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In vitro production of specialized reproductive torulose hyphae by *Frankia* strain ORS 021001 isolated from *Casuarina junghuhniana* root nodules

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Summary A *Frankia* strain (ORS 021001) isolated from *Casuarina junghuhniana* root nodules was shown to produce four types of structures *in vitro*: vegetative hyphae, sporangiospores within sporangia, N_2-fixing vesicles, and a fourth type of structure which is described in detail in this report. Structures of this latter type which we propose to call 'reproductive torulose hyphae: (RTH) result from enlargement and multiple segmentation of vegetative hyphae into torulose chains of spore-like cells. RTH differ from sporangia in three major aspects: morphology, morphogenesis and outgrowth. RTH play an important role in survival and reproduction of *Frankia* strain ORS 021001. Adding activated charcoal to the nutrient medium promotes the formation of *Frankia* colonies originating from RTH.

Introduction

Since the first successful isolation of an effective strain of *Frankia* from *Comptonia peregrina*[6], an increasing number of *Frankia* strains have been isolated from various actinorhizal plants[2,3,4,5,19]. In all these papers, the authors invariably reported that the actinomycetes belonging to the genus *Frankia* produced three main structures *in vitro*: vegetative hyphae, vesicles and polyhedral sporangiospores (spores formed within sporangia). Because vegetative hyphae of *Frankia* are frequently subject to autolysis[8,12,17], it has been throught that survival and subsequent regeneration of *Frankia* are ensured by means of sporangiospores. We know of no report on survival structures other than sporangiospores in *Frankia.*

In a previous paper reporting the isolation of five strains of *Frankia* from Casuarina root nodules[8], we noted that these strains also produced the three structures listed above. Later, in two papers describing the first successful isolation of an infective and effective strain (ORS 021001; syn. Cj1–82) of *Frankia* from *Casuarina junghuhniana* root nodules[9,11], we reported for the first time the production of a fourth type of structures consisting of elongate torulose chains of cells provisionally called 'sporangia-like structures'. These peculiar structures are now designated as 'reproductive torulose hyphae' (RTH) because of some specific characteristics presented in this report.

The aim of the present work is to report additional information

about the nature and the role of RTH and to compare their morphogenesis and germination with these processes in true sporangia. Examination of structures produced by *Frankia* strain ORS 021001 led us to propose a scheme for the *in vitro* life cycle of this strain. Special attention was given to the stimulating effect of activated charcoal on the initiation and development of *Frankia* colonies.

Materials and methods

The approach used to study the morphology, the morphogenesis and the role of RTH was to inoculate a solid medium with an homogenized *Frankia* culture and to observe the development of RTH into colonies. The study was carried out using both light and transmission electron microscopy (TEM). Our observations were restricted to strains of *Frankia* isolated from Casuarina. RTH were observed in all strains studied: ORS 020607 and ORS 020606 (syn. CeF1–82 and CeD1–82 respectively[10]). However the present paper deals only with RTH from strain ORS 021001.

Frankia *strain and inoculum preparation*

The infective and effective strain ORS 021001 used in the study was isolated from nodules of *Casuarina junghuhniana* grown in the nursery of the Thai Forest Service in Bangkok[9,11]. The culture was maintained in the laboratory in tubes (18 X 140 mm) containing 10 ml of Qmod liquid medium[15] and incubated at 28–30°C in the dark. A tube of 5-month-old culture from the collection was used to inoculate a series of tubes containing a fresh Qmod liquid medium as above. Subcultures of *Frankia* strain ORS 021001 obtained from this first step were not fluffy but exhibited several pellets consisted of compact, globose colonies *ca* 0.5 mm in diameter. Three-week, 1-month and 2-month-old subcultures described as above were then used to prepare inocula for further experiments: after decanting the supernatant medium, cultures were transferred to 125 ml flasks containing 25 ml of sterile distilled water and homogenized with a magnetic stirrer to obtain a homogenous suspension of fragments of *Frankia* colonies. The suspenion was diluted ten-fold with sterile distilled water before inoculating solid media.

Media, inoculation and numeration of colonies

Two solid media were used: Qmod agar medium and Qmod agar medium supplemented with activated charcoal (Merck, Art. 2186) at the rate of 130 mg per liter. This latter medium is designated as Qmod AC agar medium. One ml of the diluted inoculum (*ca* 0.2 µg protein) prepared as indicated above was introduced into a petri dish (10 cm in diam.) and about 20 ml of cooled molten medium was then poured. Inoculated petri dishes were incubated at 28–30°C for 2 weeks after which colonies were counted using a dissecting microscope.

Identification of the structures forming colonies

Undisturbed colonies at an early stage of growth in Qmod AC agar medium were removed from the petri dishes, stained with 1% (w/v) trypan blue in lactophenol and examined microscopically. To visualize outgrowth from RTH, colonies were crushed between glass slide and cover slip, stained as stated above and examined with objectives X 100.

Morphological and ultrastructural studies

Further structural studies were made of RTH produced in 6-week-old cultures in Qmod AC agar medium. For TEM, colonies were fixed at room temperature in 2.5% glutaraldehyde in 0.1 *M* Na cacodylate buffer, pH 7.2, for about 2 hours. They were then washed with buffer postfixed in 1% osmium tetroxide in buffer at 4°C for 2 hours and subsequently dehydrated in a graded alcohol series and embedded in Epon 812. Ultrathin sections were cut with a diamond knife and stained with uranyl acetate and lead citrate before examination with a Siemens Elmiskop 101.

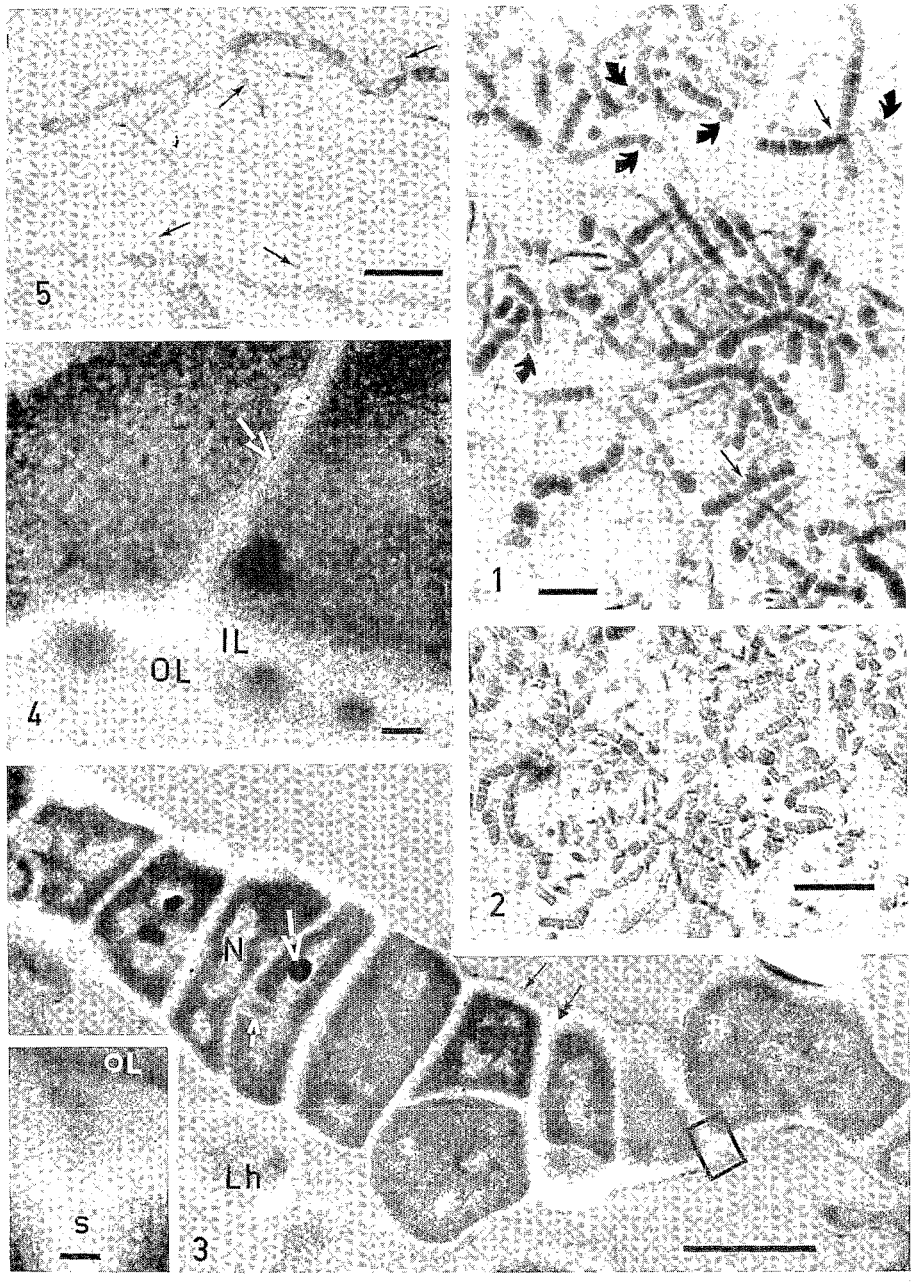
Results and discussion

Identification of the new type of structure in in vitro *cultures of* Frankia *strain ORS 021001*

General morphology. The new type of structure results from the enlargement of a vegetative hypha and the segmentation of this hypha into individual cells by means of transverse septa, the new type of structure (RTH) appears as a long chain of short cells connected to one another. This cell arrangement confers to this structure the unique aspect of a torulose hypha (Fig. 1). The length of RTH may reach 30–50 μm and width varies from 1.5 to 3–4 μm. Due to their peculiar morphology (Fig. 1, 2), RTH should not be confounded with sporangia which are generally considered as clusters of polyhedral sporangiospores. Furthermore, in contrast with sporangia, which promptly liberate sporangiospores, RTH do not break open easily but disrupt only under pressure, into single cells or groups of unicellular spore-like cells (Fig. 2). Individual cells of RTH appear in square or rectangular form in photomicrographs and measure 2–3 x 3–4 μm.

Ultrastructure and morphogenesis. Fig. 3 shows a portion of RTH. Compared with the normal hyphae, RTH are wider and exhibit a highly marked segmentation but the cell wall structure is apparently not different. RTH have a two-layered wall: (1) a thick electron-translucent layer adjacent to the plasma membrane, and (2) a relatively thin electron-dense layer at the outside. Initially, septa are a continuation of the inner layer only, but the outer electron-dense layer of the wall can invaginate the septum inwards from the periphery (Fig. 3, inset) probably contributing to the formation of a thin electron-dense layer within the septum as observed in Fig. 4. This thin electron-dense layer is the line of cell cleavage when RTH are disrupted into individual cells. Cells resulting from the rupture of RTH are thus enveloped by a two-layered wall like the parent hyphae. When the thin electron-dense layer is absent, cells remain connected in chains.

It is interesting to compare the morphogenesis of RTH with that of sporangia. According to different authors who extensively studied the morphogenesis of *Frankia* sporangia[13,16,18], the envelope surrounding the young sporangium is composed of a thin inner layer of low electron density and two distinct electron-dense layers. Complex cell wall development is then observed leading to the formation of the outer sporangial wall and the subdivision of the young sporangium into compartments by the growth of numerous transverse and longitudinal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septa. By contrast, RTH result simply from enlargement of a vegetative hypha and its subsequent segmentation by transverse septa; in addition, RTH have no outer envelop which could be assimilated to a sporangial wall. The morphogenesis of RTH cells is reminiscent of that of spores in some nocardioform actinomycetes. According to the classification of Williams *et al.*[23], RTH cells, like spores of some nocardioform actinomycetes, would result from the 'fragmentation of sheathless hyphae' whereas *Frankia* sporangia would result from the 'fragmentation of sheathed hyphae (see also Horrière *et al.*[13]). Another similarity between *Frankia* and the nocardioform group is the general development of hyphae beneath the agar medium (substrate hyphae) from which fragmentating spores are formed[14,23].

Development of transverse septa in RTH is associated with the presence of mesosomes (Fig. 3). Sometimes droplets of moderate electron density are seen embedded in the outer layer of RTH wall (Fig. 4) but their significance is not clear. During the development of vegetative hyphae into RTH qualitative changes in stored nutrients probably occur. In some RTH, each cell contains one large globose lipid body (Fig. 5) as indicated by red coloration after staining with a Sudan dye. Some cells of RTH contain spherical electron-opaque inclusions which might be polyphosphate granules (Fig. 3), since similar electron-opaque inclusions have been considered as storage bodies in fungal chlamydospores[21,22].

Fig. 1. General morphology of reproductive torulose hyphae (RTH) produced by *Frankia* ORS 021001 grown in Qmod agar medium for ca. 6–8 weeks. Note the presence of some branched RTH (small arrows) and spore-like cells obtained by disrupting RTH (large arrows). Bar: 10 μm.

Fig. 2. Rupture of RTH into spore-like cells which are reminiscent of fragmenting spores produced by certain actinomycetous groups (*e.g.* nocardioform). In the figure, these cells appear in square or rectangular form. Bar: 10 μm.

Fig. 3. TEM showing a portion of double-walled RTH comprised of an outer thin electron-dense layer (single small black arrow) and an inner thick electron-translucent layer (double small black arrow). Electron-opaque polyphosphate (?) granules (large white arrow) and mesosomes associated with septum formation (small white arrow) are seen. N: nuclear material. Lh: lysed vegetative hyphae. Bar: 1 μm. Inset: enlargement of the outlined area in Fig. 3 showing the invagination of the outer electron-dense layer (OL) into the septum (S). Bar: 0.05 μm.

Fig. 4. TEM showing a thin electron-dense layer (white arrow) within a transverse septum (S) of RTH. Note the presence of electron-dense droplets embedded in the outer layer (OL) of the RTH wall. IL: inner layer of RTH wall. Bar: 0.05 μm.

Fig. 5. Growth of new hyphae (arrows) from some RTH cells. Note the presence of a globose lipid droplet in each RTH cell as indicated by staining with a Sudan dye (see text). Bar: 10 μm.

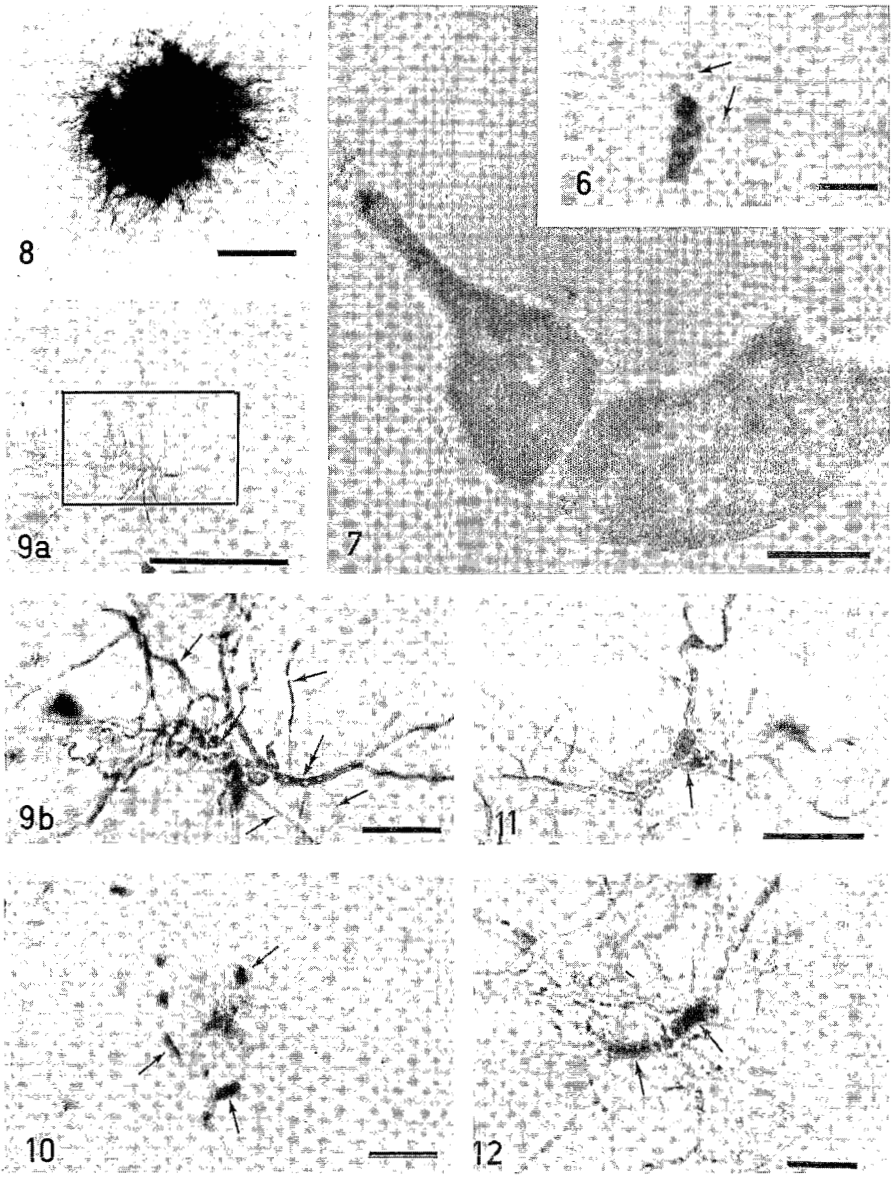


Fig. 6. Growth of new hyphae (arrows) from a fragment of RTH. Bar: 5 μm.

Fig. 7. TEM showing 2 RTH cells. One cell produces a new hypha which has the same wall as the parent cell. Bar: 0.5 μm.

Fig. 8. Large dense colony of *Frankia*.ORS 021001 (2-week-old) grown in Qmod agar medium. This colony type always derived from a large piece of an original colony used to inoculate the medium. Note the density of new hyphae formed at the periphery of the colony. Bar: 100 μm.

Fig. 9a. Small diffuse colony of *Frankia* ORS 021001 (2-week-old) grown in Qmod AC agar medium. Bar: 45 μm.

Fig. 9b. Enlargement of the outlined area in Fig. 9a showing growth of new hyphae (single arrows) from RTH (double arrows) located in the colony center. Note the presence of globose lipid droplets in RTH cells. Bar: 10 μm.

Fig. 10. Small diffuse colony of *Frankia* ORS 021001 (5-week-old) in Qmod AC agar medium. This colony type is always obtained when originated from a small fragment of original *Frankia* colonies used to inoculate the medium. Note the loose network formed by new hyphae around the colony center. Activated charcoal granules (arrows). Bar: 100 μm.

Fig. 11. Growth of new hyphae from some RTH cells (arrow) leading to *Frankia* colony formation. Bar: 10 μm.

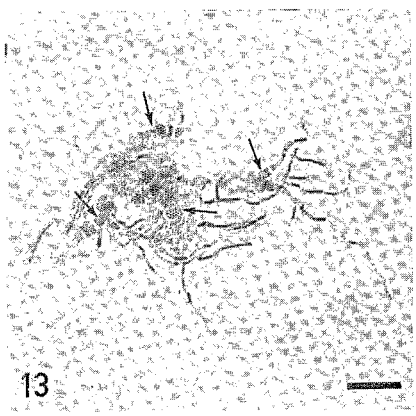
Fig. 12. Center of a small diffuse colony showing a portion of RTH (arrows) which is the origin of the colony. Bar: 10 μm.

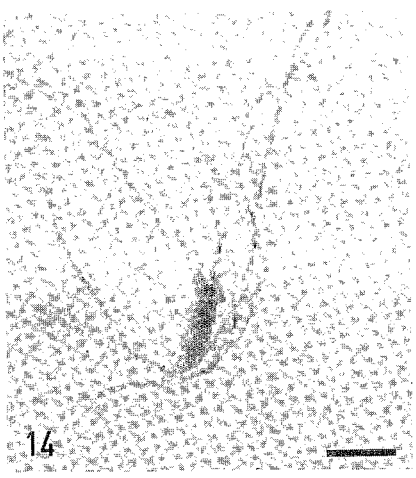
Germination versus *outgrowth.* Until now there has been few extensive work on the germination of *Frankia* sporangiospores. Microscopic observations have shown that *Frankia* sporangiospores rarely germinate, probably because they are dormant structures which require activation before germ tube growth occurs. By contrast, RTH cells readily give rise to new hyphae when deposited onto fresh media suggesting that no specific mechanism is needed prior to outgrowth of new hyphae. When observed with a light microscope, these new hyphae resemble germ tubes from fungal spores (Fig. 6), but TEM clearly shows that the wall of the parent RTH cell does not break at the emergence point so that the wall of the new hypha is a continuation of the wall of the parent RTH cell (Fig. 7). Taking into account the fact that spore germination implies a succession of specific events[1] whereas emission of hyphae from RTH cells is a simple outgrowth, we do not use the term 'germination' to designate the RTH outgrowth.

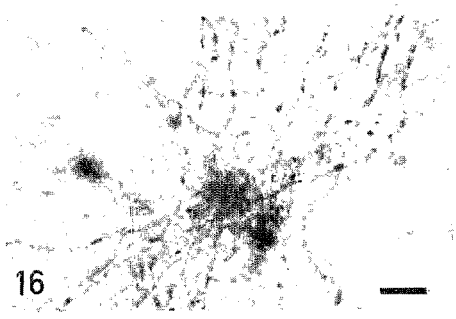
Role of RTH in in vitro *growth of* Frankia *colonies*

This role was determined experimentally by observing the origin of *Frankia* colonies developing in Qmod agar medium and Qmod AC agar medium. After 2-week-incubation, two types of colonies were obtained: (1) large dense colonies (Fig. 8) developing in Qmod agar medium as well as in Qmod AC agar medium; (2) small diffuse colonies (Fig. 9a) generally developing in Qmod AC agar medium but seldom in Qmod agar medium.

Large dense colonies. The center of the colonies (Fig. 8) was occupied by an opaque mucilaginous mass *ca* 50–200 μm in diam.







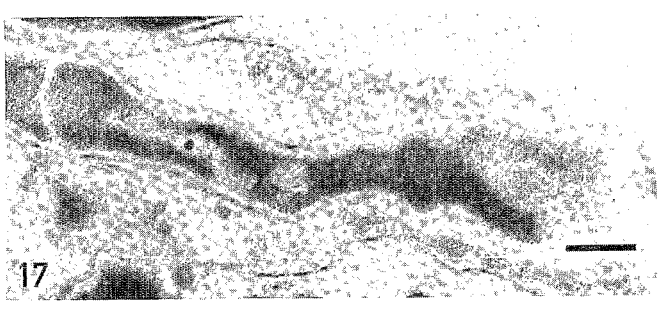


Fig. 13. New hyphae growing from a group of RTH (arrows). Subsequent development of these hyphae will probably give rise to a small diffuse colony. Bar: 10 μm.

Fig. 14. New hyphae emerging from different cells belonging to the same RTH. Note the flexuous, unbranched appearance of new hyphae. Bar: 10 μm.

Fig. 15. RTH with newly emerging hyphae (single small arrows) and a cluster of viable sporangiospores (large arrow) well stained with trypan blue. Lysed vegetative hyphae (double small arrows) not stained. Note that sporangiospores did not germinate. Bar: 10 μm.

Fig. 16. Center of a small diffuse colony of *Frankia* ORS 021001 in Qmod AC agar medium showing a concentrated mass of original *Frankia* structures probably sporangiospores. It is not possible to determine if the colony originated from sporangiospores or from other structures embedded in the mass. Bar: 10 μm.

Fig. 17. TEM showing a thin hypha growing within a wide lysed hypha of *Frankia* strain ORS 021001 (intrahyphal growth). Bar: 0.5 μm.

Table 1. Effect of activated charcoal added to Qmod agar medium on the formation of *Frankia* (ORS 021001) colonies

Age of the culture used as inoculum	No. of colonies* counted on	
	Qmod medium	Qmod AC medium**
3 weeks	10	34
1 month	82	2660
2 months	42	9400

*Number of colonies × 10^2, per ml of inoculum.
**Qmod AC medium: Qmod medium supplemented with activated charcoal (0.013%).

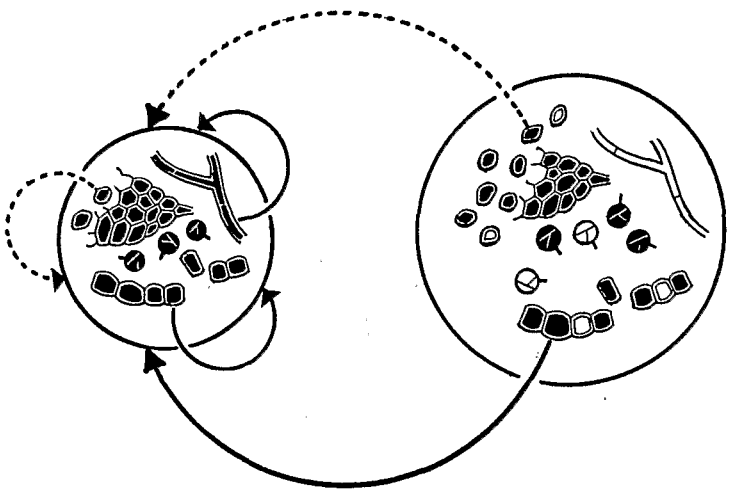


Fig. 18. Scheme of putative life cycle of *Frankia* strain ORS 021001. Black-coloured structures are viable whereas empty ones are lysed. Vesicles are only found in N-free media. Solid lines indicate the main reproduction pathway studied in this report. Dotted lines indicate the ancillary pathway which is not experimentally evidenced. Scale not observed.

At the periphery, new hyphae grew profusely forming a dense network of actively growing hyphae.

Small diffusive colonies. After 2-week-incubation, many small colonies at the early stage of growth were observed in Qmod AC agar medium. At this time, only few hyphae grew and branched in the area surrounding colony centers (Fig. 9b) which were much smaller than those of large dense colonies (Fig. 8). Later, although growth was active, the colonies were characterized by their diffuse aspect due to the loose network formed by sparse radiating hyphae around the center (Fig. 10).

Origin of the two types of colonies. The origin of large dense colonies was difficult to determine because of the concentration of entangled *Frankia* structures at the center. By contrast, small diffuse colonies always originate from a distinct and limited center which could easily be seen. Nearly all *Frankia* colonies of this type arose from a fragment (Fig. 11, 12) or a group (Fig. 13) of RTH. Hyphal outgrowth generally occurred from an individual RTH cell while still connected in the chain (Fig. 5, 6). The initial outgrowing hyphae often extended to considerable lengths before branching occurred. This explains the formation of a tuft of flexuous hyphae originating from a given RTH portion (Fig. 14). In many cases, we observed that *Frankia* colonies emerged from peculiar regions of fragments of original *Frankia* colonies. Microscopic examination of these fragments showed that RTH were abundant in these regions whereas in regions from which *Frankia* did not grow there were no RTH but only lysed hyphae. In older *Frankia* colonies (*ca* 3-month-old), clusters of sporangiospores and RTH were often embedded in a mass of lysed hyphae; thus when a piece of these colonies was exposed to a fresh medium only RTH cells could produce new hyphae (Fig. 15, arrows). No individual sporangiospores was observed to give rise to new hyphae probably because sporangiospores did not find favorable conditions required for germination. A few colonies seemed to emerge from clusters of *Frankia* structures reminiscent of clusters of sporangiospores but it was not possible to determine if this was the case (Fig. 16).

Effect of activated charcoal on Frankia *colony development*

An experiment was designed to study simultaneously (1) the influence of addition of activated charcoal to Qmod agar medium on the development of *Frankia* colonies and (2) the effect of inoculum age upon the number of *Frankia* colonies. Table 1 shows that enhanced

colony number in response to addition of activated charcoal was more marked with older cultures. We interpret these data as follows: (1) most colonies found in Qmod AC agar medium originated from specialized structures other than vegetative hyphae which were progressively lysed in old *Frankia* cultures; (2) the production of these specialized structures increased as *Frankia* cultures became older; (3) activated charcoal added to the Qmod agar medium markedly stimulated the initiation of the structures into colonies. We suppose that these specialized structures were RTH.

Activated charcoal may act as a complementary nutrient or adsorbing agent to eliminate toxic substances possibly produced in older cultures. The role of activated charcoal in the stimulation of *Frankia* growth is not yet understood. Whatever mechanisms are involved, the addition of activated charcoal to the medium is recommended to promote *Frankia* colony formation.

Concluding remarks

Multiplication of *Frankia* through live hyphae are probably the major reproduction process in the case of young cultures. In older cultures, intrahyphal growth has been proposed as an alternative mechanism of reproduction of *Frankia*[14]. Intrahyphal growth is probably not exceptional and could be important in the genus *Frankia* since it has already been observed by several authors[13,18] and ourselves (Fig. 17). In the absence of viable vegetative hyphae, sporangiospores theoretically should be the sole multiplication structures although, as far as we know, there are few specific studies on the conversion of a sporangiospore into a complete colony.

Our study of *Frankia* strain ORS 021001 has shown that colonies could frequently originate from RTH. Further observations (not reported here) have indicated that these colonies contained different typical structures of *Frankia*. Consequently, we suggest that RTH are specialized reproductive structures of strain ORS 021001 capable of giving rise to complete colonies when conditions are favorable again. As already mentioned, RTH result from a pecular transformation of vegetative hyphae, it is interesting to note that a similar transformation has been reported by Nyvall and Kommedahl[20] in the case of *Fusarium moniliforme*.

Due to the unique torulose morphology, the ability to differentiate into spore-like cells and the role as survival and reproductive structure, RTH are not normal vegetative hyphae. Are RTH chains of spores? If we refer to the definition of the term 'spore' by Cross and Attwell[7] RTH cells might be considered as such because (1) they are produced

by the modification of part of a vegetative organism; (2) they differ from the vegetative organism morphologically and (3) they may function as a unit specialized for reproduction. As reported in the present work, RTH cells differ from typical spores described in the genus *Frankia* (sporangiospores) in several respects: morphology, morphogenesis and outgrowth. Therefore, additional investigations are needed to clarify the nature of RTH cells.

The formation of RTH in strain ORS 021001 has not been studied yet but current observations frequently showed that RTH were only produced in the mucilaginous region located in the center of single colonies obtained in liquid and solid media. RTH are likely not produced when cultures are consisted of a loose network of fluffy hyphae. Environmental factors inducing the formation of RTH are not known.

Putative life cycle of Frankia *strain ORS 021001*

The examination of different sequences in the growth of *Frankia* strains ORS 021001 suggests that the *in vitro* life cycle of this strain might be depicted as is shown in Fig. 18. On the left, an actively growing colony contains four types of viable structures: vesicles, vegetative hyphae, sporangiospores within sporangia and reproductive torulose hyphae (RTH). Vegetative hyphae and RTH are able to grow into complete colonies (solid lines). Since we have not yet observed the development of sporangiospores into colonies, we have indicated this process by a dotted line in the figure. On the right, is a large colony at the stage when all vegetative hyphae are lysed, so that only viable vesicles, sporangiospores and RTH are present, some of which may be also lysed. Development of viable RTH gives rise to actively growing colonies as in the left (solid line) whereas development of sporangiospores into colonies is hypothetic as indicated above. Compared to sporgangiospores, RTH probably play the major role in the reproduction of *Frankia* strain ORS 021001 because they develop into new hyphae much more readily than dormant sporangiospores.

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