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Isolation and Characterization of *Frankia* Strains from *Alnus incana* and *Alnus glutinosa* in Finland

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Abstract

For isolation of *Frankia*, the nitrogen-fixing symbiont of actinorhizal plants, nodules from the two native alders in Finland, *Alnus incana* and *Alnus glutinosa*, were collected from different ecosystems throughout the country. Characteristic *Frankia* mycelium with branched hyphae and sporangia was obtained from half of the Sp^- type nodules but from none of the Sp^+ type nodules. In all, 118 *Frankia* isolates originating from 25 different nodules were obtained.

Isolates originating from the same nodule were generally identical, but morphologically different isolates were obtained from three nodules. The main morphological differences between the 28 strains obtained lay in the production of vesicles on N-rich media, and in the size, frequency and shape of the sporangia. Physiologically, all the strains were fairly similar to each other, in respect to utilization of carbon sources, decarboxylation of organic acids, and urease production. Irrespective of their origin, all the strains were infective and effective on both alder species.

A combination of the morphological and physiological characters can serve to distinguish some of the strains, but does not afford general identification of individual *Frankia* strains.

Keywords: actinorhizal plants, *Alnus glutinosa*, *Alnus incana*, isolation, *Frankia*, morphology, nodules, physiology

1. Introduction

The diazotrophic actinomycete *Frankia* is capable of forming N_2-fixing root nodules in symbioses with a diverse array of woody plants (Bond, 1983). In the temperate forest the most important actinorhizal plants are the alders. Two alder species, *Alnus glutinosa* (black alder) and *A. incana* (grey alder) are native in Finland.

The onset of nitrogenase activity in developing actinorhizal nodules has been correlated with the differentiation of specialized cells, the vesicles, from the hyphae. Vesicles are also produced by pure cultures in response to nitrogen limitation, with concomitant onset of nitrogenase activity (Murry et al., 1984; Tjepkema et al., 1980).

Two distinct types of *Frankia* can be recognized on the basis of nodule morphology; one forms sporangia within the actinorhizal nodules (spore-positive, Sp^+), but in the other sporulation in the nodule is repressed (spore-negative, Sp^-) (Van Dijk, 1978). The latter is the most frequent type (Torrey, 1987), except in Finland, where the Sp^+ type of *Frankia* predominates on *A. incana* (Weber, 1986).

For decades *Frankia* was considered an obligate symbiont (Becking, 1974). The first successful isolation was reported in 1978 (Callaham et al., 1978; Quispel and Tak, 1978) and by now several hundreds of isolates are available in pure culture. Although all isolates, with few exceptions (Burggraaf, 1984; Normand and Lalonde, 1982), originate from Sp^- nodules, they all produce sporangia *in vitro.*

The criteria used in classifying an actinomycete as a member of the genus *Frankia* include morphology, chemistry, infectivity and effectivity for a host plant, serology and DNA homology, but at the moment it is impossible to define species within the genus *Frankia* (Lechevalier, 1984).

Four host-specificity groups have been defined on the basis of cross-infectivity studies (Baker, 1987) and the same groups of *Frankia* were also separated by biochemical methods, such as protein analysis (Gardes and Lalonde, 1987) or sugar analysis (St.-Laurent et al., 1987). In order to differentiate strains, physiological tests have been developed (Horriere, 1984; Lechevalier et al., 1983).

In this paper we compare some morphological and physiological characters of 118 *Frankia* isolates, originating from 25 different nodules. The validity of these characters as classification parameters is discussed.

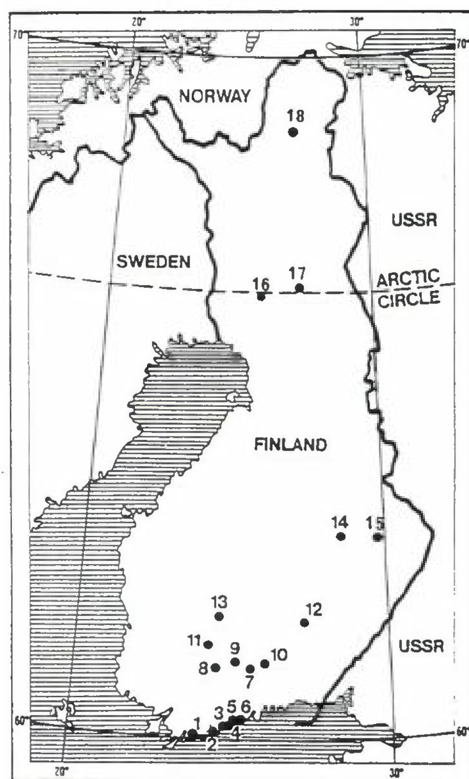

Figure 1. Map of Finland showing sites for sampling of nodules or of soil for laboratory produced nodules.

2. Materials and Methods

Nodule material

The nodule material originated from both *A. incana* and *A. glutinosa* from different ecotypes (Table 1) throughout Finland (Fig. 1). The nodules were either collected directly from the field or produced in the laboratory.

For laboratory-produced nodules, seeds of *A. incana* and *A. glutinosa* were surface sterilized for 5 min in 3% NaOClO, washed 6 times with distilled water and germinated on water agar. Axenic germlings were planted in soil taken from pure stands of either *A. incana* or *A. glutinosa* (Table 1). The plants were kept in a growth room, the light sources being one 400 W high pressure sodium lamp and one 400 W mercury lamp and the photoperiod 18 hr a day. After 3–4 months the nodules were collected and used for isolation.

Table 1. Conspectus of the attempts to isolate *Frankia* from nodules of *Alnus glutinosa* and *A. incana* collected throughout Finland

Provenience of nodule Numbers indicate site[a] on map (Fig. 1)	Soil pH[b]	Nodule type and success of isolation		
		Nodule type[c]	Success[d]	Name of isolates
Alnus glutinosa				
1 Tammisaari, sea-shore	4.3	–	1/1	Ag7
2 Siuntio, coniferous forest	3.3	–	1/3	Ag10
»	3.3	+	0/5	
swamp	3.9	+	0/2	
3 Kirkkonummi, backyard	nd	–	1/1	Ag1[t]
sea-shore	nd	–	1/1	Ag2[t]
swamp	nd	–	1/1	Ag3[t]
4 Espoo, sea-shore	5.2	–	0/2	
sea-shore*	5.2	–	1/3	Ag6
5 Helsinki, park	nd	–	1/1	Ag4
sea-shore	nd	–	0/1	
8 Hämeenlinna, experimental field**	4.9	–	1/1	Ag9
9 Lammi, alder swamp	4.6	–	0/2	
11 Pälkäne, lake shore	6.0	–	0/1	
13 Hyytiälä, alder plantation	3.6	–	1/1	Ag5
alder swamp	4.5	–	0/3	
swamp	nd	–	0/1	
14 Susijärvi, lake shore	nd	–	0/1	
17 Kemijärvi, mixed forest**	3.9	–	1/1	Ag8
»	3.9	nd	0/3	
18 Inari, mixed forest**	4.0	nd	0/12	
Alnus incana				
1 Tammisaari, sea-shore*	4.3	–	2/4	Ai11, Ai12
2 Siuntio, coniferous forest	3.2	+	0/4	
swamp	3.8	+	0/3	
4 Espoo, sea-shore*	5.2	–	2/3	Ai9, Ai10
5 Helsinki, coniferous forest	3.5	–	1/1	Ai6
6 Tuusula, backyard	4.5	–	0/1	
7 Läyliäinen, alder stand**	3.8	+	0/2	
8 Hämeenlinna, coniferous forest	3.9	+	0/10	
experimental field	4.9	–	4/6	Ai0, Ai1, Ai2, Ai3
» **	4.9	–	2/2	Ai15, Ai16
9 Lammi, alder forest	3.9	+	0/2	
	3.9	–	0/1	
10 Vierumäki, deciduous forest	3.9	+	0/2	
12 Mikkeli, deciduous forest	3.5	+	0/4	
13 Hyytiälä, coniferous forest	3.5	–	1/1	Ai8
»	3.5	+	0/1	
14 Susijärvi, coniferous forest	3.7	–	1/1	Ai7
»	3.7	+	0/1	
	3.7	nd	0/2	
15 Kontiolahti, mixed forest	3.1	+	0/5	
16 Rovaniemi, river bank	4.2	–	1/3	Ai13
»	4.2	+	0/1	
experimental field	3.1	–	1/5	Ai14
17 Kemijärvi, mixed forest**	3.9	nd	0/1	
18 Inari, mixed forest**	4.0	nd	0/9	

[a] For a detailed description of the sites, see Weber (1986)

[b] nd = not determined

[c] + = spore-positive; – = spore-negative

[d] Ratio of nodules from which isolate(s) were obtained to total nodules tried

* Laboratory-produced nodule(s) with soil from an *A. glutinosa* stand

** Laboratory-produced nodule(s) with soil from an *A. incana* stand

[t] Isolate(s) lost

The nodules were classified as Sp^+ or Sp^- through microscopical examination of 2 lobes per nodules. The presence or absence of spores was determined by investigation of fresh handcut sections, stained with diluted Fabil reagent (Noel, 1964). In addition microtome sections were occasionally prepared as described earlier (Weber et al., 1987).

Handling of nodules for isolation

Lobes detached from carefully washed nodules were cut into small pieces and soaked in 3% OsO_4 for 30 sec–4 min, washed 6 times in distilled water and transferred to sterile petri dishes (Normande and Lalonde, 1982). The sterilized pieces were either further divided with a sterile scalpel and put into tubes containing media as described below or homogenized with basal salt solution in a mortar, to release endophyte vesicle clusters. The homogenate was either directly diluted and plated or filtered through 2 nylon screens of 50 and 20 μm (Benson, 1982). The cell aggregates on the 20 μm screen were washed and diluted with basal salt solution before plating.

Media and cultural conditions

For isolation the following media were used, all based on the same basal salt solution containing per litre: K_2HPO_4, 0.3 g; NaH_2PO_4, 0.2 g; $MgSO_4 \cdot 7\ H_2O$, 0.2 g; KCl, 0.2 g; $CaCl_2 \cdot 2\ H_2O$, 0.1 g; NH_4Cl, 0.1 g; Fe-citrate, 0.01 g and minor salts according to Lalonde and Calvert (1979). The following carbon and nitrogen sources were added per litre for media a–j: (a) Tween 80, 1 ml (Blom et al., 1980), (b) Na-propionate, 1 g (Shipton and Burggraaf, 1982), (c) Na-pyruvate, 1.5 g; Tween 80, 1 ml; casamino acids, 5 g,(d) Na-pyruvate, 0.5 g; Na-propionate, 0.5 g; Tween 80, 1 ml; casamino acids, 5 g, (e) Na-pyruvate (filter sterilized), 3 g; casamino acids, 3 g (Benson, 1982), (f) Na-succinate, 3 g; casamino acids, 3 g (Benson, 1982), (g) Tween 80, 1 ml; Na-propionate, 0.8 g; casamino acids, 0.5 g, (h) medium g supplemented with glucose, 1 g, (i) medium g supplemented with alcoholic root extract (Quispel and Tak, 1978), (j) glucose, 10 g; lecithin, 0.005 g; Bacto-Pepton, 5 g; yeast extract, 0.5 g (Qmod medium; Lalonde and Calvert, 1979). Media a–f were supplemented with filter-sterilized vitamin solutions as follows: folic acid, nicotinic acid, Ca-pantothenate, pyridoxine, riboflavin, thiamine-dichloride, 0.1 mg l^{-1} final concentration, each, and biotin, 2.3 mg l^{-1}. Media g–i were supplemented with biotin alone, 2 mg l^{-1}. The pH was adjusted to 6.6–6.9 before autoclaving. For plating the medium was supplemented with 7 g agar. For each nodule 20–40 tubes or plates of the medium in question were used.

The isolates were maintained at 28°C as static liquid cultures in a Tween, propionate, casamino acid medium (TPC) containing the basal salt solution with the $CaCl_2$ concentration reduced to 0.01 mg, Fe-citrate replaced with 0.01 mg of Fe-Na-EDTA, and containing per litre 2 mg of biotin, 1 ml of Tween 80, 0.5 g of each of Na-propionate and casamino acids.

Reference strains

The strains AvcI1 (Baker and Torrey, 1980) and EuI1 (Baker et al., 1980) were kindly provided by Dr. Baker.

Physiological tests

The physiological tests comprised utilization of carbon sources, decarboxylation of organic acids and urease production, and were run in triplicate. The inocula were obtained from 150–180 ml cultures grown in TPC for 4 weeks. Cells were harvested by centrifugation, washed 3 times with distilled water, suspended in 5 ml of the basal salt solution and homogenized in a tissue homogenizer or by flushing through a needle series (20-21-23-25 G).

The ability of *Frankia* isolates to utilize different C sources was tested in the basal medium supplemented with single sources of C; the acids as 0.5 g C l^{-1} each, Tween 80 as 3.6 g and glucose as 8.0 g C l^{-1}. All media were sterilized by autoclaving, except for glucose, which was filter-sterilized and added to the autoclaved basal medium. The growth was estimated as protein content after 19 days at 25°C. The pH of the medium was determined at the beginning and at the end of the experiment.

Decarboxylation of organic acids was determined as described by Lechevalier et al. (1983), with the exception that the nitrogen in their basal medium was replaced with NH_4Cl, 0.1 g l^{-1}. Sodium salts of the organic acid were added to the medium prior to autoclaving at 0.2% (w/v) final concentration. Urease production was tested in a strongly buffered urea broth according to Horriere (1984). In both cases tubes containing 6 ml medium were inoculated with 1 ml of the inoculum suspension. The tubes were maintained at 28°C for 42 days, colour changes noted every second day, and the time needed for positive reaction in all 3 tubes was recorded.

Microscopic observations

For light microscopy, intact flocks of living cultures were mounted in water or glycerol-water and observed with dark field, phase contrast, and differential interference contrast (DIC) optics, with a Jenaval microscope from Carl Zeiss, Jena. Since it is known that both C source and temperature effects

the sporulation (Tisa et al., 1983) the growth conditions were standardized. Morphological observations were made on cultures grown on TPC at 28°C.

For electron microscopy samples were prefixed with 3% (v/v) glutaraldehyde (Leiras, Finland) in 0.1 M sodium phosphate buffer (pH 7.2) for 2 hr at room temperature and washed 3 times in the same buffer. The specimens were postfixed for 2 hr in buffered 1% (w/v) osmium tetroxide, dehydrated in a graded series of ethanol and propylene oxide, and embedded in Epon LX 112 (Ladd). Thin sections were cut with a diamond knife on LKB Ultratome I ultramicrotome and double-stained with uranyl acetate and lead citrate. The grids were examined with a Jeol JEM-1200EX electron microscope at an operating voltage of 60 kV.

Protein determination

For protein determination, cells were washed 3 times with distilled water and stored at −18°C. The frozen cells were treated as described earlier (Smolander et al., 1988). The protein content of the supernatant was measured by the Coomassie brilliant blue G 250 method of Bradford (1976), modified by Spector (1978).

Infectivity and effectivity

All the *Frankia* isolated were tested for infectivity and effectivity on *A. incana* and *A. glutinosa*. For this purpose axenic alder plants were grown in closed tubes on gravel supplied with half-strength N-free nutrient solution (Huss-Danell, 1978). The seeds had been surface-sterilized as described above.

Reisolation

For reisolation, isolates were inoculated on axenic alders. Three months after inoculation nodules were harvested and sterilized with OsO_4 as described above, and nodule pieces were incubated in Qmod medium supplemented with propionate.

3. Results

The results of the isolation attempts are shown in Table 1. The identification of isolates as *Frankia* was based on microscopical observations.

From 25 of the 53 nodules shown to be of Sp^- type, characteristic *Frankia* mycelium was detected after incubation of 4 weeks to 6 months. Young nodules gave the best result; isolates were obtained from two thirds of the

laboratory-produced nodule material, whereas less than half of the field-collected nodules provided *Frankia* pure cultures. All the isolation media (a–j) were tried, but in no case did *Frankia* proliferate in a medium lacking propionate. In some media outgrowth of typical hyphae from the nodule pieces could be detected even without propionate, but good growth was obtained only in media containing propionate.

In no case were *Frankia* isolates obtained from Sp^+ nodules. OsO_4 in combination with different isolation techniques was used unsuccessfully on a total of 42 nodules known to be Sp^+, and on the 27 nodules of unknown nodule type (Table 1).

In successful isolations more than one isolate was generally obtained from one nodule; up to half of the inoculated tubes could give growth of *Frankia* and on the plates several *Frankia* colonies were observed. In all, 118 well growing isolates were cultivated further for characterization. Isolates originating from *A. incana* were named Ai and those from *A. glutinosa* Ag. The nodules were numbered and isolates originating from the same nodule were distinguished with small letters, as suggested by Normand and Lalonde (1982). The isolates from the same nodule generally showed similarity in morphology, pigment production and physiology. In such cases, only one isolate was taken to represent the nodule in the strain collection. In 3 cases — Ai8, Ai13 and Ag5 — morphologically different isolates were obtained from one nodule.

Reisolation was undertaken with isolates from 4 nodules and was always successful. Morphologically and physiologically, these reisolates were similar to the parental strains.

A comparison of different carbon sources showed propionate, acetate and Tween 80 to be the best (Table 2). In Table 2 only two strains, Ai1 and Ai2, were included as representatives for the strains tested (data not shown). No growth could be detected on either pyruvate (except the reference strain AvcI1) or succinate, nor did the strains grow on glucose, lactate or malate (Table 2). The reference strain AvcI1 showed the best growth: in 19 days a 40-fold increase of the inoculum was obtained, whereas the corresponding values for the Finnish strains on the same media were only 12–19-fold (Table 2).

Decarboxylation activity was shown by all strains on propionate, and by none on pyruvate or succinate. Five strains did not show decarboxylation of acetate, and three of these (Ai11, Ai12 and Ag7) originate from the same alder soil (Table 3).

Table 2. Growth of *Frankia* strains on various carbon sources

C source, g l^{-1}	Protein yield, μg/10 ml culture[a] (pH)[b]		
	Ai1	Ai2	AvcI1
Acetate, 0.5	27±0.5 (+0.7)	60±4.7 (+0.8)	82±6.2 (+1.8)
Propionate, 0.5	31±0.9(+0.4)	64±4.2 (+0.4)	185±9.7 (+1.6)
Tween 80, 3.6	37±4.3 (-0.2)	94±4.2 (-1.1)	277±12.7 (-1.1)
Pyruvate, 0.5	7±0.4 (+0.2)	4±0.5 (+0.1)	19±0.2 (+0.3)
Glucose, 8.0	5±0.1 (0)	2±0.0 (0)	7±1.0 (+0.1)
Succinate, 0.5	5±0.5 (+0.1)	2±0.5 (0)	7±0.2 (+0.1)
Fumarate, 0.5	4±0.6 (+0.1)	2±0.5 (+0.1)	4±0.5 (0)
Malate, 0.5	5±0.3 (+0.1)	5±1.7 (+0.1)	7±0.6 (+0.1)
Control	3±0.3 (+0.1)	5±0.3 (0)	7±0.0 (+0.1)

[a] mean ± S.E., n≥3
[b] change in pH, during 19 days of incubation at 28°C

In media containing single sources of carbon, pH changes were observed to accompany good growth. With acetate and propionate, a rise of 0.4–1.8 pH units was observed, whereas with Tween the pH was lowered by 0.2–1.1 units. A combination of Tween and propionate gives a medium in which the pH remains stable even for longer incubation periods, and this was consequently used as standard medium (TPC).

Besides the reference strain AvcI1, only one strain (Ai6) was urease positive.

All the isolates obtained meet the morphological criteria of *Frankia* strains as previously reported in the literature (Lechevalier, 1984). Vegetative growth is filamentous with branching and septation (Fig. 2). The filament diameter varies between 0.5 and 1.0 μm, with occasional thickening to 1.5 μm. Growth of intrahyphal hyphae can be seen in some part of the mycelium, as reported by Newcomb et al. (1979).

The presence of combined nitrogen in the growth medium completely repressed formation of vesicles in some of the strains (Table 4), but the rest of the strains produced vesicles even in the presence of 30 mM nitrogen (Figs. 3 and 6a). These vesicles (Fig. 4) show the internal septation characteristic of active vesicles produced on N-free medium (Fontaine et al., 1984).

Table 3. Physiological data on *Frankia* strains

Strain	Decarboxylation of c-source[a]				Urease activity	Soluble pigment[b]
	Acetate	Propionate	Pyryvate	Succinate		
From *A. incana*						
Ai0	2	1	0	0	0	no
Ai1	2	1	0	0	0	no
Ai2	2	1	0	0	0	no
Ai3	2	1	0	0	0	no
Ai6	1	1	0	0	1	no
Ai7	2	1	0	0	0	violet[c]
Ai8a	2	1	0	0	0	no
Ai8d	3	1	0	0	0	no
Ai9	2	1	0	0	0	no
Ai10	1	1	0	0	0	no
Ai11	0	1	0	0	0	no
Ai12	0	1	0	0	0	no
Ai13a	2	1	0	0	0	no
Ai13b	2	1	0	0	0	no
Ai14	2	1	0	0	0	no
Ai15	2	1	0	0	0	no
Ai16	2	1	0	0	0	no
From *A. glutinosa*						
Ag4	0	1	0	0	0	brownish
Ag5a	3	1	0	0	0	no
Ag5b	3	1	0	0	0	no
Ag6	2	1	0	0	0	no
Ag7	0	1	0	0	0	no
Ag8	3	2	0	0	0	no
Ag9	2	1	0	0	0	no
Ag10	0	2	0	0	0	yellow
Reference strains						
Avcu I1	2	2	0	0	3	no
Euu i1	3	2	2	0	0	no

[a] 3 = positive reaction between 3 and 6 days
2 = positive reaction between 6 and 10 days
1 = positive reaction between 10 and 22 days
0 negative reaction for 42 days

[b] Pigment production recorded in TPC medium

[c] The pigment is non-soluble if Tween 80 is omitted from the medium

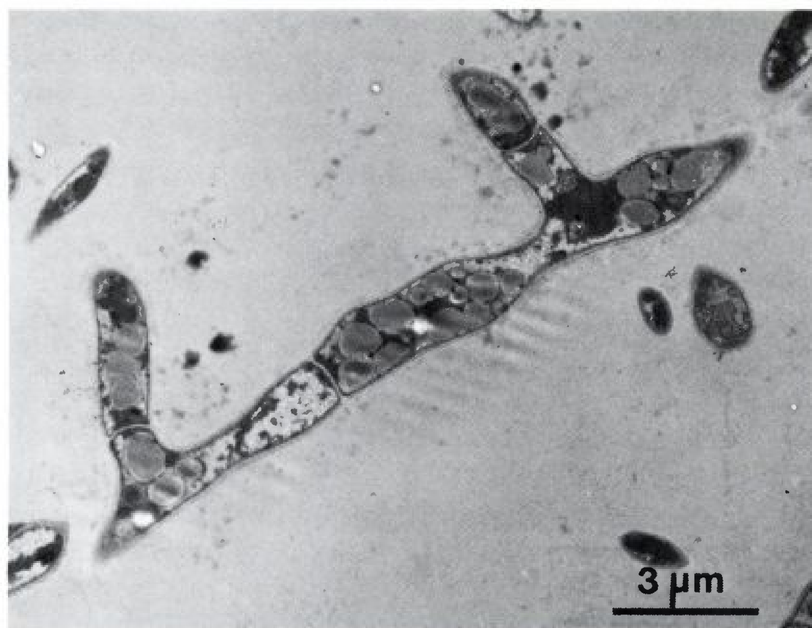

Figure 2. Electron micrograph of branched and septate *Frankia* Ai2 hypha.

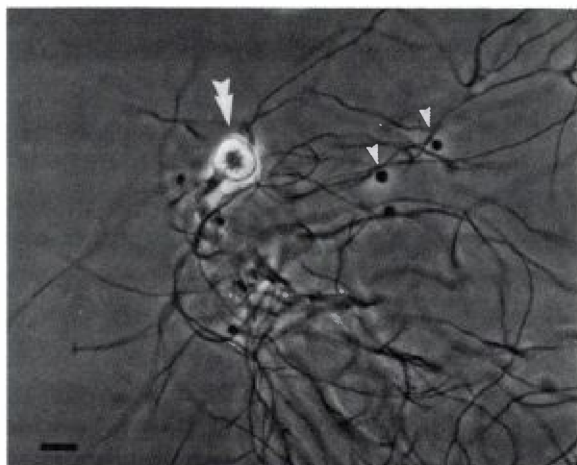

Figure 3. Several vesicles (small arrows) and a rare stalked sporangium (double arrow) on edge of living flock of strain Ai1 in TPC medium. Phase contrast optics. Bar represents 10 μm.

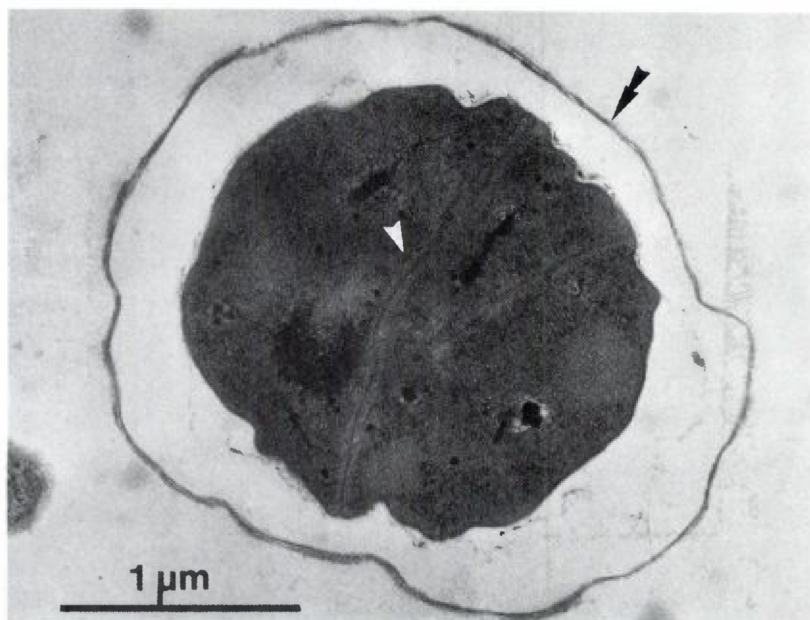

Figure 4. Electron micrograph of a vesicle produced by *Frankia* Ai6 on nitrogen containing (TPC) medium showing internal septation (arrow) and outer wall (double arrow).

Sporangia are produced by all isolates, but the size and frequency differ from strain to strain (Table 4). In some strains sporangia are hardly ever found (Fig. 3), but in others sporangia are present in every flock (Fig. 5a) or the flocks are covered with sporangia (Fig. 5b). The form of the sporangia also varies between the strains. In most strains sporangia mainly develop either as lateral or terminal swellings on the hyphae and can be of different shapes (Table 4): e.g. round (Fig. 6a), stalked (Fig. 3) or cone-like (Fig. 6b). In some strains sporangia are produced intercalarly and are of dermatophilus-like appearance, either long stretches of hyphae segregate into narrow sporangia (Fig. 6c) or numerous adjacent swellings give a calabash-like appearance (Fig. 6d). Other forms can occur besides the shapes described here, but the strains are characterised by a certain predominant sporangium shape.

All our strains were infective on both alder species, irrespective of their origin. The nodules produced were effective, since all inoculated alders grew well on N-free nutrient solution. Uninoculated controls never developed nodules and consequently died during the test.

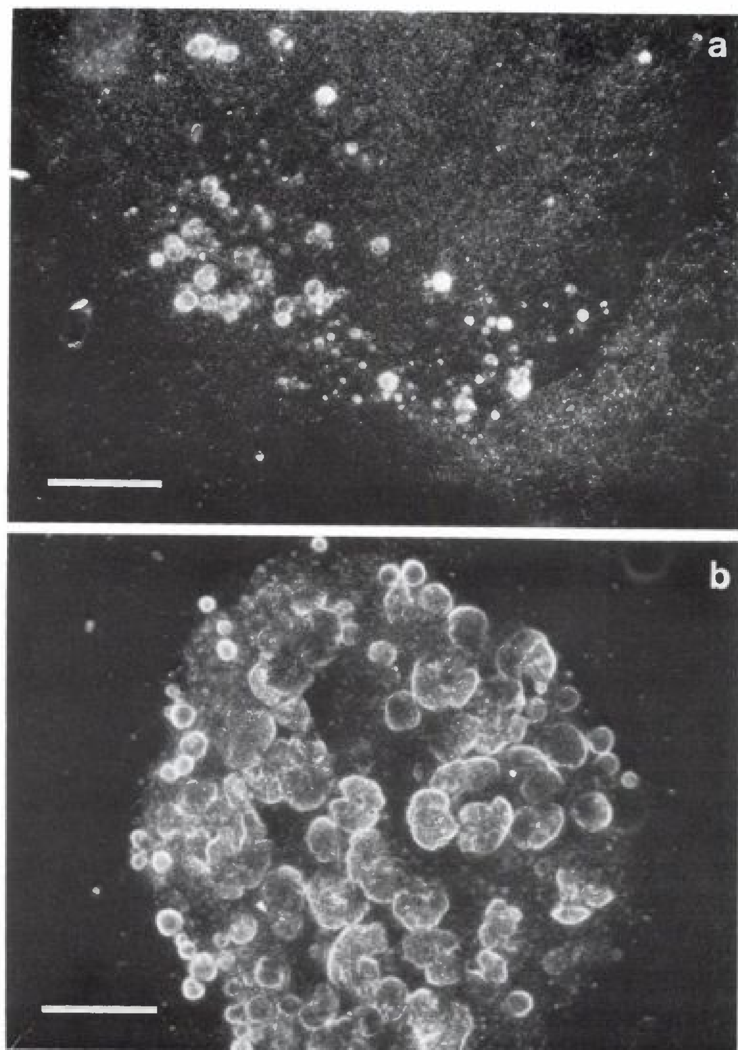

Figure 5. Low magnification overview of living flocks in TPC medium showing low frequency of small sporangia in strain Ai2 (a), and high frequency of big sporangia in strain Ai10 (b). Dark field optics. Bars represent 100 μm.

4. Discussion

As is generally the case, isolation was successful when the nodules were of the Sp^- type. In spite of many trials, using different isolation techniques and several media, no isolates could be obtained from Sp^+ nodules. Sp^+ strains

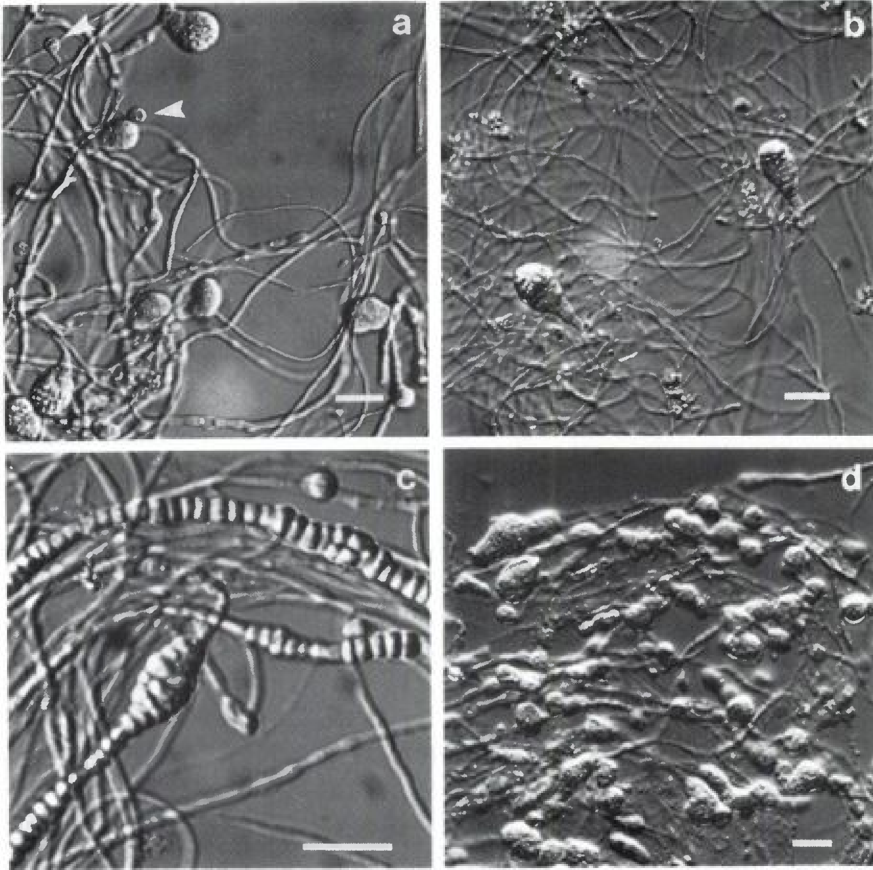

Figure 6. Examples of sporangia of different forms, all in nitrogen containing TPC medium. (a) round and cone-like sporangia in strain Ai7 (vesicles indicated by small arrows); (b) cone-like sporangium in strain Ag5a; (c) dermatophilus-like sporangia in strain Ai12; and (d) calabash-like sporangia in strain Ai15. Bars represent 10 μm.

have proved difficult to isolate and maintain, and are not available (Torrey, 1987).

All strains were physiologically very similar to each other. Propionate was used as C source by all the strains — as by *Frankia* in general. Unlike many other frankias, our strains used neither succinate nor pyruvate (Table 2); acetate was decarboxylated by most strains (Table 3), but here exceptions were found. Urease was produced by one strain only. Utilization of acetate as C source seems to be the best of the physiological tests used for differentiation of the isolates. The other physiological tests used here are of little value.

Table 4. Characterization of *Frankia* strains based on morphology and physiology

Sporangia			Pigment production	Urease activity	Acetate decarboxylation	Strains
Size[a]	Frequency[b]	Dominant shape				
Group I (vesicles in TPC medium)						
s	+	stalked	–	–	+	Ai0 Ai1 Ai3 Ai16 Ag9
s	++	stalked	–	–	+	Ai2
s	+++	calabash	–	–	+	Ai9 Ai15
b	++	cone	–	+	+	Ai6
b	++	cone	violet[c]	–	+	Ai7
b	+++	dermatophilus	–	–	–	Ai11 Ai12 Ag7
s	+	cone	–	–	+	Ag5a
Group II (no vesicles in TPC medium)						
s	+++	sarcina	–	–	+	Ai8d
s	+++	round	–	–	+	Ai8a
b	+++	round	pink[d]	–	+	Ai10
b	+++	dermatophilus	–	–	+	Ai13a
s	++	round	–	–	+	Ai13b
b	+	dermatophilus	–	–	+	Ai14
s	+	dermatophilus	–	–	+	Ag5b
s	+	round	–	–	+	Ag6
s	++	round	–	–	+	Ag8
b	+++	round	yellow[c]	–	–	Ag10

[a] s = small, $< 20\ \mu m$; b = big, 20–60 μm

[b] + = sporangium rare, found with difficulty with high power magnification; ++ = some sporangia in every flock; +++ = flock covered with sporangia

[c] soluble pigment

[d] non-soluble pigment

Although the strains originate from soils with low pH (Table 1), none of the strains tested grew in pure culture at pH 4.5 (results not shown). The pH optimum for Ai1 and Ai6 was shown to be between 6 and 7 (Smolander et al., 1988). Since the optimum pH of *Frankia* had been shown to be around 7 (Burggraaf and Shipton, 1982), our isolation media (selection) and subcultivating media (adaptation) were adjusted to pH 6.6–6.9, which might explain the reduced growth at low pH.

Vesicles have been proposed as the sites of nitrogen fixation (Noridge and Benson, 1986; Meesters, 1987). In most frankias vesicles are formed only in response to nitrogen limitation (Fontaine et al., 1984; Murry et al., 1984; Tjepkema et al., 1980), but some strains can produce vesicles even on N-rich media (St.-Laurent and Lalonde, 1987; Meesters, 1987; Tisa et al., 1983). More than half of our strains produced vesicles in the presence of high concentrations of nitrogen (Table 4). These vesicles (Fig. 4) are structurally similar to the active ones induced under nitrogen-fixing conditions only, and characterized by internal septation (Fontaine et al., 1984). The outer wall layer, believed to provide protection against oxygen for active nitrogenase (Lancelle et al., 1985), is clearly present in our strains in media containing nitrogen.

The description of the sporangia should not be taken as exclusive. Several forms are visible in our strains, but the dominant types prevail from year to year in the subcultures.

The morphological characters of the isolates allow several conclusions regarding the *Frankia* populations from which the samples were taken. The field at Hämeenlinna contains a homogeneous *Frankia* population. Five of the seven strains (Ai0, Ai1, Ai3,Ai16 and Ag9) are morphologically identical, although they were obtained from different nodules over a period of 3 years. The same homogeneity was found in a *Frankia* population by Benson and Hanna (1983), who obtained 35 (80%) indistinguishable isolates from one site.

Diversity of *Frankia* populations is also demonstrated in this study. Inoculation of one grey alder and one black alder with soil from a sea-shore (Espoo, 4) gave two *A. incana* isolates (Ai9 and Ai10) and one *A. glutinosa* isolate (Ag6) that were all different. Morphologically different isolates were even obtained from single nodules, which indicates multiple infection of the nodule, as also described by Benson and Hanna (1983).

The soil from an *A. glutinosa* stand (Tammisaari, 1) that was used for inoculation of one jar containing two grey alders and one black alder gave

isolates that were identical (Ai11, Ai12 and Ag7). Either this soil contained a homogeneous *Frankia* population or there are no differences between the two alder species in their selection of Sp^- strains, in contrast to their selection of Sp^+ strains (Weber et al., 1987).

The characters employed in actinomycete taxonomy in the past have been the size and shape of the sporangia or spores, and production of cellular or soluble pigment. For *Frankia* these characters are not considered sufficiently stable to be useful (Lechevalier, 1984). Our experience is that the morphological characteristics are stable but too diverse for identification use. Some strains can be recognized by their morphology or pigment production alone (e.g. Ai7 and Ai10), but many strains cannot be separated from each other. A combination of different observations (Table 4) can be used to separate some of the isolates, but do not allow general identification of individual *Frankia* strains.

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