tous les membres des genres *Alnus*, *Myrica* et *Comptonia* soumis à l'essai. Dans tous les cas, les symbioses développées ont été efficaces à fixer le diazote atmosphérique.

[Traduit par le journal]

Introduction

Using sucrose-density fractionation procedures, Baker *et al.* (1979) isolated from root nodules of *Alnus viridis* ssp. *crispa* (Aiton) Turrill an actinomycete which they designated *Frankia* sp. AvcI1. This bacterium was shown to infect its host plant and establish an effective nitrogen-fixing symbiosis.

Berry and Torrey (1979) using a microdissection technique isolated an actinomycete from *Alnus rubra* Bong. which they designated *Frankia* sp. ArI3 which also was able to infect its host plant and effectively reduce atmospheric dinitrogen. *Frankia* sp. ArI3 is closely similar in morphology and growth behavior to the previous isolate *Frankia* sp. CpI1, obtained from root nodules of *Comptonia peregrina* (Callaham *et al.* 1978), differing primarily in filament size and growth responses to a range of nutrient media.

In light of these findings, we describe here further investigations of this more recent *Alnus* isolate, *Frankia* sp. AvcI1, to characterize better its growth *in vitro* and its host range and to distinguish similarities to or differences from other isolated frankiae.

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Materials and methods

For routine culturing of the frankiae, a simple yeast extrac dextrose broth was used (Baker and Torrey 1979). For con venience this medium was designated *Frankia* broth.

The following additional culture media were employed fo studies of the actinomycetes *in vitro*: Bennett's agar, one-hal strength (B/2), Gordon (1968); Czapek's agar, Waksman (1961) supplemented with 0.4% yeast extract (YCz); glucose as paragine agar (GUA), Waksman (1961); glycerol asparagine aga (GYA), Shirling and Gottlieb (1966); soil extract agar (SXT) Gordon (1968); tap water agar (TAP), 1% Sigma agar; *Franki* broth agar (FRB), Baker and Torrey (1979); yeast malt (YM) Shirling and Gottlieb (1966); nutrient agar supplemented wit 0.2% Tween 80 (NTW); *Frankia* broth agar, one-fifth strengt supplemented with 5% purified potato starch (manufactured b J. T. Baker) (STR). For comparative purposes cultures of *Fran kia* sp. CpI1 (Callaham *et al.* 1978) were observed *in vitro*.

All growth media were prepared and inoculated using a pour plate technique. The cultures were incubated in the dark at 28° and examined at regular intervals. Relative growth rates, pig mentation, colony size and shape, and presence or absence c sporangia were recorded for each of the microorganisms after weeks in culture. For observation of the fragile sporangial coa of AvcI1, growth of the organism was achieved in diphasi culture. This method involved use of *Frankia* broth agar slant covered by a suspension of the microorganism in liquid *Franki* broth. After several weeks of incubation small pieces of aga which had been colonzied by the actinomycete were carefull cut from the slant, fixed in glutaraldehyde, dehydrated i ethanol, and prepared for scanning electron microscopy as de scribed below.

Cross-inoculation studies using AvcI1 were performed wit numerous actinorhizal species which included *Alnus glutinosa*

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A. incana ssp. *rugosa*, *A. incana* ssp. *tenuifolia*, *A. rubra*, *A. viridis* ssp. *crispa*, *A. viridis* ssp. *sinuata*, *Ceanothus americanus*, *Comptonia peregrina*, *Elaeagnus angustifolia*, *E. umbellata*, *Hippophaë rhamnoides*, *Myrica cerifera*, *M. gale*, and *Shepherdia argentea*. All studies were undertaken in nitrogen-free water cultures as described earlier (Baker *et al.* 1979).

Specimens for ultrastructural observation were fixed in 2% glutaraldehyde in Sorensen's phosphate buffer for 3 h at 4°C. After washing in additional buffer, the specimens were dehydrated through increasing concentrations of ethanol and then critical point dried using liquid CO_2 as an intermediate fluid. Specimens were affixed to scanning electron microscopy stubs with "double-sticky" cellophane tape and then coated with gold–palladium in a Technics Hummer II sputter coater. Root nodules were fractured with a thin razor blade before coating to expose the internal cortical regions. An AMR-1000 scanning electron microscope was used for observation.

Results

Growth in vitro

Growth characteristics of *Frankia* sp. AvcI1 are shown in Table 1. Characteristics *in vitro* of the previously isolated *Comptonia* isolate, *Frankia* sp. CpI1 (Callaham *et al.* 1978), are shown in Table 2. Growth of the two organisms was quite similar. No growth or very poor growth was evident on glucose asparagine agar, oatmeal agar, or tap water agar with either organism. Moderate growth of AvcI1 on glycerol asparagine agar was not observed with CpI1. In general growth of AvcI1 was more diffuse than CpI1 with well-defined colony formation occurring in the latter.

No growth of AvcI1 or CpI1 was observed if the cultures were incubated anaerobically and neither exhibited the ability to liquefy gelatin. Unlike *Frankia* sp. EuI1 (Baker *et al.* 1980), neither AvcI1 nor CpI1 grew on the surface of agar slants. Likewise, neither organism exhibited the ability to precipitate calcium oleate crystals from a medium containing a fatty acid supplement. No visible pigments were elaborated by cultures of AvcI1 or CpI1 on the media tested.

TABLE 1. Cultural characteristics of *Frankia* sp. AvcI1 observed after 4 weeks. All cultures were prepared by the pour-plate technique and incubated at 28°C

Media	Growth characteristics
B/2	Growth good; moderately diffuse; no sporangia
YCz	Growth poor; very diffuse; negligible sporulation
GUA	No growth
GYA	Growth moderate; diffuse; no sporangia
OAT	No growth
SXT	Growth good; diffuse; no sporangia
TAP	No growth
FRB	Growth good; moderately compact colonies; sporangia located on older parts of filaments (i.e., center of colony)
YM	Growth good; very diffuse (fungoid); no sporangia
NTW	Growth excellent; relatively compact colonies; no sporangia; no precipitation of calcium oleate
STR	Essentially no growth; no sporulation

TABLE 2. Cultural characteristics of *Frankia* sp. CpI1 observed after 4 weeks. All cultures were prepared by the pour-plate technique and incubated at 28°C

Media	Growth characteristics
B/2	Growth poor; very diffuse; occasional sporangia
YCz	Growth good; diffuse colony morphology; good sporulation
GUA	Growth very poor; very diffuse; occasional sporangia
GYA	No growth
OAT	No growth
SXT	Growth good; relatively compact colonies; moderate sporulation
TAP	No growth
FRB	Growth very good; diffuse to compact colonies; moderate sporulation
YM	Growth good; moderately diffuse colonies; extensive sporulation
NTW	Growth excellent; diffuse to compact colonies; sporulation extensive; no precipitation of calcium oleate
STR	Growth moderate; extensive sporulation

TABLE 3. Nodulation capacity of *Frankia* sp. AvcI1

Plants infected*	Plants not infected
A. glutinosa	*C. americanus*
A. incana ssp. *rugosa*	*E. angustifolia*
A. incana ssp. *tenuifolia*	*E. umbellata*
A. rubra	*H. rhamnoides*
A. viridis ssp. *crispa*	*S. argentea*
A. viridis ssp. *sinuata*	
C. peregrina	
M. cerifera	
M. gale	

*All plants developed effective nitrogen-fixing symbioses.

Host specificity of Frankia *sp. AvcI1*

Results of the cross-inoculation studies using this "*Alnus* isolate" are described in Table 3. All species of the genus *Alnus* tested as well as all members of the Myricaceae tested were infected by this organism. All plants developed effective nitrogen-fixing symbioses as was evidenced by the formation of nodules typical of each host plant and the alleviation of nitrogen deficiency symptoms after 3–4 weeks as well as rapid increase in growth rates over uninoculated control plants. The mor-

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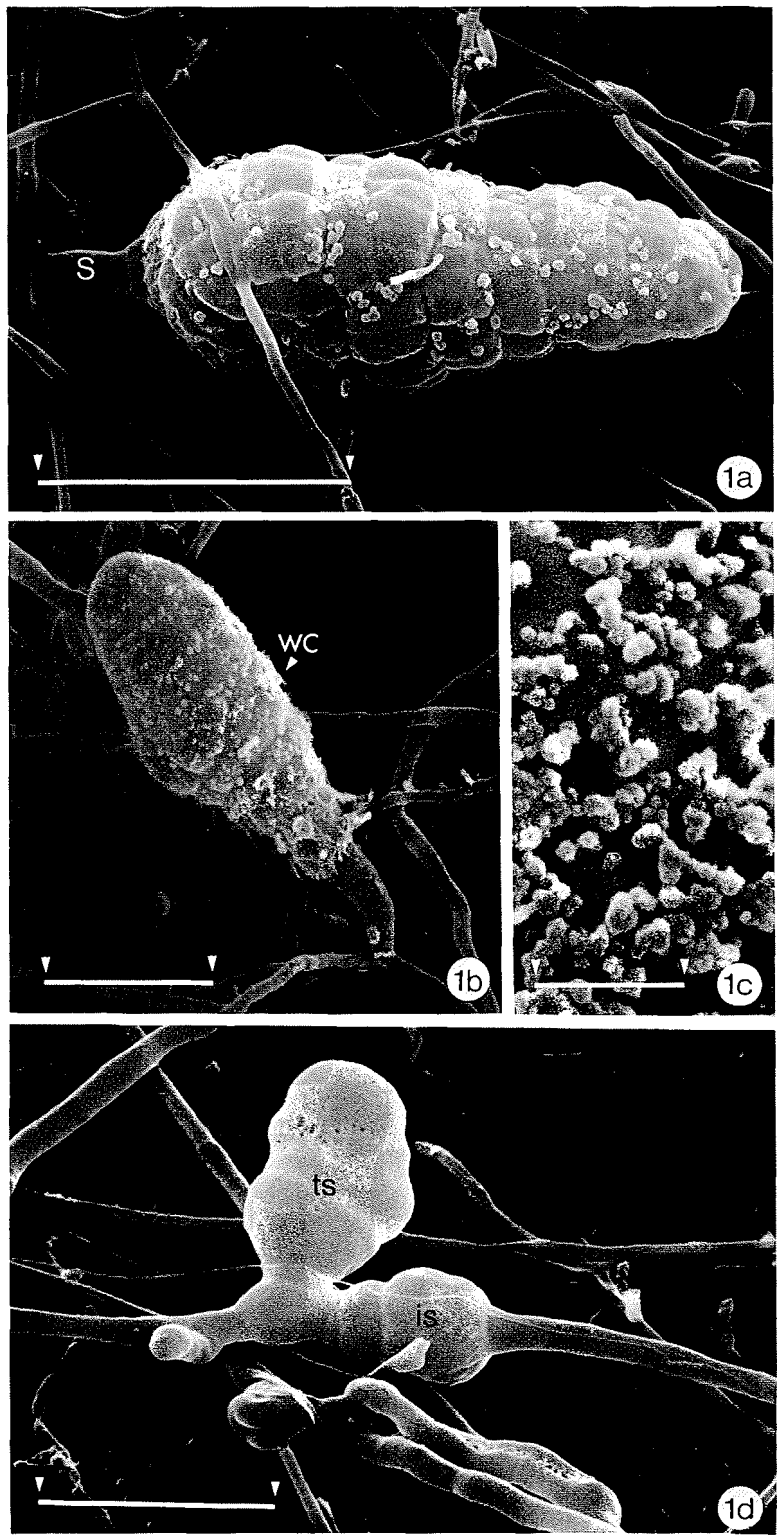

FIG. 1. Scanning electron micrographs of the actinorhizal microsymbionts *Frankia* sp. AvcI1 and *Frankia* sp. CpI1 from *in vitro* cultures. (*a*) Large sporangium of AvcI1 from broth culture; thickened sporangiophore (S) attaches sporangium to vegetative filament; bar = 10 μm. (*b*) AvcI1 from diphasic culture; fragile, warty coat (WC) surrounds the sporangium; bar = 5 μm. (*c*) Enlargement of warty coat of AvcI1; bar = 1 μm. (*d*) Sporangia of CpI1 from broth culture; terminal sporangium (*ts*) and intercalary sporangium (*is*) are characteristic of this organism; bar = 5 μm.

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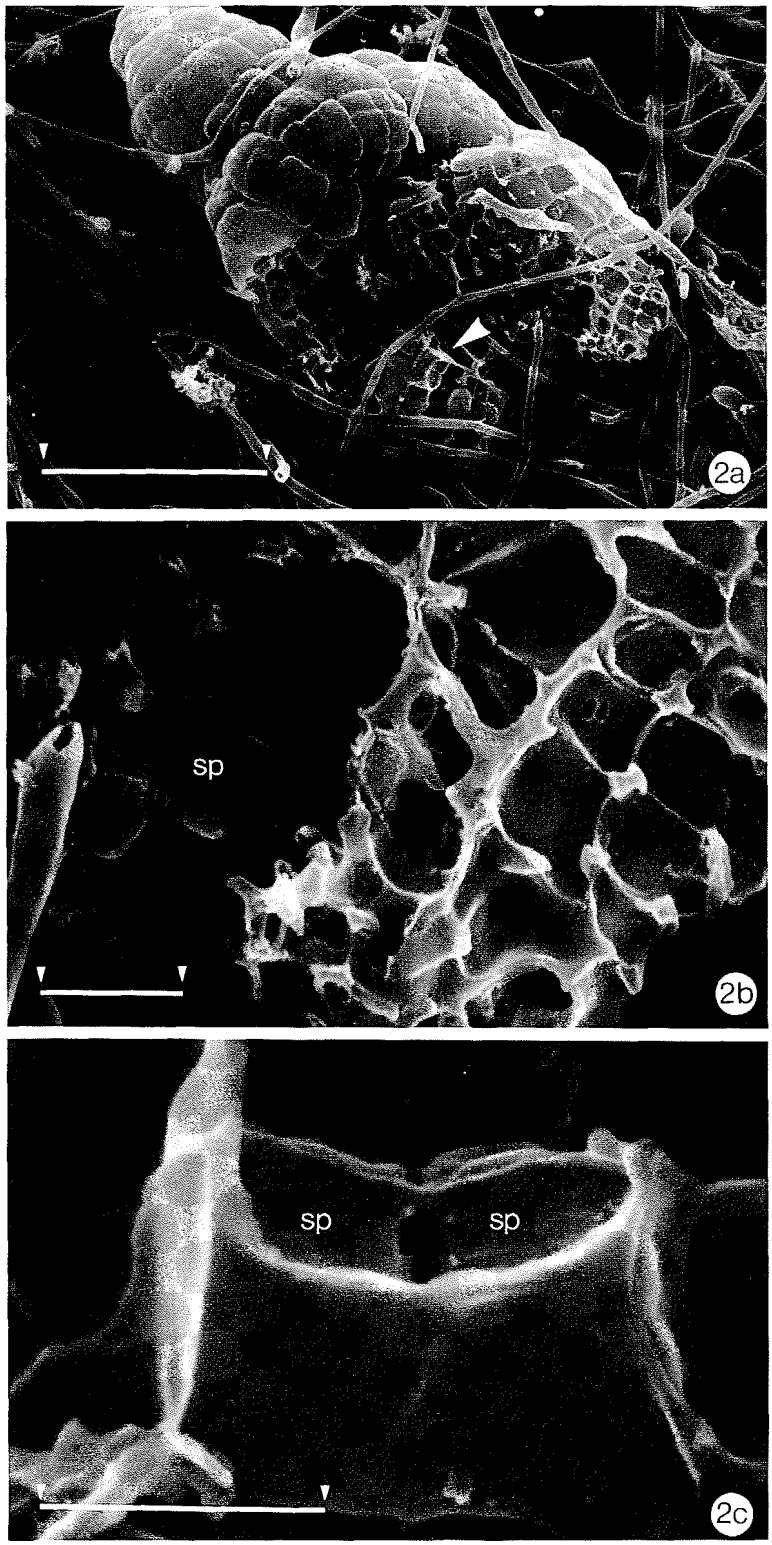

FIG. 2. Ultrastructural features of sporangial anatomy of *Frankia* sp. AvcI1. (*a*) Broken sporangium showing extensive "honey-combing" of the sporangium interior; arrow shows paired arrangement of spores; bar = 10 μm. (*b*) Enlargement of spore chambers within sporangium; spore (*sp*) has taken on the rectangular shape of the chamber; bar = 1 μm. (*c*) Enlargement of spore (*sp*) pair from (*a*) within sporangial chamber; bar = 1 μm.

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phology of the actinomycete within induced root nodules was examined by scanning electron microscopy and the organism was observed to bear numerous vesicles typical of an effective nitrogen-fixing microsymbiont. AvcI1 failed to induce nodules on tested members of the family Elaeagnaceae or on *Ceanothus* of the Rhamnaceae.

Ultrastructural observations

Cultures of *Frankia* sp. AvcI1 grown in broth and in diphasic media are illustrated in Figs. 1*a* and 1*b*. Filament diameter is typically under 1 μm and sporangia are borne on thickened sporangiophores (Fig. 1*a*). AvcI1 is superficially similar to CpI1 (Fig. 1*d*) but sporangia of AvcI1 are more consistently club-shaped than those of CpI1. Intercalary or intrahyphal sporangia commonly observed in cultures of CpI1 (Fig. 1*d*) were never observed in cultures of AvcI1.

One distinctive morphological feature of AvcI1 is the presence of a fragile warty sporangial coat. This structure entirely covers the sporangia (Figs. 1*b* and 1*c*) but due to washing during specimen preparation for scanning electron microscopy may be seen only as remnant particles which could mistakenly be considered debris (Fig. 1*a*). In careful preparations of other frankiae this striking feature has not been observed nor have others reported such a structure (Berry and Torrey 1979; Newcomb *et al.* 1979).

Another feature which distinguishes AvcI1 from other frankiae *in vitro* is the relatively more massive nature of its sporangia. In general, sporangia of AvcI1 range in size from 20 to 60 μm when mature and have a thick sporangial wall. Aspects of the sporangial anatomy of AvcI1 are shown in Fig. 2. Sporangial walls, both externally and internally, are thick (Fig. 2*a*). Extensive "honey-combing" of the internal walls creates chambers in which the spores are held (Fig. 2*b*). Like those of *Frankia* sp. EuI1 (Baker *et al.* 1980), spores are slightly less than 1 μm in size and appear to develop as pairs (Fig. 2*c*) or tetrads. The spores which become spherical or ovoid upon release are unsculptured.

Discussion

Frankia sp. AvcI1 has been shown to be an infective, nodule-inducing actinomycete that is capable of forming an effective nitrogen-fixing symbiosis. In general, the growth of this organism *in vitro* is very similar to other isolated frankiae and in particular to strains CpI1 (Callaham *et al.* 1978) and ArI3 (Berry and Torrey 1979). It is easily distinguished, however, from the other frankiae by its diffuse colonial morphology, the absence of intercalary or intrahyphal sporangia, and the presence of a warty sporangial coat.

The fact that AvcI1 as well as ArI3 and CpI1 are able to infect and nodulate all tested species of *Alnus* and all tested species of the Myricaceae is significant. This indicates that these microsymbionts probably belong to a closely related group of symbiotic nitrogen-fixing actinomycetes that includes the endophytes of all nodulated members of the Betulaceae and Myricaceae. The incompatibility of *Frankia* sp. EuI1 with members of these two plant families provides further evidence for the existence of distinct cross-inoculation groups.

Because the results of cross-inoculation studies presented here raise serious questions about the validity of the current taxonomic descriptions of the Frankiaceae (Becking 1974), further taxonomic classification of *Frankia* sp. AvcI1 cannot be made at this time. Judgements on the taxonomic status of each of the isolated frankiae should await the isolation and characterization of a greater number of the actinorhizal endophytes than at present. Additional characterization studies (Lechevalier and Lechevalier 1979) may provide important criteria upon which more thorough taxonomic evaluation can be made.

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