THE FUNCTION OF VESICLES IN THE ACTINOMYCETE FRANKIA

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THE FUNCTION OF VESICLES IN THE ACTINOMYCETE *FRANKIA*

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STELLINGEN

1. *Frankia* vesicles lijken, wat betreft celwandstructuur en functie, sterk op heterocysten van cyanobacteria.

   Torrey en Callaham (1982) Can.J.Microbiol.28,749-757
   Dit proefschrift

2. In reincultures van sommige *Frankia* stammen kunnen blaasjesvorming en stikstofbinding onafhankelijk van elkaar worden gereguleerd.

   Murry *et al.* (1985) Can.J.Microbiol.31,804-809
   Dit proefschrift

3. Het voorstel van Lalonde om een aparte soort *Frankia vandijkii* te definiëren is gebaseerd op onvoldoende onafhankelijke kenmerken.

   Lalonde (1988) Proceedings of the Intern. Congress on Nitrogen Fixation, Köln, in press

4. De uitzonderlijk hoge aktiviteiten van het enzym superoxide dismutase die Steele en Stowers meten in stikstofbindende *Frankia* cultures zijn *in planta* zelden aanwezig.

   Steele en Stowers (1986) Can.J.Microbiol.32,409-413

5. Voor de vaststelling van promoter-aktiviteit d.m.v. promoter/*lacZ*-fusies is het noodzakelijk deze constructies te integreren in het chromosoom.

   Gubler en Hennecke (1988) J.Bact. 170,1205-1214

6. Het verdient aanbeveling om in localisatie-experimenten, wanneer dit mogelijk is, zowel biochemische als microscopische technieken toe te passen.

7. Bij immuno-goudlabeling experimenten worden problemen, veroorzaakt door een te lage of te hoge specificiteit van de gebruikte antilichamen, vaak ten onrechte toegeschreven aan de goudlabelingstechniek.

8. Het feit dat het promotiereglement van de Landbouwuniversiteit slechts spreekt over "de onderzoeker, hij..." is een voorbeeld van de vaak onduidelijke redenen waarom vrouwen relatief minder vaak solliciteren op vacatures in het wetenschappelijk onderzoek dan mannen.

Promotiereglement LU Wageningen (1987)

9. Een goed commercieel computerprogramma gedraagt zich vaak als een virus: na introductie op één systeem verspreiden copieën zich zeer snel over vele systemen.

10. Het oude chinese principe, de huisarts slechts te betalen zolang men gezond is, is een logisch principe en verdient als zodanig in ere hersteld te worden.

Stellingen, behorend bij het proefschrift "The function of vesicles in the actinomycete Frankia"

Wageningen, 28 juni 1988 Titia Meesters

voor mijn ouders

voor Herman

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Introduction

Introduction

Frankia.

Frankia is an actinomycete which is found in root nodules of many non-leguminous plants. The identification of the genus *Frankia* as a member of the Actinomycetales was initially based on ultrastructural studies of endophytic material (Becking 1970). Later, this classification was confirmed by the description of *Frankia* pure cultures (Callaham *et al.* 1978, Quispel and Tak 1978, Lechevalier and Lechevalier 1979).

Frankia generally grows in the form of branched, septate hyphae. Some hyphal cells can, under certain conditions, develop into typical differentiated structures, which are called sporangia and vesicles (Becking 1974, this thesis chapter II). The organism is unique in the capability of establishing an effective endosymbiosis with many perennial, mostly woody, plants (Bond 1983). In this symbiosis, the microsymbiont fixes bimolecular nitrogen. The product, ammonia, is mainly excreted into the nodule cytoplasm (Blom 1982, Blom *et al.* 1981), while in return, *Frankia* receives carbohydrates from the plant (Huss-Danell *et al.* 1982, Schubert 1986).

Different types of root nodules have been described, in which the number of spores and the shape of the vesicles can vary: In some actinorhiza (*i.e.* root nodules with *Frankia* as endosymbiont), many spores are found, whereas in other nodules no spores could be detected (van Dijk 1978). A different endophyte vesicle morphology, with elongate vesicle-like structures, has been described for the endophytes of certain host plants like *Datisca* (Hafeez *et al.* 1984) and *Coriaria* (Newcomb and Pankhurst 1982).

For a long time, *Frankia* has been considered an obligate symbiotic organism, since cultivation of the actinomycete *in vitro* seemed impossible, due to the slow growth rate and the unknown growth conditions of the micro-organism.

The first description of what was probably a pure *Frankia* culture, was from Pommer (1959), but unfortunately his isolate was lost. Since the next first successful isolation of an endophyte from *Comptonia peregrina* in 1978 (Callaham *et al.* 1978), many hundreds of pure cultures from many different host plants have been isolated.
Untill present, an actinomycete is classified as belonging to the genus *Frankia* if: 1) it is able to induce an effective symbiosis with its host plant, 2) it is capable of formation of vesicles and/or sporangia, and 3) it can fix nitrogen. These criteria are more and more difficult to maintain, since a range of strains has become available in the past years which are in fact *Frankiae* but which have lost their ability to infect their host plants, or to fix nitrogen and to form vesicles. (Baker *et al.* 1980, Hahn *et al.* 1988). Therefore, other classification criteria are the subject of investigation, *e.g.* morphology characteristics (Lalonde 1979) and host specificity (Becking 1970). However both methods have severe limitations. The shape of the vesicles and the presence of spores can often be influenced by the host plant, and the existence of overlapping cross-inoculation groups as well as non-infective *Frankia* strains makes host specificity as (unique) classification marker inappropriate (Lechevalier 1984). Chemical composition data are better suited: *Frankiae* contain *meso*-diaminopimelic acid as a cell wall component (the cell wall is of "type III", Lechevalier and Lechevalier 1979) and 2-*O*-Methyl-D-mannose was found as a typical component of all *Frankia* strains tested (Mort *et al.* 1983). At present, the possibilities are investigated of using DNA homologies (Normand *et al.* 1983, An *et al.* 1985, Ligon and Nakas 1985, Simonet *et al.* 1985, Dobritsa 1985, Normand and Lalonde 1986), *nif*-genes restriction patterns (Normand *et al.* 1988, Ligon and Nakas 1987), protein patterns (Benson and Hanna 1983, Benson *et al.* 1984) and isozymes (Gardes *et al.* 1987), in combination

with infectivity on different plants (Normand and Lalonde 1986) for the taxonomy of *Frankia* species (Simonet *et al.*, in press).

Originally, the strains have been designated with acronyms, usually two letters and two numbers, indicating the name of the host plant, and the numbers of the isolation and the isolate. Strain CpI1 is thus isolated from *Comptonia peregrina*, isolation number I and isolate number 1. Ccl.17 is isolate number 17 from the first isolation from *Colletia cruciata*. The acronyms are sometimes confusing when the initials of the hosts are identical, *e.g.* CcI3, isolated from *Casuarina cunninghammiana* and Ccl.17, isolated from *Colletia cruciata*. Moreover, the host plant is not always typical for a strain, as stated above, and another classification has been introduced: many *Frankia* strains have been catalogued using a three letter code for the culture collection, followed by a strain number (Lechevalier 1983). According to this system, strain CpI1 should be designated HFP070101 (from: Harvard Forest, Petersham culture collection). In literature, both name systems are used interchangeably, and accordingly, one or the other designation will be used in this thesis. The subclassification of the genus *Frankia* into species has been postponed, due to lack of knowledge of the different *Frankia* strains (Lechevalier 1984). In a recent attempt, Lalonde *et al.* (in press) proposed a taxonomy of the *Frankia* strains within the host-specificity group *Alnus* and *Elaeagnus*. Two species have been proposed, *viz.* *Frankia alni* and *Frankia elaeagni*. The former species was subdivided into subspecies *pommeri* (type strain HFP070101; CpI1) and subspecies *vandijkii* (type strain ULQ 0132105009; ARgP5^{Ag}), mainly on the basis of endophytic sporulation type (sp^- and sp^+, respectively) and of different isozyme patterns (group 1 and 3,4 respectively). Strains belonging to other host-specificity groups have

not yet been classified, due to the heterogenicity within these groups.

Symbiotic nitrogen fixation

The reduction of atmospheric nitrogen to ammonia, called nitrogen fixation, is an energy consuming process. In industry, generally the "Haber-Bosch" process is used to convert molecular nitrogen into ammonia, a process that only proceeds in the presence of a catalyst, at a nitrogen pressure of 200 atm. and a temperature of 300 °C. In nature, most living organisms cannot convert atmospheric dinitrogen. Consequently they need a nitrogen supply in the form of *e.g.* ammonia, nitrate or amino acids.

A small group of prokaryotic organisms however can perform biological nitrogen fixation, which is an indispensable process to close the nitrogen cycle in nature.

The nitrogen-fixing prokaryotes can be divided into two groups, the free-living and the symbiotic organisms. The free-living species comprise *e.g.* the eubacteria *Klebsiella pneumoniae*, *Rhodopseudomonas capsulata* and *Azotobacter vinelandii* (Postgate 1981, Kennedy *et al.* 1981), various archaebacteria, *e.g. Methanosarcina barkeri* (Sibold *et al.* 1985), and cyanobacteria like *Anabaena cylindrica* (Stanier and Cohen-Bazire 1977). In this group of prokaryotes, the energy that is needed for nitrogen fixation is provided by photosynthesis, or by degradation of photosynthates from other organisms. The other group of nitrogen-fixing microorganisms consists of a limited number of genera that are capable of nitrogen fixation in symbiotic systems, *e.g.* Cyanobacteria, Rhizobia, Bradyrhizobia and Frankiae. The relation between the symbiotic partners varies, from an association in which the microsymbiont lives in cavities in the leaves of the symbiotic partner, like in the *Azolla/Anabaena* symbiosis, to a complex system in which the microsymbiont penetrates the host cells, like in actinorhiza. In the symbiotic

systems, the host plant provides photosynthates as energy source for nitrogen fixation (see below).

Among the nitrogen-fixing ectosymbionts (*i.e.* living extracellularly), several Cyanobacteria are found, which live in close relation to *e.g.* higher plants (*Gunnera-Nostoc*, Silvester 1976), ferns (*Azolla-Anabaena*, Hill 1977), fungi (lichens, Hitch and Millbank 1975) and liverworts (Hepaticae-*Nostoc*, Rodgers and Stewart 1977).

Nitrogen-fixing endosymbionts (living intracellularly) are found in root nodules of higher plants: *Frankia* can live in symbiosis with over 200 species of perennial, non-leguminous, mostly woody plants, from at least 21 genera in 8 families (Bond 1983). Rhizobia and Bradyrhizobia can infect roots of many leguminous plants and of *Parasponia* sp. (Ulmaceae). The economical importance of these root nodule symbioses has been discussed by Silvester (1977) and Akkermans and Van Dijk (1981) for *Frankia* and by Bothe *et al.* (1983) for *Rhizobium*.

The infection processes of both types of root nodule symbioses have been studied in detail. Generally, *Frankia* and *Rhizobium* both infect the roots via the root hairs. On some plant species, *e.g.* *Elaeagnus* sp., *Frankia* can also infect roots directly via the epidermis (Miller and Baker 1985). Subsequently, the cells grow into the cortex where a cluster of infected cells, a primary nodule, is formed. Near the cell cluster, a root primordium originates, and the dividing top meristem creates the root nodule in which the endophyte develops. In *Rhizobium* nodules, the cell divisions are spread over the entire meristem resulting in one outgrowing nodule; in *Frankia* nodules, only the cells next to the infected cells divide, causing the dichotomous development of a cluster of nodule lobes, the so-called corralloid nodule type. The *Frankia* infection process has been reviewed recently by Berry (1984), the *Rhizobium* infection process by Bergersen (1982) and Newcomb (1981).

In the root nodules, the endophyte cells differentiate

into nitrogen-fixing cells. The two types of endophytes have different nitrogen-fixing states:
Rhizobium cells penetrate the plant cell walls, but remain enclosed in a host derived membrane, the peribacteroid membrane. Here they differentiate into non-dividing, branched, nitrogen fixing cells, the bacteroids.
In the contrary, *Frankia* hyphae penetrate the host cells and the plasmamembrane. In the plant cells *Frankia* is enclosed by a polysaccharide capsule that is produced by the host. After outgrowth of the nodule, the tips of the hyphae develop into swollen structures with thickened cell walls, the vesicles, untill most endophyte material consists of vesicles. These clusters of vesicles can fix nitrogen (Akkermans 1971, Van Straten *et al.* 1977). The differentiation of specialized cells for nitrogen fixation is not restricted to the endosymbiotic systems: several ectosymbiotic and free-living cyanobacteria can also have special nitrogen-fixing cells, the heterocysts (Haselkorn 1978).
The differentiation into specialized cells is important for the micro-organisms in order to provide the proper conditions for biological nitrogen fixation, *e.g.* sufficient energy supply, and transport mechanisms for the excretion of fixed nitrogen products and for the uptake of carbon and energy sources. Furthermore, enough reducing power has to be available to prevent the inhibition of the nitrogenase enzyme by molecular oxygen. This protection is especially important in aerobic nitrogen fixation, as is possible in heterocystous cyanobacteria and in *Frankia*, although in these species, like in other organisms, the nitrogenase enzyme is extremely sensitive to irreversible inhibition by molecular oxygen (Haystead *et al.* 1970, Benson *et al.* 1979). The function of the differentiated cells in *Frankia*, the vesicles, in relation to their special morphology, is the subject of this thesis.
In the symbioses described above, the nitrogen fixing microsymbiont excretes the surplus of fixed nitrogen

(ammonia) which can be used by the symbiotic partner. In return, photosynthates are received from the symbiotic partner and are used as carbon and energy sources by the microsymbiont. The identity of the transported carbon compounds has been subject of many studies. In *Rhizobium*, it has been shown that bacteroids can oxidize organic acids. They contain a functional tricarboxylic acid cycle and malate synthase, an enzyme of the glyoxylate cycle (Stovall and Cole 1978, De Vries 1980). It is assumed that the transported photosynthate is a dicarboxylic acid, *e.g.* succinate (Stowers 1985). In actinorhiza, the identity of the transported carbon compound is not known. Data published untill present report varying activities of enzymes of carbon metabolism, possibly caused by strain- or culture differences. No activity of enzymes of the Embden-Meyerhof-Parnas (EMP) pathway was found in strain AvcI1 or in vesicle clusters from this strain, only enzymes of gluconeogenesis were active (Blom and Harkink 1981, Huss-Danell *et al.* 1982). However Vikman and Huss-Danell (1987) found respiration of hexoses by vesicle clusters from *Alnus incana* root nodules, with a local *Frankia* strain as well as with AvcI1 as endophyte, and by AvcI1 pure cultures. In addition, Lopez and Torrey (1985) reported that in vesicle clusters from strain ArI3, a functional glycolysis was found. Stowers *et al.* (1986) also measured activities of catabolic enzymes of the EMP pathway in a pure culture. Furthermore, a functional malate-aspartate shuttle, which might function in the transport of reducing equivalents into the endophyte has been measured (Akkermans *et al.* 1981). The hypothesis that strain differences can count for at least a part of these contradictory results, is supported by the fact that a large variety exists between strains in the capacity to utilize different carbon sources (Shipton and Burggraaf 1982). Further data of more strains are needed before some conclusions can be made concerning the transported carbon compound.

It must be noted here that information on the metabolism of *Frankia* pure cultures does not always give information about the metabolism of the endophyte: Evidence exists that metabolic changes occur if a nitrogen-fixing symbiont adapts to a free-living state. The morphological difference between endophyte and pure culture is readily visible: the endophytic vesicle clusters change into hyphal cultures, with vesicles on short side branches spread over the hyphae. Differences in metabolism have been measured as well. In strain AvcI1 *e.g.*, a functional glyoxylate shunt could be shown, whereas the endophytic state of this strain did not show these enzyme activities (Blom and Harkink 1981, Huss-Danell *et al.* 1982). In vesicle clusters, almost no nitrogen is assimilated for the use of growth and division of own cells, like in free-living cultures: the enzyme glutamin synthetase could only be detected in the plant fraction of the root nodules and not in the endophyte (Akkermans *et al.* 1983).
Another morphological and biochemical adaptation of free-living and symbiontic cells was found in cyanobacteria: the free-living state of *Nostoc* has been compared with its symbiontic state with *Gunnera* (Silvester 1976). In symbiosis, eight times more heterocysts are formed than in free living state. The vegetative cells in symbiosis do not fix CO_2, thus resembling heterocysts. Not only different environmental conditions are the reason for these metabolic changes, but also the fact that the endophytes hardly grow, and thus can use most energy for nitrogen fixation.

Vesicles and vesicle clusters

This paragraph will focus on the *Frankia* vesicles from endophytic and free-living states and the comparison of these nitrogen-fixing cells to their analogues in cyanobacteria, the heterocysts.
Untill the first *Frankia* pure culture became available in 1978 (Callaham *et al.* 1978), all information of *Frankia*

came from actinorhiza. Since long, the vesicle clusters in the nodules were associated with nitrogen fixation activity, because of the parallel occurrence of the vesicle clusters and nitrogenase activity. Further evidence for this relation came from the following observations:

1. High reducing activity, shown by formation of formazan cristals from tetrazolium salts, was found in the vesicle clusters (Akkermans 1971).
2. Acetylene reduction activity, indicating nitrogenase activity, was measured in isolated vesicle clusters (Van Straten *et al.* 1977).
3. Ineffective (non nitrogen fixing) nodules were found which contained clusters of hyphae, but no vesicle clusters (Baker *et al.* 1980).

All effective actinorhiza (with one exception of *Casuarina* nodules, see below), contain vesicle clusters, but different types of vesicle clusters can be distinguished. In *Alnus*-type nodules, spherical vesicles form a cluster with the vesicles at the periphery (Baker *et al.* 1980). In *Myrica* and *Comptonia* nodules, vesicles are also pointing outwards but they are club-shaped (Newcomb *et al.* 1978). The shape of the vesicles can be influenced by the host, as has been demonstrated for strain CpI1. Vesicles of CpI1 are spherical in *Alnus* root nodules, but in *Comptonia* they are club-shaped (Lalonde 1979). *Datisca* and *Coriaria* nodules have a completely different morphology. They contain vesicle-like, elongate structures that point towards the center of the cells (Akkermans *et al.* 1984). The only effective actinorhiza that do not contain vesicle clusters are from *Casuarina*. They contain clusters of *Frankia* hyphae, without any vesicles (Gauthier *et al.* (1981), although an aberrant type of mature non-septate hyphae has been reported which might have the same function as spherical vesicles (Berg and McDowell 1987). A

review of different types and characteristics of vesicles has been given by Torrey (1985).

The variation in vesicle types which has been described for *Frankia* endophytes is not conserved in the free-living state. In *Frankia* pure cultures, only spherical vesicles have been reported, which can be induced in most strains by omitting combined nitrogen from the culture medium. Under these circumstances both vesicle formation and nitrogenase activity are induced (Tjepkema *et al.* 1980, 1981, Tisa and Ensign 1987a), and nitrogen fixation activity can be measured mainly in the purified vesicle fractions of the cultures (Noridge and Benson 1986, Tisa and Ensign 1987b, Tisa 1988). The vesicles develop on short side branches of the hyphae, spread over the colony. Even the strains, that are isolated from *Casuarina* nodules, develop vesicles under nitrogen-fixing conditions in pure culture, although no vesicles are present in the originating root nodules (Gauthier *et al.* 1981, Murry *et al.* 1985). Some other strains show different patterns of vesicle formation: Strains ACN1ag, EAN1pec and Ccl.17 also form vesicles in media containing ammonia, although in reduced numbers (Tisa *et al.* 1983, Meesters *et al.* 1985). It has been shown that these vesicles do not contain nitrogenase (this thesis, chapters III, IV-1). The induction of vesicle differentiation seems to be similar to that in cyanobacteria where heterocyst formation is induced under nitrogen-fixing conditions, and the relative number of heterocysts can vary between the symbiotic and free-living state (Haselkorn 1978, Silvester 1976).

During the past years, more and more evidences have been published that vesicles play a role in the protection of nitrogenase, to prevent irreversible enzyme inhibition by oxygen. All nitrogenases known untill present are extremely sensitive to oxygen. Most nitrogen-fixing organisms can only fix nitrogen under microaerobic conditions, although some organisms can create intracellular reducing conditions which are sufficient for

the nitrogenase enzyme to become active. A few non-heterocystous cyanobacteria, like *Synechococcus*, *Microcoleus* and *Oscillatoria* spp. can express nitrogenase activity in air (Kallas *et al.* 1983, Stal and Krumbein 1981). Here, the nitrogenase in the cells is apparently protected against oxygen damage, mainly by high respiration rates and high nitrogenase turnover rates (Bothe *et al.* 1984).

Biochemical protection of nitrogenase against oxygen has been reported for *Frankia* cells as well. Tisa (1987) demonstrated that a calcium-dependent, CN-insensitive respiratory system is present in N_2-grown cells, which might protect nitrogenase in the vesicles. Furthermore, Steele and Stowers (1986) showed that the enzyme superoxide dismutase (SOD), which is known to protect cells against oxygen (Fridovich 1978), is present in *Frankia* cells as the manganese isozyme, but under nitrogen fixation conditions, an additional iron-SOD isozyme is present in high concentrations. However, this enzyme was induced only under relatively high oxygen concentrations, in shaken or air-sparged cultures. Therefore, the biological importance of this enzyme is not known.

Frankia vesicles are surrounded by a multilaminated outer layer, with a monolipid character (Torrey and Callaham 1982). The number of laminae in this envelope, which is 12-15 in cultures under air, can readily increase with increasing oxygen levels, *e.g.* under 40 kPa oxygen pressure 40-50 laminae have been found. Hyphae do not have envelopes with these characteristics (Parson *et al.* 1987). This phenomenon, which directly shows a relation between vesicle structures and oxygen tension, may explain the capacity of *Frankia* strains to adapt to varying oxygen concentrations (Murry *et al.* 1984a).

Another link of oxygen concentration and vesicle formation in *Frankia* is found in *Casuarina* strains. In the nodule, these strains do not form vesicles but nevertheless they fix nitrogen. The nitrogenase activity in the hyphae might

be explained by the fact that *Casuarina* cell walls are suberized, which possibly protects the endophyte cells against oxygen (Berg 1983). In addition, haemoglobins, which serve the same purpose, were found in relatively high concentrations in the *Casuarina* root nodules (Tjepkema 1983). In pure cultures, isolated from *Casuarina* nodules, the relation between the differentiation of vesicles and the concentration of oxygen could be confirmed. In strain CcI3, isolated from *Casuarina cunninghamiana* root nodules, nitrogenase is induced and vesicles develop if the cells grow under air. However, if oxygen concentrations are below 0.3 %, nitrogenase activity is present but no vesicles are formed (Murry *et al*. 1985).

Regarding the mechanical as well as the chemical oxygen barriers present in *Frankia* vesicles, a similar protection for nitrogenase is found in *Anabaena* heterocysts. *Anabaena* is, like *Frankia*, capable of nitrogen fixation if grown aerobically in culture. Nitrogenase is localized in the heterocysts (Haselkorn 1978, Peterson and Wolk 1978). It has been suggested that oxidative reactions inside the heterocysts play a role in maintaining the low oxygen concentration (Wolk 1982). Moreover, a three-layered cell envelope is found in heterocysts outside the vegetative cell membrane. The central layer of the cell envelope consists of polysaccharides, the inner layer is laminated and consists of glycolipids (Winkenbach *et al*. 1972). The inner layer is thought to be impermeable for dissolved gasses (Lang and Fay 1971, Haselkorn 1978). In accordance with this hypothesis, it has been shown that an *Anabaena* mutant, deficient in the production of the glycolipids of the cell envelope, could fix nitrogen under microaerobic conditions but not under aerobic conditions (Haury and Wolk 1978). Nevertheless, in *Anabaena cylindrica*, grown under reduced oxygen partial pressure, nitrogenase is still restricted to the heterocysts, although no protection of nitrogenase is necessary (Murry *et al*.

1984b). Still, heterocysts are not indispensable for nitrogenase activity in cyanobacteria, as was demonstrated by Stewart and Lex (1970). These authors measured nitrogenase activity in the non-heterocystous *Plectonema boryanum*, if the strain was grown under low oxygen concentrations. The information given above shows the striking resemblance between *Frankia* vesicles and *Anabaena* heterocysts.

Frankia nif-genes

Most knowledge of genes, responsible for the expression of the enzyme nitrogenase, has been obtained with *nif*-genes from the free-living nitrogen fixing bacteria *Klebsiella pneumoniae*. Three genes have been found to code for the structural nitrogenase subunits, *nif*H for component II (CII), an Fe-protein, and *nif*D and *nif*K for the two subunits of component I (CI), an FeMo-protein. The structural genes are located on one transcriptional unit. In addition, untill present 14 regulatory genes have been found. The three structural and 14 regulatory *nif*-genes are all contiguous and have a total length of 23 kb, on which seven or eight operons are located. The functions of most regulatory genes are known, and include electron transport (*nif*F,J), Mo-uptake (*nif*Q), activation (*nif*A) and repression (*nif*L) of transcription of other *nif*-genes, synthesis of FeMo-cofactor (*nif*B,N,E), substrate specificity of CI (*nif*V) and probably processing of CII (*nif*M,S,U). Reviews of *Klebsiella nif*-genes are given by Roberts and Brill (1981), Ausubel (1984) and Dixon (1984). Research on genetics of nitrogen fixation in other organisms, has been based on the information obtained from *Klebsiella nif*-genes. Corresponding names are given to genes that are structurally and functionally similar, as shown by hybridization experiments and complementation experiments. In the complementation experiments, the heterologous gene should restore the missing function in a *Klebsiella* mutant which is deficient in this gene, and

vice versa. In heterologous hybridization studies, the structural genes appeared well conserved in all nitrogen fixing organisms, although differences between strains are found in DNA analysis (Ruvkun and Ausubel 1980). Conservation of structural *nif*-genes has been shown even in the taxonomically distant archaebacteria (Sibold *et al*. 1985). In contrast, the regulatory genes appeared more variable between strains (Ruvkun and Ausubel 1980).

In *Rhizobium*, the structural *nif*-genes H,D and K, and the regulatory genes Q,B,A and E have been detected. The structural genes are transcribed under one operon (Schetgens *et al*. 1984). In addition, a series of genes have been found which are not homologous to *Klebsiella* DNA but which are also involved in symbiotic nitrogen fixation. These genes have been called *fix*-genes. Both *nif* and *fix* genes are located on a large plasmid, called the *sym*-plasmid (Rosenberg *et al*. 1981, Corbin *et al*. 1983).

In *Bradyrhizobium*, two distinct operons, comprising *nif*H and *nif*DK have been found, indicating an organization of *nif*-genes which is different from *Rhizobium* (Fisher and Hennecke 1984). Furthermore, in *Bradyrhizobium*, no megaplasmids have been found, and the *nif*-genes are integrated in the chromosome (Haugland and Verma 1981).

In *Anabaena*, *nif*-genes H,D and K have been detected. The organization is different from the *Klebsiella* and *Rhizobium* *nif*-genes. In *Anabaena*, *nif*K is separated from *nif*D by a fragment of 11 kb. The two fragments *nif*K and *nif*DH are transcribed independently. Another fragment, containing *nif*V/S, was also identified, on a separate transcription unit (Haselkorn *et al*. 1984). It has been shown, that gene rearrangements occur in differentiating heterocysts. In this process, the 11 kb fragment is removed, leaving one transcriptional unit for *nif*-genes K,D and H. The rearrangement is a site-specific recombination between two 11-bp repeated sequences on both sites of the removed fragment. A second rearrangement, near the V/S zone, was also detected but its function

could not yet be explained. The rearrangements seem to explain why in *Anabaena nif*-genes are uniquely expressed in heterocysts (Golden *et al.* 1985).

In *Frankia*, difficulties in growing cells in large quantities and lysing cells for preparing DNA have partially been solved in the past years, resulting in a stream of new information on *Frankia* genetics. *Nif*-genes H and D from five strains from *Alnus* nodules have been cloned, and in all cases they were contiguous. In three of these clones, *nif*K was located on the same fragment, contiguous with *nif*HD. The two other fragments were too small to contain *nif*K even if it was contiguous with *nif*HD (Normand *et al.* 1988). On the cloned fragment of one of these strains, also *nif*-genes A and B were located (Simonet *et al.* 1988). However, in another *Frankia* strain, which also originates from *Alnus* nodules, a different organization of *nif*-genes has been found (Ligon and Nakas 1987). Here, *nif*-genes D and K were contiguous, but *nif*H, which could be detected in total DNA, was at least 12 kb distant from *nif*DK. In this strain, *nif*E and *nif*N could be detected, but not *nif*A or *nif*B. Untill present, the designation of these *nif* genes in *Frankia* is only based on hybridization studies, no complementation studies have yet been accomplished. *Nif*H and *nif*K, from different strains from *Alnus* nodules, have been sequenced (Normand *et al.* 1988, Ligon and Nakas 1988), and homologies were found, apart from *Klebsiella* and some other strains, with *Rhizobium* (*nif*H) and *Anabaena* (*nif*H, *nif*K). Little is known about the regulation of expression of *nif*-genes in *Frankia*. Only a few regulatory *nif* genes have been identified, but since only a few DNA fragments with *Frankia nif* genes have been cloned and thus much information is still lacking, it is likely that more of these regulatory genes will be found. Still no information is available on the mechanism of repression of *nif* genes in *Frankia* hyphae. One of the possible repression mechanisms is the occurrence of rearrangement of

nif-genes, like in *Anabaena*. This possibility has been investigated and results are described in chapter V of this thesis.

Outline of the thesis

The research described in this thesis was focussed on different aspects of the function of vesicle formation in *Frankia*. At the time when the investigations of this research were started, only indirect evidence had been published for the localization of nitrogenase in the vesicles. The proposed function of this localization was, that the vesicles provide a reducing environment for the enzyme nitrogenase. Consequently, the first question to be answered was, whether the vesicles are indeed the site of nitrogen fixation in *Frankia*, and, if so, whether the localization can be influenced by varying the oxygen partial pressure. In addition, a study on the ultrastructure of some strains has been performed, in order to get more insight in the structure of the differentiated *Frankia* cells. Subsequently, the relation of the induction of vesicles and of nitrogenase was investigated using strains that induce vesicles in the presence of ammonia, when most *Frankia* strains do not form vesicles nor nitrogenase. Since the results indicated, that nitrogenase is not induced in the hyphae, when vesicles are present, a possible repression mechanism for *nif*-genes, namely DNA rearrangement in the vesicles, was investigated. The results of the investigations are presented in the following chapters:

- Chapter II describes the ultrastructure of the different *Frankia* cell types, showing electron micrographs of five *Frankia* strains.
- Chapter III gives a description of the relation of vesicle formation and nitrogen-fixation in two different strains. The results indicate that vesicle formation and nitrogenase induction are independently controlled in *Frankia* strain Cc1.17.

- Chapters IV-1 and IV-2 deal with the localization of nitrogenase using immunogoldlabelling on ultrathin cryosections. It is shown that nitrogenase is localized uniquely in the vesicles and not in hyphae of strains Ccl.17 and EANlpec.
- Chapter IV-3 gives data about the localization of nitrogenase under microaerobic conditions. It is shown that, although nitrogenase can be expressed in hyphae, in strain Ccl.17 the localization of the enzyme in the vesicles is independent of the oxygen concentration.
- Chapter V reports about the investigation of possible rearrangements of *nif*-genes during vesicle differentiation, an effort to elucidate a mechanism of regulation of gene expression in *Frankia* hyphae and vesicles. It is shown that no major rearrangements occur in strain Ccl.17.
- Chapter VI gives a summary of the presented results.

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II

Morphology of hyphae, sporangia and vesicles of Frankia species

Morphology of hyphae, sporangia and vesicles of *Frankia* species

Abstract

An ultrastructural (TEM) study was made of *Frankia* hyphae, vesicles and spores of pure cultures isolated from different host plants. Special attention was given to the outer layers of the cell wall around vesicles, vesicle stalks and hyphae. The vesicle envelope was well preserved. It is shown that the vesicle envelope is continued as the outer cell wall layer of the hyphae. The cytoplasm in the stalks of mature vesicles is often constricted, resembling cell connections between heterocysts and vegetative cells of *Anabaena*. No differences were observed between the ultrastructure of Ccl.17 vesicles formed in the presence or absence of combined nitrogen.

Introduction

The morphological characterization of actinorhiza has been described for root nodules of many host plants, e.g. for *Alnus crispa* var. *mollis* (Lalonde *et al.* 1975), *Alnus glutinosa* (Van Dijk and Merkus 1976, Lalonde 1979), *Comptonia peregrina* (Newcomb *et al.* 1979), *Hippophae rhamnoides* (Gatner and Gardner 1970). The branched, septate hyphae of the endophyte look like those of other Gram-positive actinomycetes (Becking 1974). Characteristical structures, called vesicles, and sporangia can be formed. In the nodules, the endophyte is separated from the host cell cytoplasm by a capsule. Lalonde (1979) showed that this material is pectin-like and is most probably synthesized by the host plant. He suggests that the "empty" space, seen between the

endophyte and the host cell cytoplasm is formed by this capsule. The endophyte cell wall consists of two distinct layers, an inner (peptidoglycan) layer, which also forms the septae, and an outer, more electron dense layer. In the cytoplasm, a nuclear zone, storage inclusions, mesosomes and ribosomes can be found. Around the vesicles, a multilaminate outer layer resembling monolipid layers (Parsons *et al.* 1987), called the vesicle envelope, can be seen. A "void area", found between the vesicle and its envelope, is probably the result of the bad preservation of these lipid layers in the fixation procedures used (Torrey and Callaham 1982). Relatively little has been published about the ultrastructure of *Frankia* pure cultures. Only the ultrastructure of strains HFPCcI3 (Lancelle *et al.* 1985), CpI1 (Newcomb *et al.* 1979), vesicles of CpI1 (Torrey and Callaham 1982) and vesicles of HFPArI3 (Fontaine *et al.* 1984) was described. In general, ultrastructure of pure cultures resembles the ultrastructure of the endophyte. Club-shaped vesicles, as are found in nodules of *Myrica* (Schwintzer *et al.* 1982) and *Comptonia* (Lalonde 1979), have never been described in pure cultures. Often, no mesosomes and no vesicle envelopes were found, which might be ascribed to strain differences or different methods used. Many details of ultrastructure, concerning *e.g.* the multilaminate outer layer around vesicles, the presence of mesosomes and the structure of the vesicle stalk are still unknown. This chapter gives a description of some of these structures, showing electron micrographs of hyphae, sporangia and vesicles of different *Frankia* strains.

Materials and Methods

Strains and media

Frankia strains used for transmission electron microscopy are listed in table 1. Cells were cultured for two weeks

Table 1
Strains used for electron microscopy studies

strain	host plant origin	ref.
AvcI1	*Alnus viridis* var.*crispa*	Baker and Torrey 1980
CpI1	*Comptonia peregrina*	Callaham *et al.* 1978
Agp1	*Alnus glutinosa*	Burggraaf *et al.* 1981
Hr1	*Hippophae rhamnoides*	Burggraaf *et al.* 1981
Cc1.17	*Colletia cruciata*	Akkermans *et al.* 1984

(strain Hr1: four weeks) in a medium containing propionate as carbon and energy source and N_2, or if indicated NH_4Cl 0.2 g/l, as nitrogen source, in culture tubes, as described before (Meesters *et al.* 1985).

Fixation and embedding

The cells were packed in small blocks of 2 % agar and fixed in glutaraldehyde 2.5 %, paraformaldehyde 2.0 %, at room temperature, overnight, and postfixed in osmium tetroxide 1 %, for two hours on ice. All solutions were made in 0.1 M sodium cacodylate buffer pH 7.2, washing steps between the different fixation steps were done in the same buffer. Some variations of fixation solutions were used: if indicated, only the osmium fixation was done; or ruthenium red 0.02 % was added to both fixation solutions; or $K_2Cr_2O_7$ 1 % was added to the osmium solution; or fixation was done only with $KMnO_4$ 1 % in the cacodylate buffer, 2 h on ice. After dehydration in an ethanol series, the cells were embedded in Epon 812 following standard procedures.

Sectioning, staining and electron microscopy

Ultrathin sections (70-120 nm) were made using glass knives. Formvar-coated copper grids were used. The sections were stained with a saturated uranyl acetate solution, followed by a staining with lead citrate according to Reynolds (1963). The sections were examined with a Philips EM 300 transmission electron microscope.

Results

Ultrastructure of hyphae

In all cases, a confirmation of the description of the hyphal structures as gram-positive actinomycete cells was found. Plate 1 gives examples of electron micrographs of hyphae from different strains. In the cytoplasm, the ribosomes and the nuclear zone are visible. Also membrane structures, called mesosomes are found, often near the septa (Figs. 3,4). Sometimes a connection of the mesosome with the plasma membrane is seen. Furthermore, different storage granules and/or lipid inclusions are found (Fig. 2). Outside the plasma membrane, the inner cell wall layer, a peptidoglycan layer, is visible. Outside this layer, a second, membraneous layer is visible, as was described by Newcomb *et al.* (1979). Addition of $K_2Cr_2O_7$ or fixation with only $KMnO_4$ gives the outer layer a more homogeneous or finely granulated appearance. Some pictures show that parts of this layer have become loose from the rest of the cell wall, probably due to an insufficient fixation method (Figs. 5-7). The laminate structure is especially well visible after fixation with Ruthenium Red, indicating the presence of acid polysaccharides in this layer (Thiery 1967).

Ultrastructure of vesicles

Although the relative numbers of vesicles formed varies greatly between strains (chapters 1,3), no differences in

vesicle ultrastructure could be found. The electron micrographs of the vesicles (Figs. 8-17) show all cell components described for the hyphae. Nuclear zones, ribosomes, mesosomes, plasmamembrane and the two-layered cell wall can be distinguished. In addition, the septa are often incomplete, which is typical of the vesicles (Torrey and Callaham 1982). The vesicle envelope can be seen in many cases (Figs. 9,10,11,14-17). Often the multilaminate character of the envelope is visible. In radial or transverse sections, showing the vesicle as well as the vesicle stalk, the vesicle envelope seems to be continuous along the stalk, and where visible, also along the attached hypha, as a part of the outer cell wall layer (Figs. 11,14-17). In these sections, the cytoplasm in the

pp. 33-34:
Plate 1. Figs.1-7, Hyphae.
il= cell wall inner layer, m= mesosome, ol= cell wall outer layer, n= nuclear zone, p= plasmalemma, s= septum, sg= storage granule, va= "void area". GA: glutaraldehyde/ paraformaldehyde fixation, Os: Osmium tetroxide fixation, RR: Ruthenium Red added to fixative, Cr: potassium dichromate added to fixative. Bar= 0.1 µ.
1: Hr1, GA/Os.Cr. Inner and outer layer of the cell wall well visible, 2: AvcI1, GA/Os.Cr. Hypha with storage granules, 3: AvcI1, GA/Os. Hypha with mesosome, connection of mesosome with plasmalemma indicated with arrow, 4: CpI1, GA.RR/Os.RR. Hypha with mesosome, 5: AvcI1, GA/Os.Cr. Hypha with septum and partly loose outer layer, 6: Hr1, GA/Os. Hypha with septum and loose outer layers, 7: Agp1, GA/Os.Cr. Hypha with septum and partly loose outer layer.

Plate 2. Figs.8-13, Vesicles.
Abbreviations as in plate 1. Bar= 0.5 µ.
8: AvcI1, GA.RR/Os.RR. Vesicle with vaguely visible envelope (arrows) and septa, 9: AvcI1, $KMnO_4$. Old vesicle with multilayered envelope and many septa, 10: Agp1, Os. Vesicle with multilayered envelope and one imperfect septum, 11: Agp1, GA.RR/Os.RR. Vesicle with stalk, multilayered envelope continuing around stalk (arrows), and mesosome (inset), stalk cytoplasma is constricted, 12: CpI1, GA/OsCr. Young vesicle attached to hypha, no vesicle envelope, hyphal outer layer visible, with imperfect septa, 13: CpI1, GA.RR/Os.RR. Old vesicle, envelope vaguely visible, many septa.

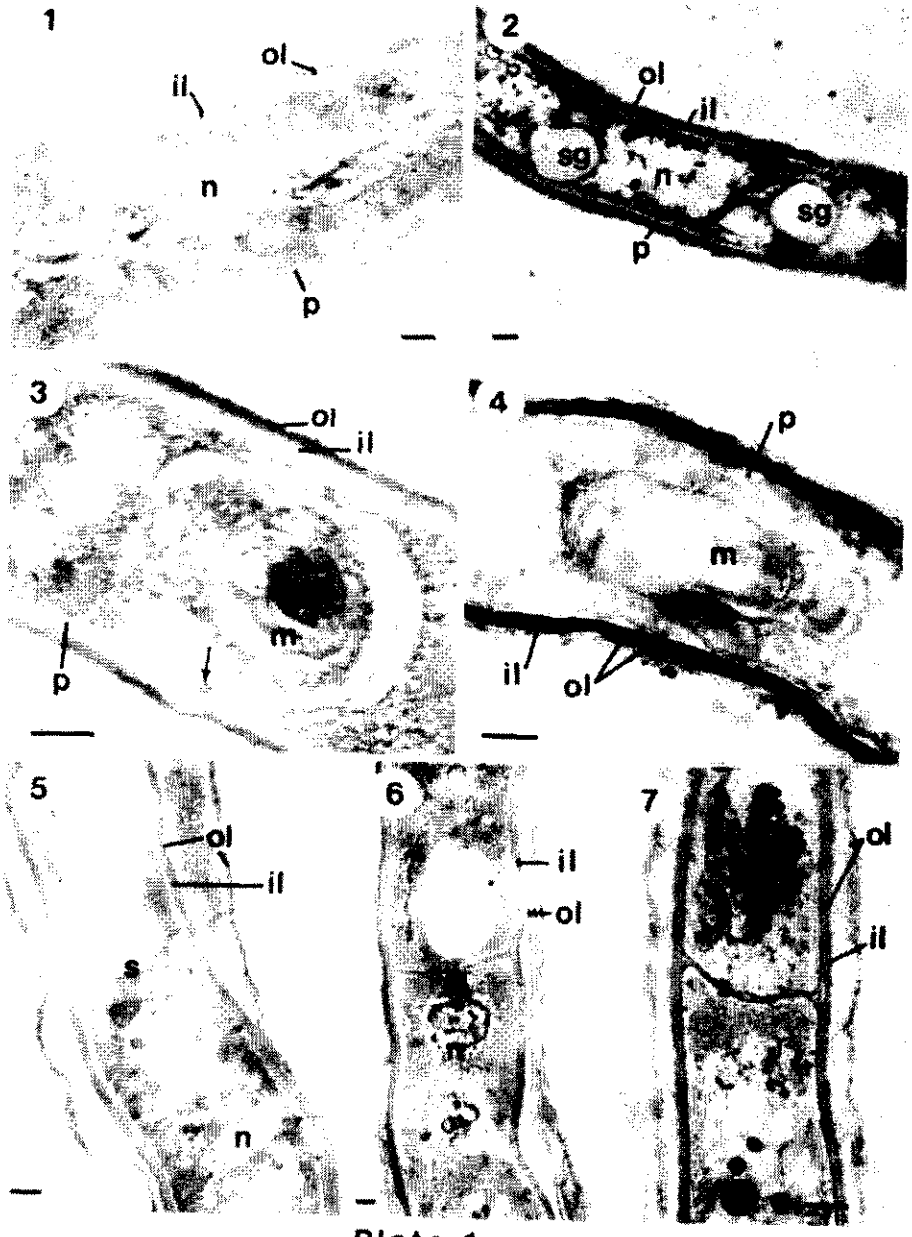

Plate 1

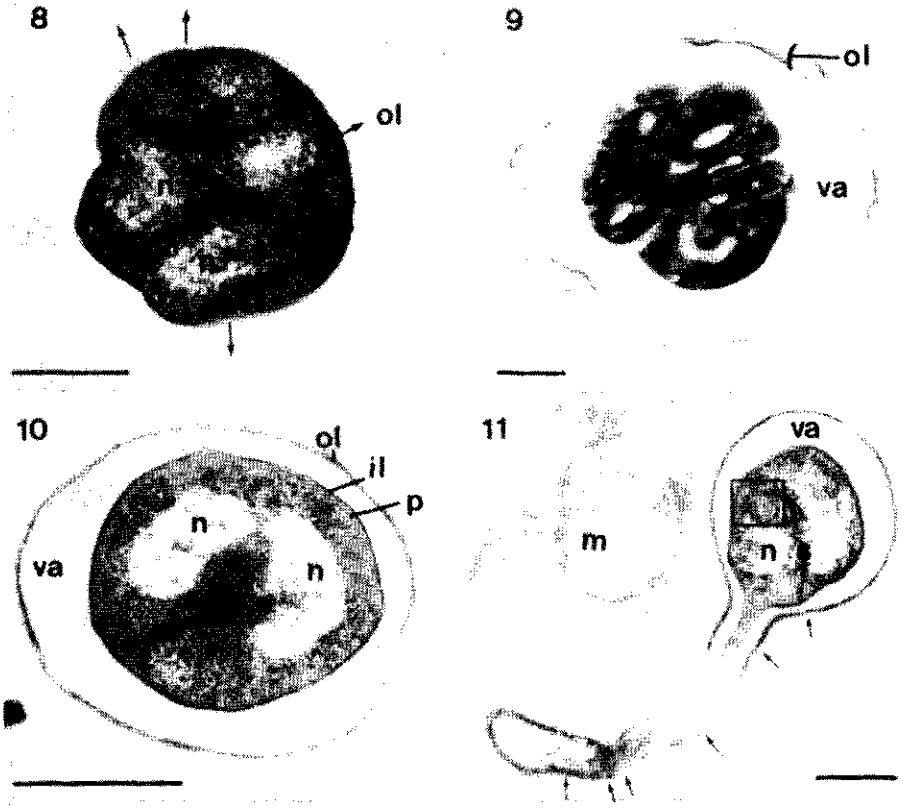

Plate 2

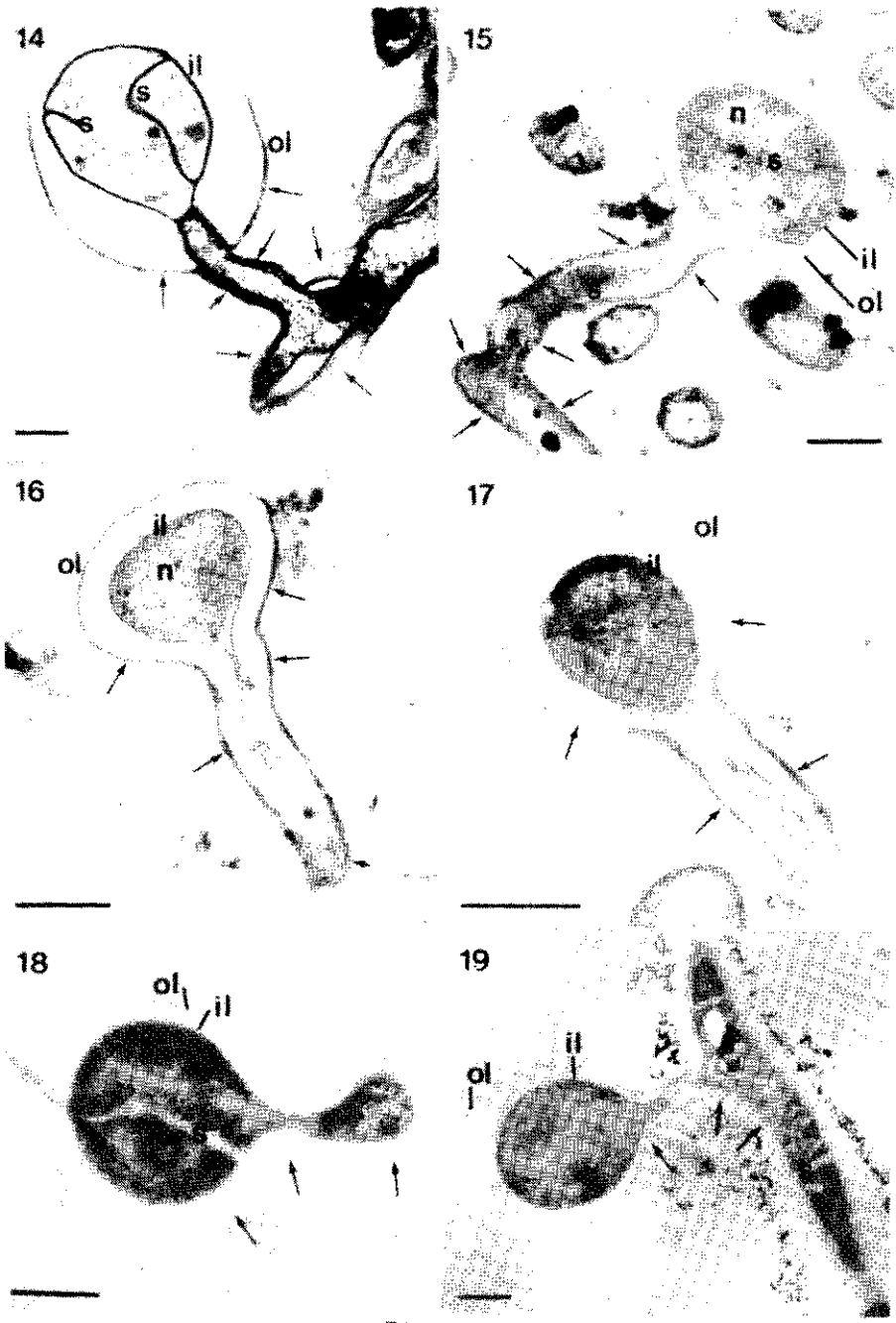

Plate 3

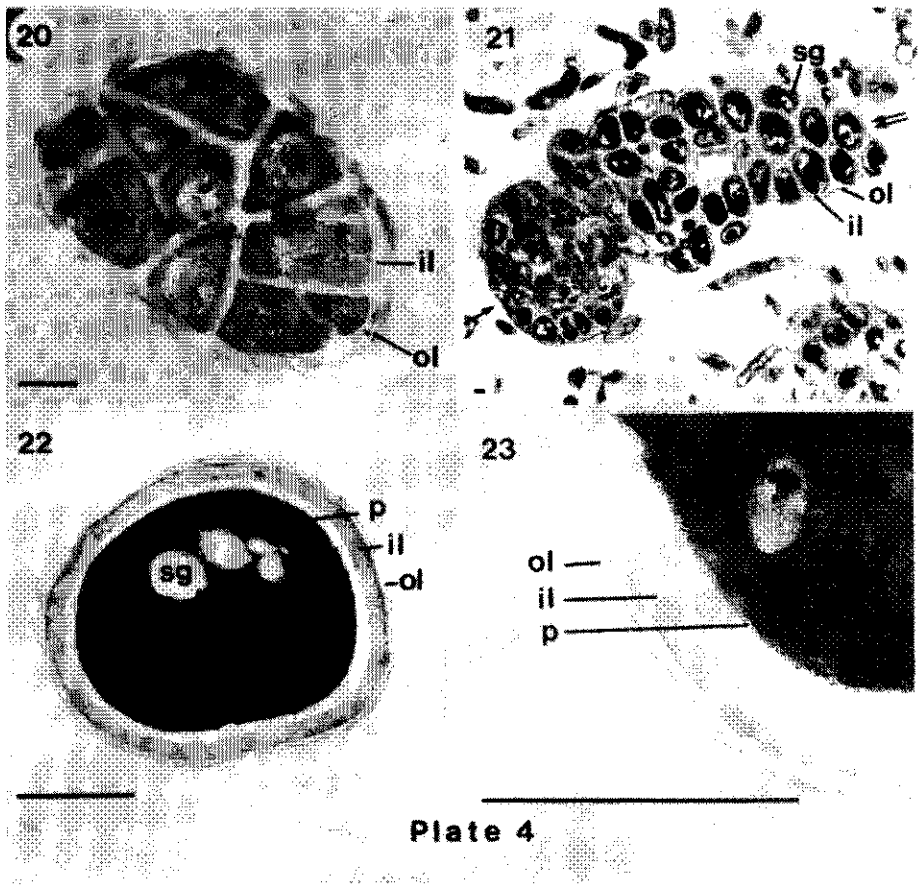

Plate 4

stalk, adjacent to the vesicle, is often constricted, also in radial sections, indicated by the sharp structure of the cell wall. This indicates the presence of a thickened cell wall on this place. In strain Cc1.17, which also forms vesicles in the presence of combined nitrogen, identical ultrastructure of vesicles was found in nitrogen-fixing and non-nitrogen fixing cultures (Figs. 18,19).

Ultrastructure of spores

Figs. 20-23 show electron micrographs of sporangia and spores from Hr1 and Agp1. No differences were found with regard to descriptions of the ultrastructure of *Alnus glutinosa* endophyte spores (Van Dijk and Merkus 1976). A membrane system on the outside of the typical thickened cell wall can be seen. The two-layered cell wall from the hyphae is also visible around the sporangia. The inner peptidoglycan layer, first forming the septa of the dividing hyphal cells, later forms the thickened cell wall of the spores. Especially in the spores, many storage granules are present (see description of hyphae above). The same results were obtained for all five strains.

pp. 35-36:
Plate 3. Figs. 14-19, Vesicles with attached stalks. Cytoplasm constricted in the stalks. Outer layer continuously around vesicle, stalk and hypha (arrows). Abbreviations as in plate 1. Bar= 0.5 µ.
14: Agp1, GA.RR/Os.RR, 15-17: Agp1, GA/Os.Cr, 18: Cc1.17, GA/Os. Vesicle from nitrogen-fixing culture, 19: Cc1.17, GA/Os. Vesicle formed in medium with NH_4Cl (0.2 g/l).

Plate 4. Figs. 20-23, Sporangia and spores.
Abbreviations as in plate 1. Bar= 0.5 µ. 20: Agp1, $KMnO_4$. Young sporangium. 21: Hr1, GA/Os. Sporangium with young spores at the base (single arrow) and mature spores at the top (double arrow), 22: Hr1, GA/Os.Cr. Mature spore, with thickened wall, 23: detail of Fig. 22.

Discussion

This chapter gives an overview of the ultrastructure of the different *Frankia* cell structures, using five pure cultures. All strains form the same type ("*Alnus*-type") of nodules in their host plants (Akkermans *et al.* 1984). The five strains investigated in the present study showed similar results to those already published earlier. No differences between the five strains could be found. It was suggested that mesosomes in *Frankia* are fixation artefacts, because they are not found in freeze-substituted material (Lancelle *et al.* 1985). The connection of these mesosomes with the plasmalemma (Fig. 3), and the frequent localization of the mesosomes near the septa, make this hypothesis less probable. The two-layered cell wall around hyphae is continuous with the cell wall around vesicles and sporangiae (Figs. 11, 16-19). The outer layer has a laminate structure, as was reported by Newcomb *et al.* (1979) (Figs. 9, 10, 14). The laminated spore wall, comprised of two or three outer layers (Figs. 22, 23), is typical of *Frankia* (Berg and Lechevalier 1985). The composition of this cell wall layer is not known. Lalonde (1979) showed that it could be stained with a PAS staining, but not with the polysaccharide stain Calcofluor White. The outer layer forms the vesicle envelope, by multiplying the number of layers. (Parsons *et al.* 1987). Thus, the vesicle envelope seems to be an extension of already existing structures, instead of being present uniquely around the vesicles. By using Nomarski interference microscopy and scanning electron microscopy, Torrey and Callaham (1982) showed, that this multilayered vesicle envelope must be in contact with the inner layers of the vesicle cell wall. The "void area" seen between these layers (Figs. 9-11) is an artefact caused by washing away a big part of the multilaminate outer layer, and stretching and shrinking of the rest of the outer layer and the vesicle respectively.

In transverse sections, cut on one side of the vesicle, the "void area" appears also larger than it really is. The relatively small size found for the vesicles indicates that shrinking does occur in these preparations.
No influence of ammonia on vesicle ultrastructure could be found in Ccl.17 vesicles which were formed in the presence of ammonia (Figs. 18,19). Huss-Danell *et al.* (1982) showed that vesicles in root nodules of *Alnus incana* were severely damaged by addition of 20 mM NH_4Cl. Fontaine *et al.* (1984) found a similar effect of ammonia on *Frankia* strain HFPArI3. Since this alder strain is not able to form vesicles in the presence of combined nitrogen, like strain Ccl.17, a different effect of ammonia can be expected. The same is probably true for the endophyte of *Alnus incana* which was used by Huss-Danell, but since crushed nodules were used for inoculation in that experiment this could not be confirmed.
Untill present, little is known about the connection between vesicles and hyphae. Since nitrogen fixation is localized in the vesicles (Noridge and Benson 1986, Meesters 1987) transport of nitrogen compounds from the vesicles to the hyphae must take place. It is not known whether these compounds are transported through the vesicle stalks or via excretion by the vesicles and uptake by the hyphae. Regarding the structure of the vesicle envelope, the former possibility seems the most probable. In *Anabaena*, the connection between heterocysts and vegatative cells is typically a septum with reduced diameter and with small transport channels, called microplasmadesmata (Lang and Fay 1971, Haselkorn 1978). No special structures like these have been seen in *Frankia*. The electron micrographs presented here provide indications that the cytoplasm in the vesicle stalk is constricted, leaving a narrow opening between vesicle and hypha (Figs.11, 15-17, 19). In accordance with the theory about the "void area" around the vesicles, the relatively thick void area around this constricted region might be

caused by a thickened cell wall which is washed away during fixation. The phenomenon of constricted vesicle stalks can be found in other published electron micrographs, in ultrathin sections and in freeze-fracture preparations from vesicles from different *Frankia* strains (Lalonde 1979 Fig.12, Lalonde and Devoe 1976 Fig.10, Lalonde *et al.* 1975 Fig.18, Gatner and Gardner 1970 Figs.5,7a). The function of this constriction is not known, it might play a role in control of oxygen concentration in the vesicle, as well as in vesicle-hypha transport. In all cell structures, but most abundant in spores, storage granules can be seen as unstained round structures (Figs. 2, 21, 22). The storage meterial, *e.g.* glycogen (Lopez *et al.* 1984, in endophytes: Newcomb *et al.* 1978) or lipid inclusions (Lalonde 1979, Lancelle *et al.* 1985), or trehalose (Lopez *et al.* 1984) is washed out leaving the white structures. The identity of the inclusion material is not known.

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III

Growth, acetylene reduction activity and localization of nitrogenase in relation to vesicle formation in Frankia strains Cc1.17 and Cp1.2

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Growth, acetylene reduction activity and localization of nitrogenase in relation to vesicle formation in *Frankia* strains Cc1.17 and Cp1.2

Abstract

A comparative study was conducted on the effect of NH_4Cl on growth, vesicle formation and formation of nitrogenase of *Frankia* strains Cc1.17 and Cp1.2, derived from root nodules of *Colletia cruciata* and *Comptonia peregrina*, respectively. On a medium without combined nitrogen ("P-N"), both strains formed spherical cells, called vesicles, like many other *Frankia* strains. Data are presented on the number of vesicles per mg protein, after cultivation in media with sodium propionate as C-source without combined nitrogen ("P-N") or with 0.2 g NH_4Cl/l ("P+N"). Strain Cp1.2, as many other *Frankia* strains, showed on "P+N" medium a very strong reduction of vesicle formation of 99 % relative to the number of vesicles formed on "P-N" medium, after 11 d growth. However, in strain Cc1.17 this reduction was only 70 %. The occurence of relatively large numbers of vesicles in "P+N" media has not yet been reported for other *Frankia* strains. No acetylene reduction activity was found in NH_4^+-grown cells. The regulation of induction of nitrogenase in *Frankia* by NH_4Cl was tested by immuno-gelelectrophoresis using antisera against nitrogenase of *Rhizobium leguminosarum* PRE. The component I of the enzyme showed crossreactivity while the component II had only a weak crossreaction. The experiments indicated that no nitrogenase was detectable in the NH_4^+-grown cells. For the localization of nitrogenase, relative amounts of the enzyme were compared in whole cells and vesicle-enriched fractions. Western blots showed a significant enrichment

of nitrogenase in the vesicle fractions, which indicated that most of the nitrogenase was localized in the vesicles.

Key words: Actinomycetes, *Frankia*, Vesicles, Nitrogenase, Localization, Induction

Introduction

In actinorhizal root nodules as well as in free-living cultures of *Frankia* strains, several authors have related nitrogenase activity with the differentiation of spherical cells, called vesicles. In root nodules, reducing activity was shown with tetrazolium chloride to be localized in the vesicle clusters (Akkermans, 1971). Acetylene-reducing activity was also shown to be present in the vesicle clusters (Van Straten *et al.*, 1977). In isolated *Frankia* strains, no vesicle clusters are formed, but in nitrogen-poor media vesicles differentiate from terminal swellings of hyphae spread over the colony. In these cultures, it was shown that the amount of vesicles was correlated with the level of nitrogenase activity. These cultures neither formed vesicles nor showed acetylene-reduction on nitrogen-rich media. (Tjepkema *et al.*, 1980, 1981). Thus the hypothesis was suggested that nitrogenase is localized in the vesicles. This hypothesis is supported by the observation of a thick cell-envelope around the vesicles, that might protect the oxygen-labile nitrogenase from too high oxygen-levels (Torrey and Callaham, 1982). Murry *et al.* (1984) showed that nitrogen-fixing cells, in contrast to NH_4^+-grown cells, have a diffusion-limitation for oxygen and concluded that this limitation is due to the vesicles. However, up till now no direct evidence has been given to proove that nitrogenase is indeed localized in the vesicles. On the other hand, these vesicles are not always necessary for nitrogen fixation. Tyson and Silver

(1979) described the existence of effective *Casuarina*-nodules with a *Frankia*-endophyte without any vesicles. No isolate with these characteristics has been described, but also in isolates the correlation between vesicle formation and acetylene reduction activity is not strictly positive. This is illustrated by the description of *Frankia*-strains that do form vesicles but do not show acetylene-reduction activity on nitrogen-rich media. (Gauthier *et al.*, 1981). The activity of nitrogenase in these strains is probably regulated by glutamine synthetase, GOGAT or a product of their reaction.(Gauthier, 1983). Recently we have isolated a *Frankia* strain, Cc1.17, from *Colletia cruciata* which produces large numbers of vesicles in N_2-grown cultures (Akkermans *et al.*, 1984). However, significant numbers of vesicles are also present in NH_4^+-grown cultures of this strain. This study presents data about the number of vesicles and the relative amounts of nitrogenase in vesicles and in whole cells of strain Cc1.17 in N_2- or NH_4^+-grown cultures. Strain Cp1.2, derived from *Comptonia peregrina*, is used as a reference. To test the possible localization of nitrogenase in vesicles of *Frankia*, we used immuno-electrophoresis-techniques with antisera against nitrogenase from *Rhizobium leguminosarum* PRE (Bisseling *et al.*, 1980).

Materials and Methods

Strains

Strain Cc1.17 was isolated by Baas and Akkermans as a single colony from root nodules of *Colletia cruciata*, which were grown as exotic plants in the Netherlands (Akkermans *et al.*, 1984). Cp1.2 is a clone from one colony of the original CpI1 culture which was isolated from *Comptonia peregrina* by Callaham (Callaham *et al.*, 1978).

Culture conditions

Precultures were subcultivated about twice a week on the same medium for two months to provide cells that were well adapted to the medium. Composition of the medium: "P-N": (g/l) sodium propionate (1.0); $MgSO_4.7H_2O$ (0.2); $CaCl_2.2H_2O$ (0.1); K_2HPO_4 (1.0); $NaH_2PO_4.2H_2O$ (0.67); Fe-EDTA and trace elements according to Allen and Arnon (1955); and the following vitamins: biotin, folic acid, nicotinic acid, Ca-pantothenate, cyanocobalamine, pyridoxine-HCl, riboflavin, thiamine dichloride, each 0.1 mg/l final concentration. "P+N" has the same composition but with 0.2 g NH_4Cl/l. The pH of the media was 6.8 and remained constant during the incubation time. Cultures were grown at 28 °C in non-shaken tubes containing 9.5 ml of medium and were routinely inoculated with 5 % cell material.

Growth experiment

Tubes (150x17 mm) with 9.5 ml medium (P+N or P-N) were inoculated with 0.5 ml of inoculum containing 10 µg of *Frankia* protein from 5 d old precultures of the strains Cc1.17 or Cp1.2. Daily, the cell contents of three replicate tubes were harvested by spinning the cells down (10 min, 2000xg), reducing the volume to 0.5 ml. Two tubes were stored at 4°C for protein estimation. The cell content of one tube was washed twice with phosphate buffered saline (PBS) and the cell suspension (0.5 ml) was sonicated in 1.5 ml Eppendorf-vials for 50 s, 20 W with a Branson sonifier (model B12) with microtip, and counted in a Burker-Turk counting chamber at 400x magnification with phase-contrast microscopy. Spherical cells with greyish colour, stalk and a diameter of more than 1 µm were counted as vesicles. After counting, 200 µl-samples were taken for protein estimation. Samples were taken during a period of two weeks of incubation with intervals of one day in the first, and two days in the second week. In a parallel experiment on "P-N" medium, acetylene-reduction

activity was measured in duplicate tubes. The cotton plugs were pushed further into the tubes and the tubes were closed with suba-seals. Acetylene was added to a final concentration of 10 % of the air-volume above the medium and tubes were put with an angle of 45° on a reciprocal shaker at 28°C during the incubation period. Etylene production was recorded gaschromatographically after 1, 4 and 21 h. Specific activity was calculated as nmol ethylene per mg protein per hour between 4 and 21 hr.

Protein measurements

Protein contents were measured according to the modification of the Lowry method as described by Moss and Bard (1957).

Vesicle preparation

Cells from Erlenmeyer flasks (300 ml) with 100 ml "P-N" or "P+N" medium were harvested after 4 d growth and washed two times with PBS. During the following procedure, the tubes were kept on ice. After homogenisation in a Potter tissue homogeniser of 10 ml, the cells were pushed through a needle (0.5 mm thickness) and sonicated 40 s, 20 W with a Branson sonifier (model B12) with macrotip. During this procedure most vesicles are detached from the hyphae and remain structurally intact. One part, for vesicle preparation, was passed through a filter of fine glasswool. The filtrate was spun down and the pellet was washed 3 times in PBS (5 min 3000xg), and resuspended in PBS. This preparation and the original material were used to determine the protein content, to count the number of vesicles and to make a Western blot.

SDS-polyacrylamide-gelelectrophoresis and Western blotting

Slabgels of 1.0 mm thickness were made essentially according to the method of Laemmli (1970) with the following final compositions: Running gel: Acrylamide 10% w/v; bisacrylamide 0.16% w/v; sodium dodecyl sulfate (SDS)

0.1% w/v; Tris/HCl 0.4 M; N,N,N',N'-tetramethyl-ethylene-diamine (TEMED) 0.1%; ammonium persulfate 0.075% w/v; pH= 8.8. Stacking gel: Acrylamide 3% w/v ; bisacrylamide 0.15% w/v ; SDS 0.1% w/v; Tris/HCl 0.125 M; TEMED 0.1% w/v; ammonium persulfate 0.1 % w/v; pH= 6.8. Samples were sonicated with a Branson sonifier with microtip for 2 min at 20 W. Then they were suspended in the solubilization buffer with the following composition (final concentrations): Tris/HCl 0.0625 M; SDS 2% w/v; 2-mercaptoethanol 5% v/v; bromophenolblue 0.005% w/v; pH= 6.8. Samples were stored at -20°C until use. Running of the gels was overnight at 55 V. Electrophoresis buffer was 0.025 M Tris/glycin buffer with 0.1% w/v SDS; pH 8.3. Proteins were transferred from gels to nitrocellulose by electroblotting overnight at 50 V. The nitrocellulose was incubated with antisera against nitrogenase component I (CI) and component II (CII) from *Rhizobium leguminosarum* PRE (Bisseling *et al.*, 1980). The sera were visualised by either 1.: binding 125-I-labelled protein A to the antibodies and making an autoradiogram, or 2.: binding goat-anti-rabbit/ horseradish-peroxidase conjugate ("GAR-HRP") and staining the blot with an enzyme-reaction with 4-chloro-1-naphtol as a substrate according to a protocol developed by J.Visser *et al.* (unpublished). The second method was used for routine work, the first if a very sensitive staining was necessary.

Results

Growth characteristics and nitrogenase activity

A comparison was made between *Frankia* strains Cc1.17 and Cp1.2. In one experiment, growth and number of vesicles were determined for both strains. Figure 1a,b shows protein yields from the two strains on "P+N" and "P-N" medium. There is no significant difference in growth rate between the two strains and growth is almost linear over

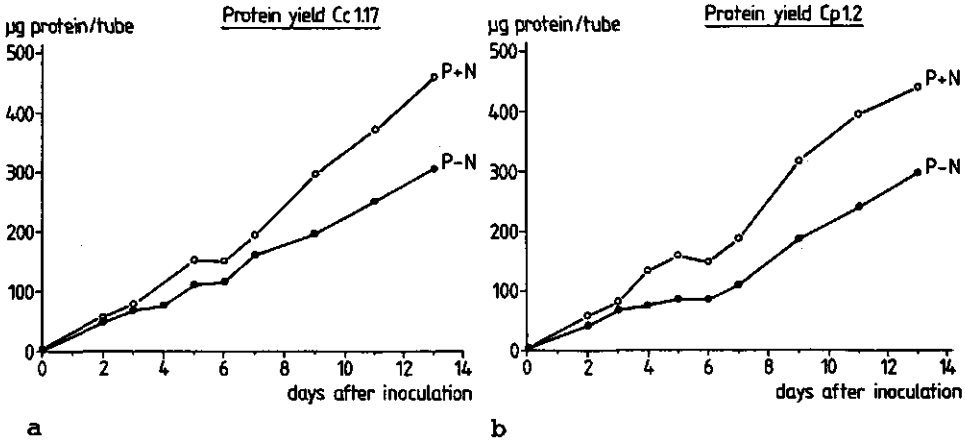


Fig.1. Protein yields of strains Cc1.17 (a) and Cp1.2 (b) on "P-N" and "P+N" medium

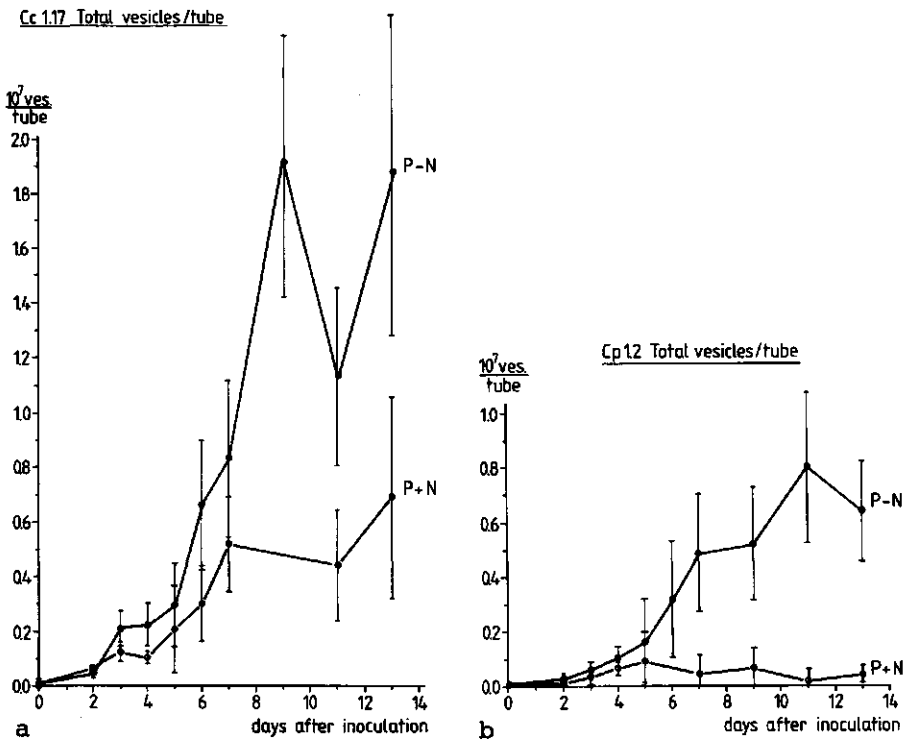


Fig.2. Total amounts of vesicles per tube of strains Cc1.17 (a) and Cp1.2 (b) on "P-N" and "P+N" medium. Vertical bars show standard deviations

the period of measurements. In "P+N" medium, growth is about 50 % higher than in the medium without combined nitrogen, which suggests a sub-optimal nitrogenase activity in the "P-N" medium. In one tube from this series, numbers of vesicles were determined. Figure 2a,b shows the total amounts of vesicles per tube. In the "P-N" medium, the maximum value counted for strain Ccl.17 was 1.9×10^7 vesicles/tube; for strain Cpl.2 this number was ca. two times lower. In the "P+N" medium, Ccl.17 still produced vesicles, this in contrast to Cpl.2. After 13 d, the number of vesicles in strain Ccl.17 was 0.7×10^7/ tube, while in strain Cpl.2 this number was more drastically reduced to 0.04×10^7 / tube. This difference remains when relative amounts of vesicles are compared: After 7 d, Ccl.17 had $(2.65 \pm 0.88) \times 10^7$ vesicles per mg protein, which was a reduction of 72 %, while in Cpl.2 the number was reduced to $(0.33 \pm 0.51) \times 10^7$, a reduction of 97 % (Table 1a,b).

Statistical analysis with Student's t-tests showed significant differences in numbers of vesicles per mg protein of both strains on the different media, with a confidence level of 0.01 for all days except day 5 for strain Ccl.17 (not significant) and day 3 for strain Cpl.2 (confidence level 0.05). Differences in numbers of vesicles of the two strains on "P+N" medium were significant with a confidence level of 0.025 on the 5th day and 0.01 from the 7th to the 13th day.

The repression of vesicle formation by NH_4Cl in Ccl.17 of only ca. 60 % was also registered in cultures grown in 200 ml flasks, where oxygen diffusion did not limit nitrogenase activity in the "P-N" medium and growth rates were the same on "P+N" and "P-N" medium (Raj, pers. comm.) Under these conditions the specific activity was higher than in tubes: i.e. 500-700 nmol ethylene / mg protein x h in "P-N" medium, while the activity in the tubes was about 100 nmol ethylene / mg protein x h. Figure 3a,b gives representative phase-contrast micrographs which show

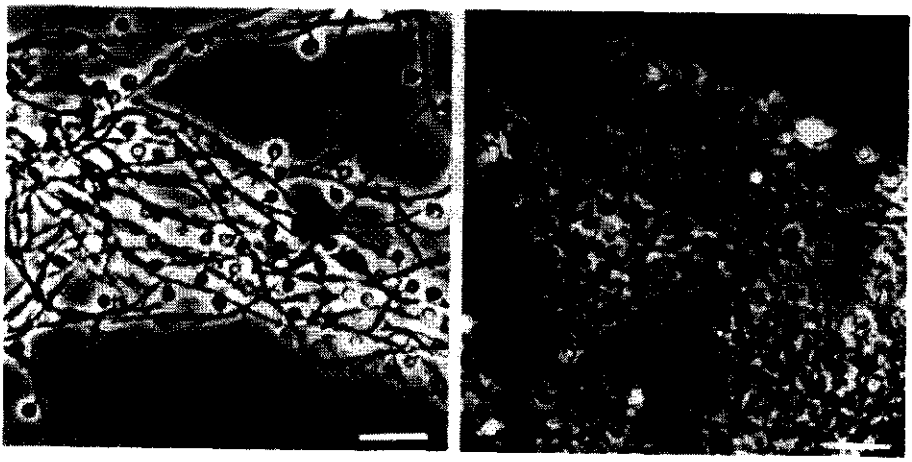
TABLE 1a,b: Numbers of vesicles of strains Cc1.17 and CpI1.
(standard deviations between brackets). --- : not counted. Statistical analysis of the figures is presented in the text

a: Numbers of vesicles of strain Cc1.17

Day	Medium		
	P-N 10^7 ves/mg prot.	P+N 10^7 ves/mg prot.	P+N % repression of vesicles
0	0.87 (1.23)	---	--
2	1.72 (0.62)	1.14 (0.12)	34
3	5.60 (1.77)	2.07 (0.62)	63
4	4.67 (1.67)	1.61 (0.34)	66
5	4.50 (2.30)	2.76 (2.13)	39
6	9.95 (3.51)	2.69 (1.22)	73
7	9.42 (3.24)	2.65 (0.88)	72
9	11.48 (2.90)	---	--
11	5.40 (1.59)	1.60 (0.73)	70
13	5.69 (1.77)	1.41 (0.75)	75

b: Numbers of vesicles of strain Cp1.2.

Day	Medium		
	P-N 10^7 ves/mg prot.	P+N 10^7 ves/mg prot.	P+N % repression of vesicles
0	---	---	--
2	0.94 (0.72)	1.44 (1.13)	--
3	2.37 (1.16)	0.90 (0.80)	62
4	4.14 (1.54)	1.65 (0.68)	60
5	4.43 (4.26)	1.25 (1.51)	72
6	7.42 (5.02)	---	--
7	10.76 (4.76)	0.33 (0.51)	97
9	2.93 (1.17)	0.40 0.43)	86
11	3.27 (1.11)	0.04 (0.12)	99
13	2.67 (0.76)	0.09 (0.06)	97



a b

Fig.3. Phase-contrast micrographs of Cc1.17 grown on "P-N" (a) and "P+N" medium (b). Bar= 10 µm

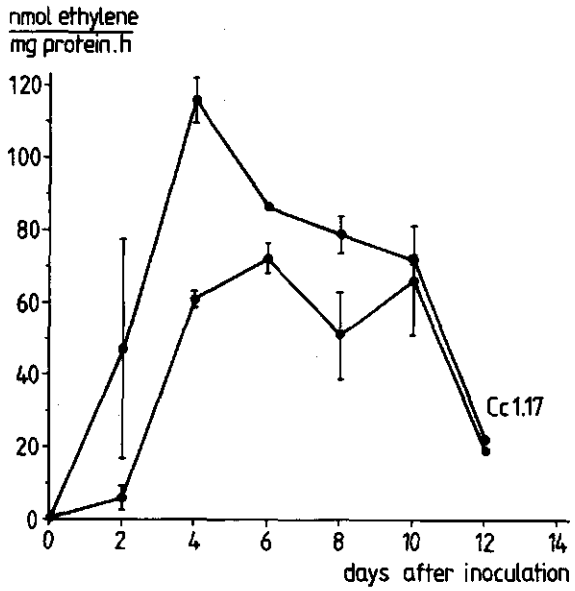


Fig.4. Acetylene reduction activity of strains Cc1.17 and Cp1.2 on "P-N" medium. Each value represents the activity in one tube.

cultures of Cc1.17 on the two media. In a parallel experiment, similar to the one described above, acetylene reduction activity of the strains was registered in "P-N" medium (Fig.4). Strain Cc1.17 had somewhat higher nitrogenase activity than Cp1.2. Both strains do not show any significant acetylene reduction on "P+N" media.

Identification of nitrogenase on Western blots

Anti-*Rhizobium*-nitrogenase serum was used to identify *Frankia* nitrogenase CI and CII on Western blots. Figure 5 shows an autoradiogram of Cc1.17 grown on "P+N" and "P-N" medium. One band from a protein with a molecular weight of about 58000 is visible, only in the "P-N"-grown culture (lane 3). This is most likely to be the CI of the *Frankia* nitrogenase. When the autoradiogram was exposed for a longer period, a second band with a molecular weight of about 34000 became visible in the "P-N" medium (lane 5) When the blot was incubated with only the anti-CII serum, only this band became visible (not shown). Therefore this 34000-protein band is probably the *Frankia*-nitrogenase CII. This protein shows less cross-reactivity with the *Rhizobium*-antiserum than the CI. Another band of intermediate size (MW about 46000) which binds to the antiserum is mainly visible in the "P+N"-grown culture. This band was not always visible in other experiments. On silver-stained gels some minor differences in band pattern are visible on both media, however this is not easily detected because of the high number of visible bands (not shown).

Localization of nitrogenase

Table 2 shows the results of a preparation of a vesicle-enriched fraction of strain Cc1.17. Variations in relative vesicle yields are mainly due to differences in cell density during sonication and in

Table 2: Vesicle enrichments in cell fractions of Strain Cc1.17 (standard deviations between brackets)

preparation	medium	10^7 ves/ mg prot
whole cells	P-N	4.40 (1.30)
vesicle prep.	P-N	15.30 (2.18)
whole cells	P+N	1.42 (0.74)
vesicle prep.	P+N	9.13 (1.46)

compactness of the glasswool filter. Equal amounts of proteins of whole-cell- and of enriched vesicle-preparations were used to show the presence of nitrogenase on Western blots (Fig.6). The results indicate that there is relatively more nitrogenase CI present in the vesicles than in the hyphae. In lane 4 where a lower amount of protein from the vesicle preparation was put on the gel, with the same amount of vesicles as the whole-cell preparation, a much weaker band is visible. This can be due to leakage of nitrogenase out of the vesicles during the preparation of the vesicle-enriched fraction, or to the presence of some nitrogenase in the hyphae. Cells and vesicle preparations from "P+N" (lane 5-7) showed no nitrogenase, although many vesicles were present in these cultures. The unknown band with molecular weight of 46000 is also visible in these preparations. Since this band is more pronounced in the whole cells than in the vesicle preparation, it is apparently more present in the hyphae than in the vesicles. *Frankia* Cp1.2 was also used to make blots of whole cell- and vesicle preparations. Similar results were obtained as with strain Cc1.17 (data not shown).

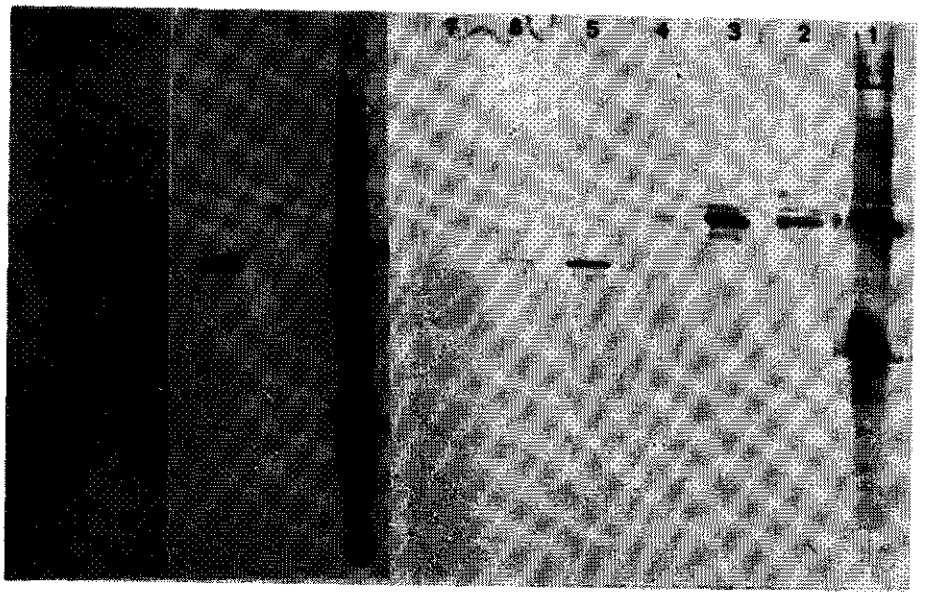


Fig.5 Fig.6

Fig.5. Autoradiogram of proteins from *Frankia* strain Cc1.17 incubated with anti-nitrogenase serum from *Rhizobium leguminosarum* nitrogenase. Lane 1: *Rhizobium* bacteroid suspension (reference); 2: Cc1.17 grown on "P+N" medium; 3: Cc1.17 grown on "P-N" medium; 4 and 5: same as lane 2 and 3 resp., but the film was exposed for a longer period

Fig.6. Western blot; nitrocellulose with serum visualized with peroxidase. Lane 1: *Rhizobium* bacteroids (reference); 2: whole cell proteins from culture from "P-N" medium, 20 µg protein, 8.9 x 10^5 vesicles; 3: Vesicle preparation from cultures from "P-N" medium, 20 µg protein, 30.5 x 10^5 vesicles; 4: as 3, 5.8 µg protein, 8.9 x 10^5 vesicles; 5: whole cell proteins "P+N" medium, 20 µg protein, 2.8 x 10^5 vesicles; 6: Vesicle preparation "P+N" medium, 20 µg protein, 18.2 x 10^5 vesicles; 7: as 6, 3.1 µg protein, 2.8 x 10^5 vesicles

Discussion

In earlier reports about the role of vesicle differentiation in *Frankia* isolates, most attention has been paid to activity of nitrogenase in the cells. Comparisons were made between cultures with and without vesicles, which was the same as cultures with and without acetylene reduction activity (Tjepkema *et al.*, 1980; Murry *et al.*, 1984). In the experiments described here, data are given from a strain which also forms many vesicles under conditions that nitrogenase is repressed. This opens the possibility to study the metabolic activity of vesicles and hyphae independent of the presence of nitrogenase. It is shown in the results of the growth experiment that there was no difference in protein yields of both strains, and both strains showed higher yields on the nitrogen-rich medium under these conditions. The culture conditions in the tubes, however, were not optimal for growth, probably because of the low oxygen-concentrations on the bottom of the tubes which may limit acetylene reduction activity. The vesicle numbers are in the same order of magnitude as those published by other authors. Burggraaf and Shipton (1983) reported vesicle amounts of about 10^8 vesicles per mg protein for strain AvcI1, which is similar to the data we found in strain Cc1.17. However, these data were significantly higher than the numbers of vesicles from strain AvcI1 as counted in our laboratory (Raj, unpublished). Culture conditions were also different and the micrograph from the AvcI1-culture published by Burggraaf and Shipton (1983) shows indeed a very high vesicle density on agar. Murry *et al.* (1984) reported vesicle densities of 5 - 8 x 10^8 *ves./mg protein for* strain ArI3. To be able to do a profound study on vesicle functions it is very important to select a suitable strain and standardize the culture conditions. On the blots shown in this report we have used an antiserum that was raised against nitrogenase of a *Rhizobium* strain. The serum shows

many bands if a blot is made with *Rhizobium* bacteroids. The two components of the nitrogenase complex can be recognised in these lanes as thicker bands. The other bands from this preparation are supposed to be aggregation- and degradation products of nitrogenase, because none of these bands are present if a fix$^-$ mutant is used to make such a blot (Schetgens *et al.*, 1984). The anti-CII serum has a lower titer than the anti-CI serum, even for *Rhizobium*, but there is also a lower cross-reactivity from this serum with the *Frankia* nitrogenase-CII. This suggests that the CII from the Frankia nitrogenase, which contains the electron transport function, is more different from the *Rhizobium*-CII than the component I. The molecular weights of the two components are only slightly different from the *Rhizobium* proteins i.e. 58000 and 34000 for the CI and CII respectively. The results indicate that on "P+N" medium nitrogenase is not induced, as is common to nitrogen-fixing organisms. The fact that nevertheless vesicles are formed under these circumstances, raises the question what can be the role of these structures if no nitrogenase is present in the cells. It seems that vesicles, are the favourable sites for nitrogenase. However they might play just a general role in cell metabolism that happens to create these favourable conditions. This role would probably consist of providing an oxygen protection for the enzyme (Torrey and Callaham, 1982). It is not clear yet whether active nitrogenase is also present in the hyphae. A strain with nitrogenase activity but without vesicles would be very helpful in these investigations.

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IV-1

Localization of nitrogenase in vesicles of Frankia sp. Cc1.17 by immunogoldlabelling on ultrathin cryosections.

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Localization of nitrogenase in vesicles of *Frankia* sp. Cc1.17 by immunogoldlabelling on ultrathin cryosections.

Abstract

Immunogoldlabelling on ultrathin cryosections of *Frankia* sp. Cc1.17 showed specific labelling of nitrogenase in the spherical cells called vesicles. No label was found in the hyphae in any cells grown on a medium with combined nitrogen, nor in those to which no specific antiserum was added. Similar results were obtained with cultures grown under high (20 %) and low (2 %) oxygen tension in the gas phase.

Keywords: Actinomycetes, nitrogen fixation, symbiosis, immunocytochemistry, ultracryotomy.

Abbreviations:
BSA: Bovine serum albumin fraction V (Sigma)
PBS: phosphate buffered saline. Posphate buffer 0.1M, 8 g NaCL/1, 0.2 g KCl/1.
PIPES: Piperazine-1,4-bis(ethane sulphonic acid)

Introduction

Actinomycetes belonging to the taxon *Frankia* are capable of inducing the formation of root nodules in many non-leguminous plants (Bond 1983). The organisms grow in hyphae and form, in addition, typical spherical cells, called vesicles. It has been suggested that in root nodules as well as in free-living *Frankia* cultures, vesicles are the site of nitrogen fixation. Nitrogen reducing activity could be demonstrated in vesicle clusters in root nodules of *Alnus glutinosa* and *Hippophae*

rhamnoides (Akkermans 1971) and in isolated clusters obtained from root nodule suspensions (van Straten *et al.* 1977). In pure cultures of strain CpI1, obtained from root nodules of *Comptonia peregrina* vesicle formation can be induced by growing the cultures under nitrogen limitation (Tjepkema *et al.* 1980, 1981). In these cultures, most of the nitrogenase (Meesters *et al.* 1985) and nitrogen fixing activity (Noridge and Benson 1986) could be detected in the vesicles. The outer cell wall of the vesicles was shown to be multi-layered. Torrey and Callaham (1982) hypothesize that such a structure might play a role in the protection of the oxygen labile enzyme. However, nitrogen fixing activity and vesicle formation does not seem to be strictly related. Murry *et al.* (1985) found in strain CcI3 derived from *Casuarina cunninghamiana* nitrogenase activity in the absence of vesicles when cells were grown under very low oxygen tensions. The contrary was described by Meesters *et al.* (1985) in strain Cc1.17 isolated from *Colletia cruciata*. This organism did form many vesicles under conditions when no nitrogenase was induced. To obtain more information about this phenomenon, nitrogenase immunolabelling on ultrathin cryosections was performed in strain Cc1.17. The data of this study are presented in the following.

Materials and Methods

Strain and media

Strain Cc1.17 was isolated from *Colletia cruciata* as described before. (Akkermans *et al.* 1984). The cells were cultured in tubes containing 9.5 ml of medium to which 0.5 ml of a homogenized culture was added as inoculum. For growth of cells under reduced oxygen tension, 100 ml bottles with 25 ml of medium were flushed with argon and closed with rubber stoppers. The closed bottles were supplied with 1 % of CO_2 and 2 % O_2 (v/v). The bottles

were inoculated with 2 ml of a homogenized culture. After 5 d of growth, the cells were harvested. Media contained propionate as only carbon and energy source and in some experiments ammonium chloride as nitrogen source. The composition of the culture medium was the same as described before (Meesters *et al.* 1985) except for sodium propionate which was added in a lower concentration, namely 0.5 g/l. "P+N" medium contained ammonium chloride (0.2 g/l); "P-N" was the same medium but without ammonium chloride.

Protein content

The protein content of the purified antibody preparation was estimated from the absorbance at 280 nm, using the specific extinction coefficient of rabbit IgG (Johnston and Thorpe 1982). Protein concentrations of the preparations for gel-electrophoresis were determined according to a modified Lowry method (Moss and Bard 1957) using bovine serum albumin as standard.

Antiserum

Antiserum against *Azotobacter vinelandii*- nitrogenase component II, kindly supplied by A.Braaksma (Dept.of Biochemistry, Wageningen) was used for the localization experiments. Purification of the nitrogenase and preparation of the antiserum were performed as described by Braaksma *et al.* (1983) and Voordouw *et al.* (1982). An aliquot of 0.5 ml of antiserum was purified by precipitation with 2 M ammonium sulfate (Clausen 1981), followed by a further purification using a DEAE-sepharose-CL-6B column (Pharmacia, Uppsala, Sweden). The column was eluted with 0.02 M sodium-phosphate buffer pH 8.0. Fractions of 1 ml were collected and the absorbance at 280 nm was registered. This procedure gave one protein peak that came off from the column with the void volume.

Gelelectrophoresis and Western blotting

SDS-acrylamide gels and Western blots were prepared as described before (Meesters *et al.* 1985). 40 µg of purified antiserum was applied to a gel to test the purity of the preparation. Gels were stained with 0.15 % Coomassie Brilliant Blue R250 (CBB) in 45 % methanol / 10 % acetic acid / 0.5 % w/v $CuSO_4.7H_2O$. Destaining of the gel was done in the same solution but without CBB. For the blotting experiment, 40 µg of cell proteins from Cc1.17, grown on "P+N" or "P-N" medium, were applied to the gel. The nitrocellulose blots were incubated with the purified anti-nitrogenase CII IgG preparation (45 µg IgG /10 ml buffer) Immunostaining of the blots was performed with peroxidase as described by Hawkes *et al.* (1982).

Fixation and cryosectioning

Cells were fixed with 6 % paraformaldehyde in 0.2 M PIPES buffer at pH 7.2 for 16 h. Subsequently the cells were washed with the buffer and embedded in the same buffer containing 4 % gelatin. After a short postfixation (1 h, 6 % paraformaldehyde and 0.25 % glutaraldehyde) the embedded cells were stored until use at 4°C in PIPES buffer with 2% paraformaldehyde and 1M sucrose. Before sectioning, small pieces (ca. 0.5 mm^3) of embedded cells were cut with a razor blade and incubated 60 min in 2.3 M sucrose. Cryosectioning was done essentially according to the method of Tokuyasu (1980). The pieces were mounted on copper blocks, excess of sucrose was removed with filter paper and the blocks were frozen in liquid nitrogen. Ultrathin sections were cut on a Reichert FC 4D ultracryotome with a glass knife at a temperature of -95°C. Copper grids, 100 mesh, covered with carbon-coated formvar film and glow-discharged, were used. Sections were transferred to the grids on the lower surface of a drop of sucrose 2.3 M in a small loop. The grids were placed sections-down on a block of 2 % gelatin in a petri-dish on ice.

Immunolabelling and staining

The gelatin was melted at room temperature leaving the grids floating on the surface. The grids were then incubated with the subsequent solutions by floating them on the surface of small drops and transferring them from one drop to another with a forceps. The sequence of the incubations was as follows: 4 x 5 min PBS/gly (PBS with 0.02 M glycin); 3 min 0.02 M ammonium chloride in PBS; 3 min 0.015 M sodium borohydride in PBS; 2 h either purified specific antiserum, diluted as indicated in the text with PBS/BSA (PBS with 1 % w/v BSA) or only PBS/BSA; 5 x 4 min PBS/gly; 45 min anti- rabbit IgG, conjugated to colloidal gold 10 nm (Janssen Pharmaceutica, Beerse, Belgium), 20 x diluted in PBS/BSA; 5 x 5 min PBS; 1 min milli-Q filtered water; 10 min 2 % uranyl acetate oxalate pH 7.2 and 10 min 3 % uranyl acetate, according to Tokuyasu (1980). The grids were covered with a film of methylcellulose (Fluka Methocel 300 cP, 1 % w/v) to prevent drying damage of the sections (Tokuyasu 1978). The sections were studied with a Philips TEM 301 at 60 or 80 kV.

Results

Purification of antiserum

The peak fractions (2 x 1 ml) that came off the column had an estimated protein content of 0.9 mg/ml. 40 µg of this fraction was applied to a SDS-PAA-gel. Only one IgG-band was visible on the Coomassie-stained gel, other serum proteins could not be detected (not shown).

Specificity of the antibodies

To test the binding of the antibodies to the cell proteins, extracts of *Frankia* cells with or without nitrogenase activity (grown on "P-N" or "P+N" medium) were used to make Western blots from SDS- polyacrylamide gels. Only one band from nitrogenase component II is visible

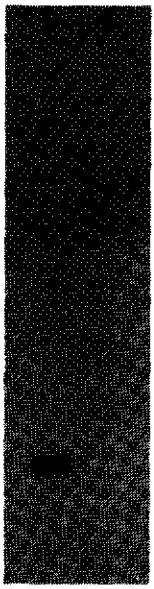

Fig.1.
Immunoblot of *Frankia* proteins incubated with anti- nitrogenase CII. Lane a: cells grown on P-N medium, lane b: cells grown on P+N medium

(Fig. 1, lane a) and no antibody binding can be detected in absence of nitrogenase (Fig. 1 lane b).

Localization of nitrogenase

In all nitrogen fixing cultures of strain Ccl.17 many vesicles were found that were densely labelled with gold. No significant labelling could be seen in the hyphae. No significant label was found in cells grown on "P+N" medium. Figure 2 a,b and e shows micrographs of labelled vesicles and not labelled hyphae from cells grown with 20 % oxygen in the gas phase. The same labelling in vesicles

p. 68:
Fig.2. Electronmicrographs of immunolabelled cells. H= hypha, V= vesicle. Bars represent 0.5 µm. Goldlabel is visible as black spots in all vesicles from cultures grown on P-N medium (a-e). a: vesicle with goldlabel of average density, hypha without goldlabel. Cells grown under 20 % oxygen; b: hypha without label, 20 % oxygen; c: labelled vesicle and unlabelled hypha grown under 2 % oxygen; d: vesicle with very dense goldlabel, 2 % oxygen; e: vesicle with gold particles laying around the cell, 20 % oxygen; f: immunolabelled vesicle and hyphae grown on P+N medium, without significant labelling.

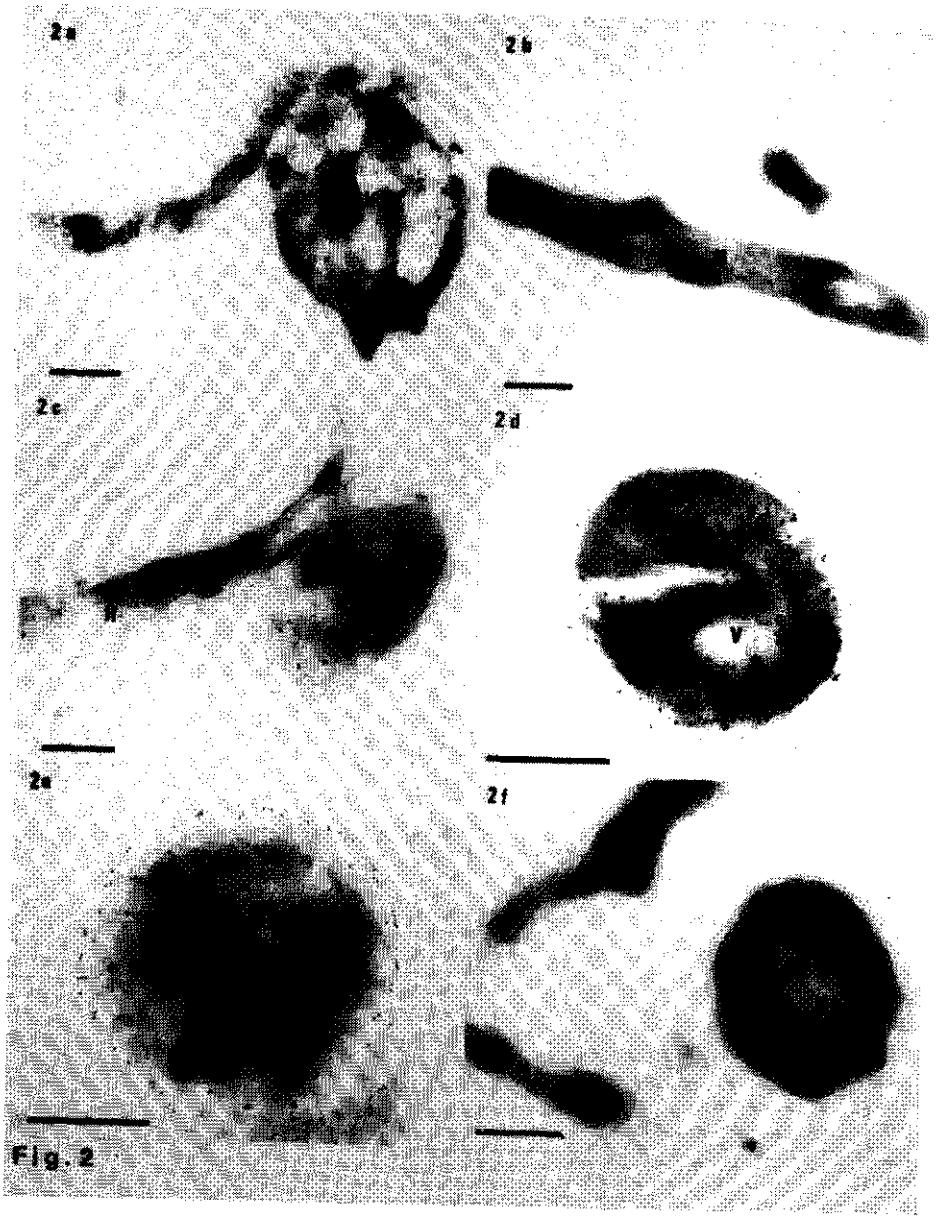

Fig. 2

and not in hyphae was found in cells grown with only 2 % oxygen in the gas phase. Figure 2 c,d shows examples of labelled vesicles, and hyphae without label. Again no significant labelling could be detected in cells grown on "P+N" medium. Nonspecific labelling outside the cells was also neglectable. The results described above were obtained with a 100 x diluted antibody preparation. Nonspecific binding of antibodies was present on sections incubated with too high (tenfold higher) antibody concentration, as is common in immunolabelling. This was clear from the presence of goldlabel in cells grown on "P+N" medium and, to a lower degree, in the spaces in between the cell sections (not shown). Goldlabel disappeared from both places by diluting the antibody solution with only a slight decrease of label density in the labelled vesicles from cells grown on "P-N" medium. A further dilution of the antibodies (tenfold lower) resulted in a drastical decrease of the specific labelling. Incubation in PBS/BSA without antibodies did not give any significant labelling (not shown).

Discussion

In *Frankia* sp. Cc1.17 the nitrogenase component II (CII) can only be detected in the vesicles but not in the hyphae. One of the main problems in the immunotechniques is to get a very specific antiserum. The specificity of the used antibodies, which were raised against nitrogenase CII from *Azotobacter vinelandii*, was tested with two different methods. First, immunoblotting of *Frankia* cell extract gave only one band that was stained by the reaction with the antibodies, while control preparations, of cells without induced nitrogenase, did not show any reaction. This means that the antibody preparation was highly specific. Second, labelling of cryosections of

cells without induced nitrogenase did not show any goldlabel in the cells. This means that the antiserum reaction in the chosen procedure for immunolabelling on the grids is also specific. In former experiments (Meesters *et al.* 1985) *Frankia* nitrogenase component I (CI) was visualized on Western blots using an antiserum against *Rhizobium* CI. Those experiments showed that most of the CI in *Frankia* sp. Cc1.17 is present in the vesicles. This anti-CI serum crossreacted not only with CI but also with some *Frankia* proteins that were present in cells grown on "P+N" medium. Assuming therefore that these proteins are not nitrogenase, this antiserum could not be used for EM localization. Although it can not be excluded that, though all CII in *Frankia* sp. Cc1.17 is localized in the vesicles, a minor part of CI is present in the hyphae. The results presented here, in combination with the former results make it very likely that all nitrogenase in strain Cc1.17 is present in the vesicles. If localization of nitrogenase in the vesicles is functional because of oxygen protection for the enzyme in the vesicles, as was suggested by many authors (*e.g.* Torrey and Callaham 1982, Murry *et al.* 1985, Tjepkema and Murry 1985), it is possible that nitrogenase will be expressed in hyphae as well if no oxygen protection is necessary because of reduced oxygen concentration. To test this possibility, cells were grown under reduced (2%) oxygen tension. A still relatively high oxygen concentration was chosen to allow some growth of the cultures. Under these circumstances, no expression of nitrogenase in the hyphae could be detected. This indicates that either expression of the enzyme in the hyphae is not possible in this strain, due to an unknown regulation mechanism, or still lower oxygen concentrations are required for expression. The latter possibility is still under investigation. In the labelling procedure that was chosen, an optimal preservation of the antigenicity of the nitrogenase was favoured by using a very mild fixation. Using stronger

fixatives with 0.5 % glutaraldehyde resulted in a clearly decreased label density. The ultrastructure of the sections was therefore of lower quality than that which can be achieved by other EM techniques. Also low-temperature Lowicryl embedding, which gives better ultrastructural preservation is known to give a lower antigenic preservation. Since vesicles and hyphae are clearly to distinguish in the method used here, this procedure is the most informative for this kind of work. However, it is not clear from these results whether nitrogenase is present as a membrane-bound enzyme or not (see Fig. 2d,e). Therefore more experiments will be needed to find an optimal procedure to preserve membrane structures with enough preservation of nitrogenase antigenicity.

Acknowledgements

The antiserum was prepared by A. Braaksma, Dept. of Biochemistry, Wageningen. W. van Vliet prepared the cell cultures and assisted with immuno- gelectrophoresis. Immunocytochemistry experiments were done at the Dept. of Plant Cytology and -Morphology, Wageningen. Helpful advice for immunogold-labelling techniques was given by H.Kieft from this Department. The author thanks also A.D.L. Akkermans and A.J.B. Zehnder, Dept. of Microbiology, Wageningen, for helpful discussions and critically reading the manuscript. These investigations were supported by the Foundation for Fundamental Biological Research (BION), which is subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO).

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IV-2

Nitrogenase is restricted to the vesicles in Frankia strain EAN1pec

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Physiol. Plantarum (1987) 70, 267-271

Nitrogenase is restricted to the vesicles in *Frankia* strain EAN1pec.

Abstract

The presence of nitrogenase in vesicles and hyphae of *Frankia* EAN1pec was investigated by using immunogold labelling on ultrathin cryosections for electron microscopy. These studies resulted in the specific labelling of nitrogenase in the vesicles of nitrogen fixing cultures. No significant label could be found in the hyphae, indicating a strong repression of nitrogenase in the hyphae.

Keywords: Actinomycetes, cell differentiation, immunocytochemistry, immunogold labelling, localization, nitrogen fixation

Abbreviations: CI, CII: Components I and II of nitrogenase.

Introduction

Frankia cultures, isolated from nitrogen-fixing root nodules from many non-leguminous plants (Bond 1983), form thin, branched hyphae with a diameter of ca. 0.5 - 1 µm. In addition, many strains can form typical spherical structures, called vesicles, which are formed from the tips of short side branches of the hyphae. The vesicles generally have a diameter of 2-4 µm. In accordance with observations in vesicle clusters in root nodules (van Straten *et al.*, 1977), vesicles are the proposed site for nitrogen fixation (Tjepkema *et al.* 1980, 1981, Noridge and Benson 1986). The necessary protection of nitrogenase against oxygen is possibly provided by the vesicle structures (Torrey and Callaham 1982, Murry *et al.* 1984,

1985, Tjepkema and Murry 1985). Noridge and Benson (1986) recently described a method to purify vesicles from *Frankia* strain CpI1. They found all nitrogenase activity almost exclusively in anaerobically purified vesicle fractions. Low activity in the hyphae fractions could be due to vesicle contamination. Recently, we could localize nitrogenase only in the vesicles of *Frankia* strain Cc1.17 by using immunogold labelling on cryosections (Meesters 1987). In this study, the presence of nitrogenase in vesicles and hyphae in strain EAN1pec was studied. An improved method of preparing the cryosections was used, which resulted in a better ultrastructural preservation of the *Frankia* cells.

Materials and methods

Strain and media

Frankia strain EAN1pec was isolated from *Elaeagnus angustifolia* L. by Lalonde *et al.* (1981). A subculture of the strain was obtained from L.S. Tysa, Univ. of Wisconsin, Madison, USA. The cells were cultured in tubes containing 9.5 ml of medium to which 0.5 ml of a homogenized culture suspension was added as inoculum. After 5-8 days of growth, the cells were harvested. The composition of the culture media was the same as described before (Meesters *et al.* 1985), except for the carbon source which was 20 mM fructose. The nitrogen source was either N_2 or 3.74 mM NH_4Cl.

Antisera

Antisera against both components of nitrogenase from *Azotobacter vinelandii* Lipman 1903 were kindly supplied by A. Braaksma (Dept of Biochemistry, Wageningen Agricultural Univ.) and purified as described by Meesters (1987). Antiserum against ATPase from mitochondria from *Neurospora crassa* Shear & Dodge was kindly supplied by M. Schwaiger

(Institut für Physiologische Chemie, München Univ., FRG). This serum binds specifically to the β-subunit of the F1-part from the membrane-bound ATPase (M. Schwaiger, personal communication). The antiserum was purified in the same way as the anti-nitrogenase sera. All antisera were raised in rabbits.

Fixation and cryosectioning

Fresh cells were washed and fixed with 8 % (w/v) paraformaldehyde in 0.2 M PIPES buffer (Piperazine-1,4-bis(ethane sulphonic acid), sodium salt) at pH 7.2, 4°C, for at least 4 h. The cells were stored in the fixation solution at 4°C until use (not longer than 14 days). The cell suspension was centrifuged in Eppendorf vials for 10 min and small clumps of cells were immersed in 2.3 M sucrose in phosphate buffer (0.1 M Na- phosphate, with 0.137 M NaCl and 2.68 mM KCl, pH= 7.3) for 15-30 min (G. Griffiths, personal communication). These clumps were then mounted on copper blocks, excess of the sucrose containing buffer was removed with filter paper and the blocks were frozen in liquid nitrogen. Cryosectioning was done according to Tokuyasu (1980). Ultrathin sections were cut on a CryoNova (LKB, Uppsala, Sweden) ultracryotome with a glass knife at -95°C. Copper grids, 100-150 mesh, covered with carbon-coated formvar film and glow-discharged, were used. Sections were transferred to the grids on the lower surface of a drop of 2.3 M sucrose in the phosphate buffer (see above) in a small loop. The grids were stored sections-down on a solution of 1 % (v/v) newborn calf serum on ice.

Immunolabelling and staining

Immunolabelling, staining and drying of the sections were performed as described by Griffiths *et al.* (1984). Incubation with specific antiserum or, in controls, with 1 % (v/v) newborn calf serum, was performed for 2 h. A second incubation was performed for 45 min, with

anti-rabbit IgG conjugated to colloidal gold particles, 6 nm diameter, prepared according to Slot and Geuze (1985). Antisera and gold particles were diluted in 1 % (v/v) newborn calf serum in phosphate buffer (see above). Several washing steps with the buffer were performed between the incubations. To prevent damage of the sections due to air-drying, the grids were covered by a thin film of methyl cellulose 25 cP (Fluka AG) with 0.15 % (w/v) uranyl acetate. The sections were observed with a Philips 301 Transmission Electron Microscope at 80 kV.

Results

Ultrastructural preservation

In comparison with the results on cryosections of *Frankia* strain Cc1.17 (Meesters 1987), the preservation of the ultrastructure of the EAN1pec cells in these cryosections was clearly improved. In many cells, most of the plasma membrane was visible (Fig.1). Similar results have later been obtained also with strain Cc1.17 (T.M., unpublished results).

Localization of nitrogenase on cryosections

Sections of strain EAN1pec (Fig.1), labelled with anti-nitrogenase CII purified serum, showed many densely labelled vesicles if the cultures were grown on N-free medium. Using the proper antibody concentration (9 µg IgG ml^{-1}), the gold particles were only visible in the vesicles from these cultures. No significant labelling was found in hyphae from N-free medium (Fig. 1b,c), or in any cells from NH_4Cl-medium (not shown). If no specific antibody was added, no labelling was found (Fig.1a). Although these results clearly indicate the occurrence of nitrogenase only within vesicles, the intracellular localization is still unclear. In some sections, however, but not always, the gold particles were located along the


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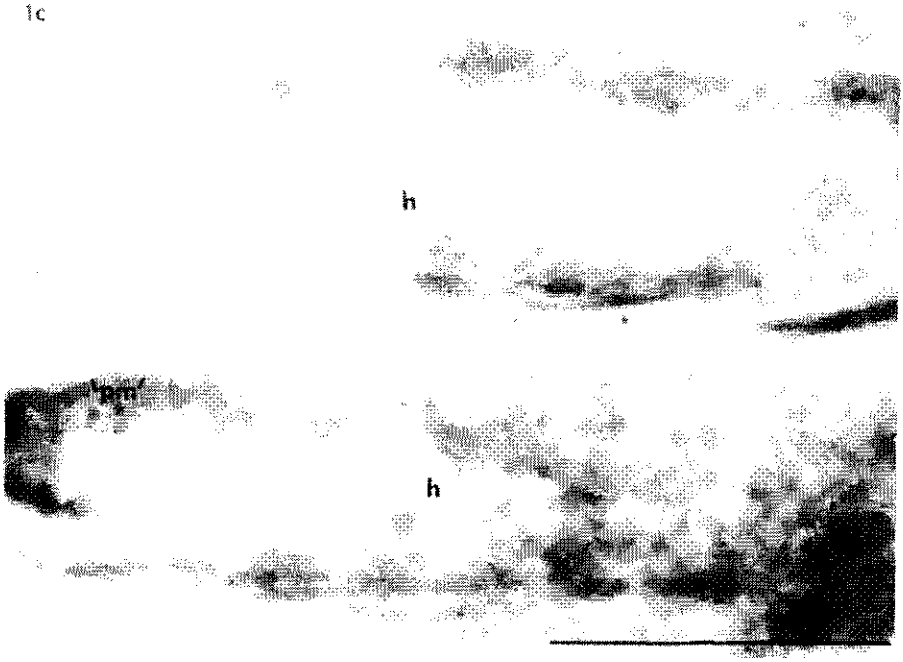

pp. 80-81:
Fig.1. Immunolabelled vesicles and hyphae from *Frankia* strain EAN1pec. Gold particles are visible as black spots. a, Control. Cells grown on N-free medium, incubated without specific anti-*nitrogenase* CII antibodies but with gold particles. No significant non-specific labelling is visible; b and c, cells grown on N-free medium, labelled vesicles and unlabelled hyphae. h, hypha, pm, plasma-membrane, v,vesicle. Bars represent 0.5 µm.

membranes (data not shown). Similar differences in localization of nitrogenase have also been observed on sections of gelatin-embedded cells of *Frankia* Ccl.17 (Meesters 1987). Immunolabelling of (presumably) membrane-bound ATPase, however, showed also the same variable distribution of gold particles (not shown). This indicates that delocalization of proteins might have occurred during the procedure.

Discussion

The localization of nitrogenase CII only in vesicles of *Frankia* strain EAN1pec was demonstrated by electron microscopy on immunogold-labelled cryosections. Recently we reported the labelling of nitrogenase in vesicles of *Frankia* strain Ccl.17 (Meesters 1987) by using cryosections of gelatin-embedded cells. Although these techniques gave good and specific labelling in vesicles and not in hyphae, the experiments were limited by difficulties in preservation of the ultrastructure of the cells. The results presented here demonstrate that the ultrastructural preservation can be improved, without changement of the immunolabelling, by omitting the embedding step. This improvement might be an indirect effect of heating the gelatin, since the embedding material does not enter the cells.

The results of the immunolabelling experiments are to a great extent dependent on the specificity of the antisera used. It is important but often neglected, that microscopic localization studies should be coupled with studies of immuno- gelelectrophoresis. In previous experiments we have used antisera against *Rhizobium* nitrogenase, from which anti-nitrogenase CII showed only a weak crossreaction with *Frankia* nitrogenase CII and anti-nitrogenase CI showed some unspecific crossreactions (Meesters *et al.* 1985). Also the antiserum against

Azotobacter vinelandii nitrogenase CI showed some unspecific reactions on Western blots. The antiserum against *Azotobacter* nitrogenase CII is highly specific as was demonstrated by the Western blotting technique (Meesters 1987). This antiserum is a good marker of nitrogenase CII in immunogold-labelling experiments. One may wonder to what extent the distribution of nitrogenase CII covers that of CI. Additional immunogelelectrophoresis experiments, not shown here, have indicated that both nitrogenase CI and CII are only expressed in the vesicles and not in the hyphae. The specific localization of nitrogenase CI and CII that has now been demonstrated by independent techniques, indicates a strong repression of nitrogenase in the hyphae. The mechanism of this repression is still unknown and needs further studies at the molecular genetic level.

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IV-3

The influence of low oxygen concentration on the localization of nitrogenase.

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The influence of low oxygen concentration on the localization of nitrogenase.

Abstract

The localization of nitrogenase in microaerobically grown *Frankia* strain Ccl.17 was investigated. Using immuno-gelelectrophoresis, evidence is provided that nitrogenase is still localized in the vesicles, although protection of the enzyme against oxygen is not needed under these conditions. Immunogoldlabelling of nitrogenase in the cells appeared impossible, caused by aspecific labelling of mucilage that was excreted by the cells.

Introduction

It has been shown in the chapters IV-1 and IV-2 of this thesis, that in the presence of oxygen, *Frankia* nitrogenase is localized in the vesicles. It has also been shown that nitrogenase is still localized in the vesicles in cultures grown under 2 % oxygen. The localization of nitrogenase in the specialized cells probably protects the enzyme against inactivation by oxygen (Murry *et al.* 1984a, 1985, Parsons *et al.* 1987, Torrey and Callaham 1982, this thesis chapter 1). *Frankia* strain CcI3, isolated from *Casuarina cunninghamiana*, is able to fix nitrogen in the absence of vesicles, in the hyphae, under 0.3 % oxygen, when no protection of nitrogenase is necessary (Benson 1979, Murry *et al.* 1985). However, in aerobically grown cultures of the strain, many vesicles are present.
For *Anabaena cylindrica*, localization of nitrogenase under microaerobic conditions was investigated by Murry *et al.* (1984b). She provided evidence, that under 0.3 % oxygen, nitrogenase is still restricted to the heterocysts in *Anabaena cylindrica*.

Frankia Strain Ccl.17, isolated from root nodules of *Colletia cruciata*, does form vesicles under microaerobic conditions, although in reduced numbers. In the present study, we will focus on the localization of nitrogenase in *Frankia* Ccl.17 grown under 0.3 % oxygen.

Materials and Methods

Cultivation of the strain

Frankia strain Ccl.17, isolated from *Colletia cruciata* (Akkermans *et al.* 1984) was cultivated in 10-l. culture flasks with 5 l. "P-N" medium (Meesters 1987), which were sparged with a gas mixture of 0.3 % O_2/ 0.5 % CO_2/ balance N_2, or with air. The flasks were continuously stirred with a magnetic bar. After one (20 % oxygen), or two weeks (0.3 % oxygen), the cells were harvested and vesicles and hyphae were separated on a sucrose gradient as was described by Noridge and Benson (1986).

Gelelectrophoresis and Western blotting

Nitrogenase contents of the fractions was analysed on Western blots as described before (Meesters 1987). The nitrocellulose filters were incubated with crude anti-*Azotobacter vinelandii* nitrogenase component II serum.

Immunogoldlabelling and electron microscopy

Immunogoldlabelling and electron microscopy were performed as described before (Meesters *et al.* 1987).

Results

Vesicle preparations

Under 20 % and 0.3 % oxygen, vesicles developed in the cultures, although less vesicles were seen under low oxygen concentration (Figs. 1,4). The separation of

vesicles and hyphae on sucrose gradients resulted in vesicle preparations that were almost devoid of hyphal fragments (Figs 2,5), and hyphae preparations that consisted mainly of hyphae (Fig. 6), although a few vesicles were present (not shown).

Localization of nitrogenase

Using immuno-gelelectrophoresis, evidence was provided that in both cultures, grown under 20 % or 0.3 % oxygen, nitrogenase was present in the vesicle fractions of the cultures (Figs. 3,7). Almost no enzyme could be deteced in the hyphae fractions, indicating that most, and probably all nitrogenase was localized in the vesicles, under aerobic and under microaerobic conditions.

The localization of nitrogenase in the vesicles could not be confirmed using immunogoldlabelling on ultrathin cryosections. The cultures produced high amounts of mucilageous material, which reacted aspecifically with the specific antiserum (data not shown).

p. 89:
Figs. 1-3.
Localization of nitrogenase in *Frankia* strain Cc1.17 grown under 0.3 % oxygen.
Fig.1, Culture of Cc1.17, with vesicles (v); 2, Vesicle preparation; 3, Nitrocellulose blot with nitrogenase component II bands (arrow), Lane a, total cell proteins, Lane b, proteins from hyphae fraction, Lane c, proteins from vesicle fraction. Bars represent 10 µ.

Figs. 4-7.
Localization of nitrogenase in *Frankia* strain Cc1.17 grown under 20 % oxygen.
Fig.4, Culture of Cc1.17, with vesicles (v) and young sporangia (s); 5, Vesicle preparation; 6, Hyphae preparation; 7, Nitrocellulose blot with nitrogenase component II bands (arrow), Lane a, total cell proteins, Lane b, proteins from hyphae fraction, Lane c, proteins from vesicle fraction. Bars represent 10 µ.

fig.1

fig.2

fig.4

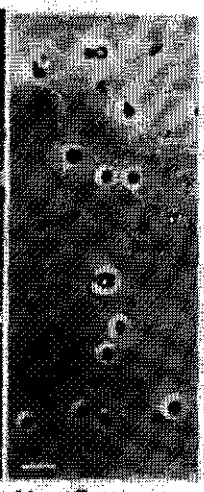

fig.5

fig.6

Discussion

Different from *Casuarina* strain CcI3, *Frankia* strain Cc1.17, isolated from root nodules from *Colletia cruciata*, does form vesicles under microaerobic conditions, though in reduced numbers. Using immunoblotting techniques, we found that in strain Cc1.17, grown under 0.3 % oxygen, nitrogenase is still localized in the vesicles (Figs. 1-7).

Unfortunately, we were not able to localize nitrogenase in the vesicles by immunogoldlabelling techniques, caused by several problems. The concentration of nitrogenase is probably very low in these cells, regarding the low enzyme activities and low growth rates of the cultures (data not shown). Another problem was strongly interfering with immunolabelling: The cultures produced and excreted a large amount of mucilageous material. After immunogold-labelling, we found a dense, aspecific goldlabelling on the outer layer around the vesicles, regardless whether the vesicles were formed in the absence or presence of ammonia (data not shown). The aspecific reaction was most probably caused by the mucilageous material. Also aerobically grown Cc1.17 cultures, used in the same experiments, formed a mucilage, though for unknown reasons. The labelling of CcI3, grown under air, was carried out as well. Since this strain does not form any vesicles in the presence of ammonia, it was impossible to check if the same aspecific labelling was present in these cells too. The unexpected occurrence of aspecific labelling shows again the importance of good controls in immuno- labelling experiments. The results presented here do not interfere with conclusions drawn from the experiments described in chapters IV-1 and IV-2, because of the fact that neither the large mucilage, nor the aspecific labelling of vesicles, grown in the presence of ammonia, has ever been

observed in those experiments, while labelling on vesicles without nitrogenase was always carried out.
In conclusion, it seems that nitrogenase in *Frankia* is always localized in the vesicles, when vesicles are present. For nitrogen fixation under aerobic growth conditions, vesicles are indispensable. At least one *Frankia* strain is able to fix nitrogen, under microaerobic conditions, in the hyphae.

Acknowledgements

Antiserum against *Azotobacter*-nitrogenase CII was kindly provided by A. Braaksma (Dept. of Biochemistry, Wageningen, The Netherlands). Immunocytochemistry was performed at the Dept. of Plant Cytology and Morphology, Wageningen. These investigations were supported by the Foundation for Fundamental Biological Research (BION), which is subsidized by the Netherlands Organization for the Advancement of Pure research (ZWO).

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V

A study of the possibility of DNA rearrangement in *Frankia* vesicles.

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A study of the possibility of DNA rearrangement in *Frankia* vesicles.

Abstract

Since differences exist between expression of nitrogenase in *Frankia* vesicles and hyphae, investigations were carried out to detect whether differences could be found between *nif*-genes from vesicles and from hyphae. Total DNA was isolated from *Frankia* endophytic vesicle clusters and from the free-living hyphal stage, both from strain ArI3. DNA restriction analysis on agarose gels revealed that no differences in DNA restriction patterns from vesicles and hyphae could be found. Hybridization of total DNA with the homologous *nif*-genes HDK and AB from hyphae as probes resulted in similar band patterns for DNA from vesicles and hyphae. It was concluded that no important DNA rearrangements of *nif*-genes occur during the differentiation of hyphae into vesicles.

Introduction

In different *Frankia* strains it has been shown that nitrogenase is only present in the vesicles (this thesis, Noridge and Benson 1986). This is not only true if the cells are grown under ambient oxygen concentration, but in certain strains (see ch.IV-3) also under micro-aerobic conditions. Thus, the regulation of the expression of *nif*-genes is not strictly oxygen-directed. Also in vesicle clusters in root nodules, nitrogenase is localized in the vesicles (Akkermans 1971, van Straten *et al.* 1977). The mechanism of this regulation in *Frankia* is unknown. Several other nitrogen-fixing organisms have also differentiated cells for nitrogen fixation. In legume root nodules, nitrogen fixation is found in *Rhizobium*

bacteroids. However, these bacteroids are not formed in pure culture and cannot protect the cells against oxygen. In *Anabaena*, heterocysts are formed which have a multilaminate cell wall like *Frankia* vesicles (Lang and Fay 1971). In these cells, nitrogen can be fixed aerobically (Haselkorn 1978).

In several cases of differentiation of cells or proteins it has been shown that rearrangement of DNA fragments occurs. (*e.g.* Antibody specificity: Kataoka *et al.* 1980; phage Mu: Toussaint and Resibois 1983). In *Anabaena*, DNA rearrangements cause the transcription of *nif*-genes in heterocysts (Golden *et al.* 1985). However, in *Rhizobium trifolii* bacteroids, no DNA rearrangement could be detected (Scott *et al.* 1984).

Since vesicles develop from hyphal cells, eventual differences in DNA composition between vesicles and hyphae can only be caused by rearrangement of DNA. This chapter describes a study of the possible occurrence of DNA rearrangement in *Frankia* vesicles. Recently, the *nif*-genes HDK and AB from *Frankia* strain ArI3 have been cloned and described (Normand *et al.* 1988, Simonet *et al.*, in prep). These data open the possibility to study such rearrangement in *Frankia*. In this study, DNA was purified from endophyte material (vesicle clusters) from *Frankia* strain ArI3. These DNA preparations were compared to DNA that had been purified from the same pure culture, but grown *in vitro* as hyphae only. Both total DNA restriction enzyme patterns and hybridizations with homologous *nif*-HDK or *nif*-AB genes are compared in hyphae and in vesicles.

Materials and Methods

Strains and media

Frankia strain ArI3 (HFP013003) (Berry and Torrey 1979) was used to inoculate seedlings and to extract DNA from pure cultures. "P+N" medium was described before (Meesters

1987). Infection medium: $CaCl_2$ 55 mg/l; KCl 752 mg/l; $MgSO_4.7H_2O$ 504 mg/l; $NaH_2PO_4.2H_2O$ 198 mg/l; FeEDTA and trace elements according to Allen and Arnon (1955). In some cases $NaNO_3$ (65 mg/l) was added to the medium.

Plant inoculation

Seedlings of *Alnus glutinosa* (L.) Gaertn., type Weerribben (Hahn *et al.* 1988) were grown on perlite in plastic containers at 25 °C, on infection medium. Only the first two weeks, sodium nitrate was added to this medium. After one month, 2400 seedlings were inoculated with *Frankia* strain ArI3 from a 5 l.-culture, grown on "P+N" medium and washed with nitrogen-free infection medium. Using a syringe, 2 ml of this culture was added near the roots of each plantlet. After three months, the nodules were harvested by taking them from the roots using forceps. The nodules were kept on ice for at maximum 1 h, and frozen in liquid nitrogen. The material was kept frozen until use.

DNA purification

DNA from vesicle clusters from root nodules was purified as follows: Portions of 18 g of nodules were homogenized with 100 ml TE8* (Tris 50 mM/EDTA 50 mM/sucrose 0.5 M; pH8) and 0.1% PVP in a Virtis mixer at maximum power, 4 x 1 min., on ice. The homogenate was centrifuged 5 min. 1000xg. Supernatants were stored, the pellets were combined, resuspended in 100 ml TE8* and recentrifuged 5 min. 1000xg. The supernatants of both centrifugation steps were combined and centrifuged 10 min. 22000xg. The pellet, containing most of the vesicle clusters, was washed once with 100 ml TE8* and resuspended in 100 ml TE8 (Tris 50 mM/EDTA 20 mM; pH8) with 0.5 M sucrose. Lysozyme (10 mg/ml) and achromopeptidase (1 mg/ml) were added and the cells were incubated at 37 °C. After 60 min., 10 ml SDS 20 % (w/v) was added and the mixture was incubated 20 min. at 80 °C to complete lysis. DNA was further purified following standard procedures (Maniatis *et al.* 1982)

including phenol extractions, ethanol precipitations and two $CsCl_2$ gradients. The purified DNA from 75 g nodules was finally dissolved in 0.5 ml TE (Tris 10 mM/EDTA 1mM; pH7.5)

DNA gelelectrophoresis and Southern blotting

DNA was digested with restriction enzymes and analyzed on agarose gels and transferred to nitrocellulose filters according to Maniatis *et al.* (1982). Restriction enzymes: Bam HI, Pstl, BglII, HindIII were obtained from Boehringer (Grenoble, France), Sstl was from BRL (Cergy-Pontoise, France). Plasmids pFQ148 and pFQ149, (Simonet *et al.*, in prep) containing *Frankia nif*-genes HDK and AB, respectively, were nick-translated with 50 µCi of 32-P dCTP and used as probes. DNA hybridization reactions were done as described by Simonet *et al.* (1986) using 25 % v/v formamide, at 53 °C. The nitrocellulose filters were washed with 0.2x SSC solution at 65 °C (Maniatis *et al.* 1982). Autoradiograms were made using Kodak X-Omat AR films with intensifying screen, at -80°C.

Results

Nodule production

2400 alder seedlings were inoculated. The yield, three months after inoculation, was 150 g (fresh weight) of nodules. Not inoculated control seedlings were not nodulated and remained small and yellow, indicating nitrogen fixation activity in the nodules of the inoculated plants.

DNA purification and analysis

Endophyte DNA was purified in two portions of 75 g nodules. The final yield was, roughly estimated, 2x 250 µg DNA. DNA from endophyte was digested with several restriction enzymes and compared on agarose gels. Total

DNA patterns did not show clear differences between pure culture and endophyte DNA (data not shown). This indicates that the major part of the endophyte preparation does indeed consist of *Frankia* DNA and not of contaminating plant DNA. The digested DNA was transferred to nitrocellulose filters and hybridized with homologous *nif*-genes. Fig.1 shows hybridization patterns with *nif*-HDK as a probe. The size of the hybridizing bands from pure culture DNA was in accordance with previously obtained results with the cloned DNA fragment from pure culture ArI3 hyphae that was also used as probe. (Simonet *et al.*, in prep). The corresponding bands are indicated with arrows. Fragments that are not indicated were not completely located on the analysed fragment, therefore their size could not be chequed with these data. Some extra bands found are most probably the results of imperfect digestion. The results of hybridization of endophyte DNA with the *nif*-HDK probe did not show any differences in hybridization patterns as compared to pure culture DNA. Fig.2 shows results of hybridization of pure culture DNA and endophyte DNA with homologous *nif*-AB used as a probe. The same results are found as with the *nif*-HDK probe. No differences between pure culture and endophyte DNA can be found in hybridizing bands. Bands as expected

p.99:
Fig 1.
Hybridization patterns of total DNA from ArI3 pure culture (A) and ArI3 endophytic vesicle clusters with *Frankia nif* HDK as probe. Restriction enzymes: 1: BamHI; 2: SstI; 3: PstI; 4: BglII. Arrows: hybridizing bands as expected in pure culture DNA (see text)

Fig 2.
Hybridization patterns of total DNA from ArI3 pure culture (A) and ArI3 endophytic vesicle clusters with *Frankia nif* AB as probe. Restriction enzymes: 1: BamHI; 2: SstI; 3: PstI; 4: BglII. Arrows: hybridizing bands as expected in pure culture DNA (see text)

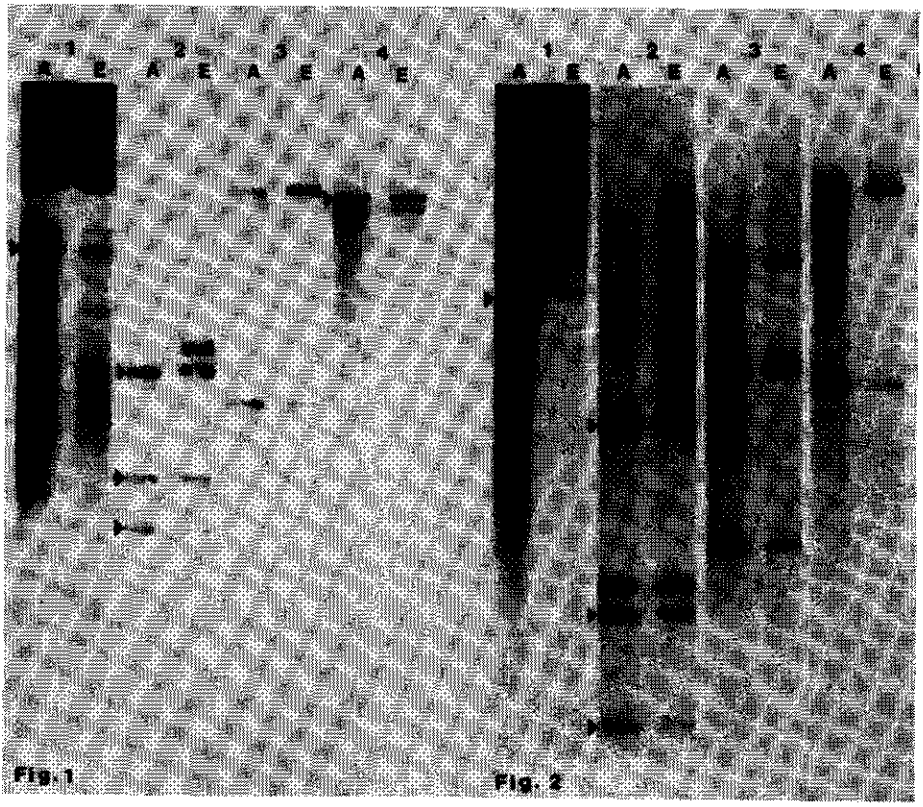

Fig. 1 Fig. 2

from pure culture DNA (Simonet *et al.*, in prep) are indicated with arrows. Thus no major DNA rearrangements are found in *Frankia* endophyte vesicle clusters.

Conclusions and discussion

In this study, a comparison has been made between *Frankia* DNA from vesicles and from hyphae. Up to now, it is not known why the *Frankia* nitrogenase genes are not expressed in hyphae. The possibility of regulation of gene expression by DNA rearrangement was studied because of the functional resemblance of the vesicles with *Anabaena* heterocysts (this thesis chapter IV-3), where the occurrence of DNA rearrangement was shown. In *Anabaena* heterocysts, a fragment of 11 kb is excised between *nif*H and *nif*DK and is found as a circle (Golden *et al.* 1985). No functions of genes on this fragment are known, but it seems that the fragment interrupts the *nif*D coding region, making expression impossible. After rearrangement, *nif*K, *nif*D, and *nif*H are contiguous and can be transcribed under the same operon. This rearrangement is a site-specific recombination of two repeated sequences on both ends of the excised fragment. The involvement of a specific restriction enzyme was suggested but could not be confirmed (Golden *et al.* 1985).

Apart from many examples of aspecific DNA rearrangements as adaptation mechanism in prokaryotes and eukaryotes (*e.g.* insertion sequences, transposons, see: Shapiro (1983)), some other specific DNA rearrangements have been described: In phage Mu, viral DNA can move to different places on the bacterial genome to recombine with host DNA. These recombinations are essential for replication of the virus (Toussaint and Resibois 1983). In *Salmonella*, site-specific DNA recombination can change the flagellar phase, and the serotype, of the cells, which is probably a mechanism to evade immunological defenses of the hosts

(Silverman and Simon 1983). Both specific rearrangements are used to regulate gene expression, as is the case in *Anabaena*.

Like in *Anabaena*, in *Klebsiella* and *Rhizobium*, *nif*-genes K, D and H are contiguous and transcribed under one operon (Golden *et al.* 1985, Dixon 1984, Corbin *et al.* 1983). Recently, it has been shown that also in *Frankia* strain ArI3 the *nif*-genes H, D and K are contiguous (Simonet *et al.*, in prep.). The same was found in four other *Frankia* alder strains (Normand *et al.* 1988). Ligon and Nakas (1987) however found that in *Frankia* strain FaCl *nif*H and *nif*D were not contiguous. For *Bradyrhizobium*, two distinct operons for *nif*H and *nif* DK were found (Fuhrmann and Hennecke 1982). Only in the case of *Rhizobium trifolii* it was reported, that no rearrangement of *nif*-genes could be detected (Scott *et al.* 1984). It should be noted here, that *Rhizobium* bacteroids are different from nitrogen-fixing *Frankia* vesicles or *Anabaena* heterocysts, because all bacteria in legume root nodules differentiate into bacteroids, and no separation of fixing and non-fixing cells is made.

The study presented here was performed to elucidate the possibility of DNA rearrangements involved in differentiation of vesicles and onset of *nif*-genes in the differentiated cells. The results show that no major rearrangements of *nif*-genes play a role in vesicle differentiation in *Frankia* strain ArI3. However, rearrangements of DNA, allowing the transcription of nitrogenase genes, will not necessarily extend to relatively large fragments. Smaller changes in the *nif*-genes cannot be excluded by these results. The possibility of DNA rearrangement has recently been suggested by Dobritsa and Tomashevskii (1988). However, from the results reported here, it can be concluded that major DNA rearrangements are not essential for the repression of expression of *nif*-genes in *Frankia* hyphae.

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VI

Summary

Summary

The actinomycete *Frankia* is a symbiotic nitrogen fixer, living in root nodules of many non-leguminous plants. A typical characteristic of this endophytic organism is the formation of specialized swollen cell structures, called vesicles. *Frankia* vesicles have been brought in relation to nitrogen fixing activity, but many questions about their formation and their function still had to be answered. The present study was done to investigate the function of these vesicles. First, an electronmicroscopy study has been initiated to investigate the ultrastructure of *Frankia* cells. The vesicle envelope was studied in detail, and shown to be a multilaminate structure, which was in accordance with other published results. The vesicle envelope was continuous with the hyphae cell wall. It was also shown that no difference was found in the ultrastructure of vesicles with, or without induced nitrogenase (chapter II). The formation of vesicles in different *Frankia* strains was investigated by comparing a strain with, and one without vesicle formation in the presence of ammonia. In the presence of ammonia, nitrogenase is generally repressed. The vesicles that were formed in the medium with ammonia did not contain nitrogenase (chapter III).

Frankia nitrogenase could be detected by using antisera against nitrogenase from *Rhizobium leguminosarum* or from *Azotobacter vinelandii*. The latter appeared to be very specific, and was used for localization of nitrogenase on ultrathin cryosections. It could be shown that nitrogenase is present uniquely in the vesicles in *Frankia* strains Cc1.17 and EAN1pec (chapters IV-1, IV-2). In these cells, probably a reducing environment is maintained, thus preventing inhibition of nitrogenase by oxygen. In accordance herewith, it has been reported that one *Frankia* strain (CcI3) was able to fix nitrogen without forming

vesicles, under microaerobic conditions. The influence of microaerobic conditions on the localization of nitrogenase in strain Cc1.17 was tested. In this strain, no change of the localization of nitrogenase in the vesicles with reduced oxygen partial pressure has been observed (chapter IV-3). From these results, it can be concluded that nitrogenase is generally located within the vesicles, although vesicle formation and nitrogenase induction can be controlled independently in some strains. The mechanism of regulation of the strict localization is still unknown. One of the possibilities of repression of *nif*-genes is the occurrence of DNA rearrangement during vesicle differentiation. This possibility has been investigated by comparing DNA from vesicle clusters from nitrogen fixing root nodules with DNA from hyphae from the corresponding pure culture. No differences could be detected in the *nif*-genes from these two preparations. The results mean that the question, how *nif*-gene expression is repressed in the hyphae of *Frankia*, still need to be answered. The progress made in the past years in *Frankia* molecular genetics promises that many still open questions about regulation mechanisms in *Frankia* will be answered in the near future.

VII

Samenvatting

Samenvatting

De actinomyceet *Frankia* is een symbiontische stikstofbinder die leeft in wortelknollen van veel niet-vlinderbloemige planten. Typerend voor deze endofyt is het vermogen om gespecialiseerde opgezwollen celstructuren te vormen. Deze gespecialiseerde cellen worden blaasjes (vesicles) genoemd. Deze *Frankia* blaasjes zijn vaak in verband gebracht met de stikstofbindingsaktiviteit. Er bleven echter nog veel vragen over over de vorming en de functie van deze cellen. Dit onderzoek werd uitgevoerd om de functie van deze blaasjes nader te onderzoeken. Er werd een onderzoek gedaan naar de ultrastructuur van *Frankia* cellen. De celwand van de blaasjes werd gedetailleerd bestudeerd. Deze wand bleek een gelaagde structuur te bezitten, wat ook door anderen was gevonden. De celwand van de blaasjes bleek door te lopen als hyfencelwand. Verder werd geen verschil gevonden tussen de ultrastructuur van blaasjes met en zonder het stikstofbindend enzym, nitrogenase (hoofdstuk II). De vorming van blaasjes werd onderzocht aan de hand van twee stammen waarvan de een wel, en de ander geen blaasjes maakt in de aanwezigheid van ammonia. In aanwezigheid van ammonia wordt nitrogenase in het algemeen niet gemaakt. De blaasjes, gevormd in aanwezigheid van ammonia, bleken geen nitrogenase te bevatten (hoofdstuk III).

Het *Frankia* nitrogenase kon worden aangetoond door gebruik te maken van antisera tegen nitrogenase van *Rhizobium leguminosarum* of van *Azotobacter vinelandii*. Het laatste serum bleek zo specifiek te zijn, dat het kon worden gebruikt voor localisatie experimenten met ultradunne vriescoupes. Er kon worden aangetoond dat nitrogenase uitsluitend aanwezig is in de blaasjes van de stammen Ccl.17 en EANlpec, en niet in de hyfen (hoofdstukken IV-1 en IV-2). De functie die men voorstelt voor de localisatie

van nitrogenase in de blaasjes is, dat in deze cellen een reducerende omgeving wordt gehandhaafd, waardoor het nitrogenase niet wordt geinactiveerd door zuurstof. Er is ook geplubiceerd dat één *Frankia* stam in staat was stikstof te binden zonder blaasjes te hebben, maar alleen bij zeer lage zuurstofconcentratie. De invloed van deze zeer lage zuurstofconcentratie op de localisatie van nitrogenase in stam Cc1.17 werd getest. In deze stam bleek de zuurstofconcentratie geen invloed te hebben op de localisatie van nitrogenase in de blaasjes (hoofdstuk IV-3). Uit deze resultaten kon de conclusie worden getrokken dat nitrogenase in het algemeen alleen in blaasjes wordt geinduceerd en niet in hyfen, hoewel in sommige stammen de vorming van blaasjes en de inductie van nitrogenase onafhankelijk kan worden gecontroleerd. Het mechanisme van de regulatie van deze localisatie is nog onbekend. Een mogelijkheid om de expressie van *nif*-genen te onderdrukken in de hyfen en niet in blaasjes, is het herrangschikken van het DNA tijdens de differentiatie van blaasjes. Deze mogelijkheid werd onderzocht door DNA uit blaasjesclusters uit stikstofbindende wortelknollen te vergelijken met DNA uit hyfen van de overeenkomstige reinculture. Er werden geen verschillen gevonden tussen deze twee DNA preparaten. Dat betekent dat de vraag, hoe de *nif*-genen in *Frankia* hyfen worden onderdrukt, nog niet kon worden beantwoord. De vooruitgang die de laatste jaren werd geboekt in de moleculaire genetica van *Frankia* belooft echter dat in de nabije toekomst veel vragen op dit gebied zullen kunnen worden opgelost.

Curriculum vitae

Op 9 januari 1958 ben ik geboren te Velsen. In 1976 werd het einddiploma Gymnasium B behaald op het Mendelcollege te Haarlem en begon ik met de studie Biologie aan de Rijksuniversiteit te Leiden. In 1980 behaalde ik de kandidaatsexamens B1 (Biologie) en B4 (Biochemie). In 1983 werd het doctoraalexamen behaald met als hoofdvak Biochemie en als bijvak Botanische Voedingsfysiologie. In datzelfde jaar ben ik begonnen bij de vakgroep Microbiologie van de Landbouw Universiteit Wageningen, met het promotie-onderzoek waarvan de resultaten beschreven zijn in dit proefschrift. Dit onderzoek werd financieel ondersteund door het BION. Vanaf april 1988 ben ik werkzaam bij de vakgroep Biochemie van de Katholieke Universiteit Nijmegen, in het kader van een door het Nederlands Reumafonds gesubsidieerd onderzoeksproject.

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