ARBON AND NITROGEN METABOLISM OF FREE-LIVING *FRANKIA* SPP. AND OF
RANKIA-ALNUS SYMBIOSES

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CARBON AND NITROGEN METABOLISM OF FREE-LIVING *FRANKIA* SPP. AND OF *FRANKIA-ALNUS* SYMBIOSES

Proefschrift

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STELLINGEN

I

Het verdient aanbeveling dat auteurs van overzichtsartikelen zich beperken tot die gebieden waarop zij zelf werkzaam zijn.

"Root nodule associations occur between organisms other than legumes and *Rhizobium*. (....) It appears that the symbiont is an actinomycete, however, the organism has not been isolated and cultured in the free-living state."
L.Beevers (1981) in: Biology of inorganic Nitrogen and Sulfur. H.Bothe and A.Trebst, Eds. p. 16. Springer Verlag, Berlin, Heidelberg, New York.

II

De conclusie van Kurz en Larue dat "the coincidence of high isocitric dehydrogenase activity with the peak of nitrogen fixation is presumptive evidence that it provides reductant for fixation" wordt niet gesteund door hun experimenten.

W.G.W.Kurz and T.A.Larue (1977) Can.J.Microbiol. 23,1197-1200.

III

De door Matsumoto en medewerkers waargenomen "repressie" van nitraatreductase in komkommerbladeren ten gevolge van calcium-deficiëntie is geen echte repressie, maar veeleer stoppen van de enzym-synthese ten gevolge van het afsterven van de plant.

H.Matsumoto, K.Teraoka and T.Kawasaki (1980) Plant and Cell Physiology 21, 183-191.

IV

De conclusies van Boland en medewerkers betreffende de (sterke) affiniteit voor ammoniak van glutamine-synthetase uit wortelknollen van vlinderbloemige planten, worden niet gesteund door hun waarnemingen.

M.J.Boland, A.M.Fordyce and R.M.Greenwood (1978) Aust.J. Plant Physiol. 5,553-559.

V

Ten onrechte concluderen Schubert en medewerkers uit hun labelling- en pulselabelling-experimenten met $^{13}NH_4^+$, dat in elzeknollen NH_4^+ niet via de GS/GOGAT route geassimileerd wordt.

K.R.Schubert, G.T.Coker III and R.B.Firestone (1981) Plant Physiol. 67,662-665.

VI

Het is aan twijfel onderhevig of de actinomyceet-stammen die volgens Gauthier en medewerkers acetyleen reduceren, werkelijk *Frankia*-stammen zijn.

D.Gauthier, H.G.Diem and Y.Dommergues (1981) Appl. Env. Microbiol. 41,306-308.

VII

Het gebruik van "radio-immuno-assays" ter bepaling van het gehalte aan diethylstilbestrol in de urine van mestvee kan leiden tot "vals-positieve" uitkomsten.

J.Blom (1981) Chem. Weekbl. 77-12, 97
J.Blom (1981) Chem. Weekbl. 77-24, 215.

VIII

In tegenstelling tot de bewering van Brill (1981) werden bacteriën van het geslacht *Rhizobium* voor het eerst geïsoleerd door Beijerinck (1890) en niet door Hellriegel en Wilfarth (1888).

W.J.Brill (1981) Scientific American 245 (3),146-156
H.Hellriegel und H.Wilfarth (1888) Beilageheft zu der Zeitschrift des Vereins f.d. Rübenzucker-Industrie d.D.R. p. 83
M.W.Beijerinck (1918) Proceedings of the Section of Sciences, Kon. Akademie van Wetenschappen, Amsterdam. XXI, 183-192
Index Bergeyana (1966) p. 246. The Williams and Wilkins Company, Baltimore Md. 21202, U.S.A.

IX

Aangezien belastingheffing niet meer uitsluitend wordt gezien als het doen bijdragen van de belastingplichtigen aan de financiering van de traditionele overheidstaken, doch mede als een van de instrumenten waarover de overheid beschikt bij de door haar gewenste herverdeling van de beschikbare middelen, verdient het aanbeveling bepaalde giften van die belastingplichtigen in mindering te brengen op de door hen verschuldigde belasting, in plaats van op hun belastbaar inkomen, voor zover deze giften passen in het herverdelingsbeleid van de overheid.

X

Door medewerking te verlenen aan het zogenaamde "damesschaak", maakt de Koninklijke Nederlandse Schaakbond zich medeplichtig aan discriminatie en achterstelling van de vrouw.

Er is geen einde aan het maken van
veel boeken, en veel doorvorsen is
afmatting voor het lichaam. Van al
het gehoorde is het slotwoord:
Vrees God en onderhoud zijn geboden,
want dit geldt voor alle mensen.
(Pred. 12:12-13)

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Chapter I

INTRODUCTION

I.1 Symbiotic nitrogen fixation

Although nitrogen occurs on earth in large amounts, the growth of many organisms is limited by inadequate supply of the element. This is due to the fact that most organisms cannot utilize atmospheric nitrogen (N_2), but require combined nitrogen as it is incorporated in nitrate, ammonium and amino acids. Only very few organisms can fix N_2, *i.e.* form ammonium from N_2.

Industrial nitrogen fixation, often occurring according to the Haber-Bosch process, proceeds at a N_2-pressure of 200 atmospheres and a temperature of 300°C. It is responsible for the fixation of 30×10^6 metric tons of nitrogen *per annum* (Hardy and Havelka, 1975; Burns and Hardy, 1975). Biological nitrogen fixation proceeds at a N_2-pressure of 0.8 atmospheres and a temperature usually not exceeding 30°C; it produces 175×10^6 metric tons of fixed nitrogen annually.

Nitrogen-fixing organisms have at their disposal a catalytic system *viz.* the enzyme nitrogenase that does not require the extreme conditions of the Haber-Bosch process. Detailed information on the nitrogenase system is given by Orme-Johnson *et al.* (1977) and Emerich *et al.* (1981).

Up till now, nitrogen fixation has been observed to occur only in prokaryotic organisms, either free-living or living in symbiosis with eukaryotes or with other prokaryotes. In the present section, attention will be given to symbiotic nitrogen fixers only.

Symbiotic nitrogen-fixing organisms are divided into three groups of prokaryotic organisms, *viz.* cyanobacteria, rhizobia and actinomycetes. These microorganisms can occur in symbiosis with a number of eukaryotic organisms, either as ectosymbiont or as endosymbiont (Table 1). Associations of cyanobacteria with bacteria do also occur.

Table 1. N_2-fixing symbioses (from Mulder, 1981)

N_2-fixing symbiont	Non-N_2-fixing partner
Rhizobium spp.	Leguminous plants (with a few exceptions)
Actinomycetes of the genus *Frankia*	Non-leguminous plants
Cyanobacteria	Angiospermous plants (*Gunnera* spp.)
	Gymnospermous plants (*Cycas* spp.)
	Liverworts
	Mosses
	Small fresh water ferns (*Azolla* spp.)
	Fungi (lichens)
	Bacteria

In the case of ectosymbioses the N_2-fixing symbiont lives extracellularly, be it in close relation to the host. Of these ectosymbioses especially lichens are known to contribute significantly to the N-input of their ecosystem, particularly in regions having an extremely dry or cold climate; *e.g. Stereocaulon* sp., a lichen occurring in the North-Swedish pine forests (Huss-Danell, 1977).

In the case of endosymbioses the N_2-fixing symbiont is found within the cells of the host. Some primitive forms of endosymbiosis include cyanobacterium associations with phycomycetes, diatoms and some gymnospermous and angiospermous plants. However, the root-nodule symbioses with either rhizobia or actinomycetes as N_2-fixing symbiont are of much wider ecological importance.

The *Rhizobium* symbioses have so far gained much more attention than the actinomycete symbioses. In the following part of this section, a comparison will be made between some aspects of the *Rhizobium*-legume symbioses and those of the actinomycete-non-legume symbioses. Attention will be paid mainly to differences between the two types of symbiosis.

In non-leguminous plants, similar to legumes, infection is preceded by deformation of root hairs (Hiltner, 1903; Pizelle, 1972). The actinomycete (*Frankia* sp.) in the form of hyphae passes through a deformed root hair and penetrates into the cells of the cortex (Angulo, van Dijk and Quispel, 1976; Pommer, 1956; Taubert, 1956). Cortical cells close to the infected cells start to divide, causing a slight swelling of the root. The hyphae penetrate the dividing cells and form clusters of hyphae within the host cells. The hyphae tips at the periphery of the clusters are swelling and develop into spherical vesicles. By invading new dividing cells, a cluster of infected host cells is formed which is called primary nodule. In the neighbourhood of the primary

nodule, a root primordium, initiated within the pericycle, grows into the cortex where it is invaded by the endophyte. This infected root primordium continues to grow and develops into a visible root nodule. Due to dichotomous division of the top meristem, a cluster of lobes is formed (Angulo, 1974; Angulo *et al.*, 1976).

In non-leguminous plants the meristematic activity is thus initially restricted to the immediate vicinity of the site of infection, whereas in leguminous nodules cell divisions spread to other cells, causing the outgrowth of the nodule (Angulo *et al.*, 1976).

It is presumed that in non-leguminous nodules nitrogen fixation takes place in the vesicles, which therefore may be compared with the bacteroids in the nodules of leguminous plants. Evidence as to this presumption is as follows:
(a) When alder root nodules are homogenized and the homogenate is filtered through a 20 μm filter, the distribution between the residue and filtrate of the N_2-ase activity corresponds with the distribution of the numbers of vesicles and of the amount of diamino pimelic acid (an endophyte cell-wall marker) (Akkermans, 1978; Akkermans, Roelofsen and Blom, 1979).
(b) In ineffective nodules (*i.e.* nodules not capable of fixing nitrogen) no vesicle clusters are found (Mian, Bond and Rodriguez-Barrueco, 1976; Baker, Newcomb and Torrey, 1980).
(c) In free-living *Frankia* CpI1 (*cf* Chapter I.2) nitrogen fixation is reported to be attended with the development of vesicles (Tjepkema, Ormerod and Torrey, 1980 and 1981).

In both the *Rhizobium* and the actinomycete symbioses, the microsymbiont obtains sources of carbon and energy from the host. The transport of newly formed photosynthates to the root nodules of *Pisum sativum* plants has been shown by using $^{14}CO_2$ (Bach, Magee and Burris, 1958; Lawrie and Wheeler, 1973). The chemical nature of the carbon and energy source for the nitrogen-fixing microsymbiont is the subject of research for both types of symbiosis.

It has been shown that some citric acid cycle intermediates, *viz.* succinate, fumarate, malate and oxaloacetate, can support respiration of *Rhizobium leguminosarum* bacteroids isolated from root nodules of *Pisum sativum* (Tuzimura and Meguro, 1960; Houwaard, 1979), whereas citrate, glucose and pyruvate did not stimulate respiration (Houwaard, 1979). The uptake of succinate by free-living and bacteroidal forms of *Rhizobium* spp. has been shown by Glenn, Poole and Hudman (1980). Succinate-dependent N_2-fixation was found to occur in bacteroids

from soybean (Bergersen and Turner, 1967) and in bacteroids from pea plants (Houwaard, 1979).

Bacteroids of *Rhizobium japonicum* contain a functional citric acid cycle (Stovall and Cole, 1978) and an Entner-Doudoroff pathway (Keele *et al.*, 1969; Martinez-de Drets and Arias, 1972; Ronson and Primrose, 1979). The presence of the Embden-Meyerhof pathway is disputed (Mulongoy and Elkan, 1977; Martinez-de Drets and Arias, 1972; Ronson and Primrose, 1979).

From the data known so far it is generally assumed that the microsymbiont of the *Rhizobium*-legume symbiosis obtains some dicarboxylic acid, *e.g.* succinic acid from the plant.

Less is known about the physiology of the vesicle clusters in actinomycete symbioses. Akkermans and Roelofsen (1980) found that NADH supports the respiration of vesicle clusters isolated from root nodules of *Alnus glutinosa*. This suggests the presence of an NADH dehydrogenase on the outer side of the membrane surrounding the vesicle clusters, or partial damage of this membrane during the homogenization of the nodules.

Recently, it has been found in our laboratory that vesicle clusters of alder root nodules contain the enzymes isocitrate dehydrogenase, succinate dehydrogenase, fumarase and malate dehydrogenase (Akkermans, Huss-Danell and Roelofsen, Physiol. Plant., in press). The same authors found that addition of malate plus glutamate plus NAD to a suspension of vesicle clusters resulted in increased oxygen consumption, whereas the addition of only one or two of these compounds was ineffective (Huss-Danell, Roelofsen, Akkermans and Meyer, submitted). In addition, they found activity of glutamate-oxaloacetate transaminase in both vesicle clusters of the endophyte and the cytoplasm of the host cells. They suggest that this enzyme, in cooperation with malate dehydrogenase, may function in transporting excess reducing equivalents from the cytoplasm of the host into the vesicle clusters, analogous to the mitochondrial-cytoplasmatic malate-aspartate shuttle (*cf* Lehninger, 1975).

Vesicle clusters probably do not contain a functional glycolysis. Huss-Danell *et al.* (manuscript submitted) found in vesicle clusters only glycolytic enzymes catalyzing reversible reactions (*i.e.* enzymes also functioning in gluconeogenesis) *viz.* aldolase, enolase, 3-phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase, but no enzymes catalyzing the irreversible glycolytic reactions, *viz.* hexokinase, pyruvate kinase and pyruvate dehydrogenase. This suggests that the source of carbon and energy which the microsymbiont in non-leguminous nodules obtains from the plant cannot be a compound which is broken

down through glycolysis. No positive indications with respect to the chemical nature of this compound have so far arisen from investigations with intact nodules or with the microsymbiont (vesicles) isolated from nodules. A possible way to tackle this problem was to investigate the growth requirements of *Frankia* spp. grown *in vitro* (*cf* Chapters I.2 and II).

*I.2 Cultivation of the root nodule endophytes (*Frankia *spp.) of non-leguminous plants.*

Research on the actinomycete symbioses in root nodules of non-leguminous plants was adversely affected until recently by the lack of actively growing pure cultures of the microsymbiont. Though some isolations of these actinomycetes (*Frankia* spp.) have been reported in the past, no reproducible tests were made to confirm their identity with the microsymbiont (Quispel and Burggraaf, 1981); in one instance the isolated strains were lost rapidly after isolation (Pommer, 1959). Only since 1978 the reproducible isolation and cultivation of *Frankia* spp. from root nodules of non-leguminous plants have been reported (Callaham, Torrey and del Tredici, 1978; Lalonde, 1978; Quispel and Tak, 1978; Baker and Torrey, 1979; Baker, Torrey and Kid, 1979; Berry and Torrey, 1979; Lechevalier and Lechevalier, 1979; Quispel, 1979; Baker and Torrey, 1980; Baker, Newcomb and Torrey, 1980). Various isolation techniques have recently been reviewed by Baker and Torrey (1979) and Quispel and Burggraaf (1981). The media used for cultivation of *Frankia* spp. by the above-mentioned authors all have in common a marked complexity. The *Frankia* broth medium (Baker and Torrey, 1979) contains, among other things, yeast extract, dextrose and Casamino acids. The medium of Quispel and Tak (1978) contains peptone, glucose and alcoholic root extracts. The QMOD medium (Lalonde and Calvert, 1979) contains yeast extract, peptone, glucose and a lipid supplement, consisting of 22% pure phosphatidyl choline.

Cell yields and growth rates of the *Frankia* spp. on these media have originally not been determined or at best measured indirectly, using the infectivity of a culture as a device for estimating the density of *Frankia* cells (Quispel and Tak, 1978). However, the cultivation of an organism on defined media and the definite determination of its cell yield and growth rate are prerequisites for ascertaining its growth requirements (*cf* Chapter II of this thesis).

Frankia spp. grown in pure culture are slow-growing (*cf* Chapter II.1), aerobic to microaerophilic actinomycetes, with long, branching, septate hyphae. The hyphal wall is primarily Gram-positive, although short regions of Gram-negative

wall do occur (Berry and Torrey, 1979). The hyphal diameter is variable, ranging from 0.3 to 1.0 µm. Sporangia develop terminally on main hyphae, terminally on short side branches or in an intercalary fashion (Berry and Torrey, 1979).

Up till now little information is available on the physiology and biochemistry of *Frankia* spp..In Chapter II of this thesis research concerning the carbon and nitrogen requirements, the growth rate and growth yield, and some enzyme activities of *Frankia* spp. will be reported.

I.3 Ammonia assimilation in nitrogen-fixing organisms

The ammonia formed from nitrogen fixation is ultimately assimilated into proteins, nucleotides and other nitrogen-containing compounds. The pathways of this assimilation differ for the various nitrogen fixers. Nagatini, Shimizu and Valentine (1971) showed that in the free-living nitrogen fixers *Klebsiella pneumoniae*, *Azotobacter vinelandii*, *Clostridium pasteurianum* and *Rhodospirillum rubrum* the primary ammonia assimilation proceeds *via* the GS/GOGAT pathway:

$$\text{glutamate} + NH_3 + \text{ATP} \xrightarrow{\text{GS}} \text{glutamine} + \text{ADP} + P_i$$

$$\text{glutamine} + \alpha\text{-ketoglutarate} + \text{NAD(P)H} + H^+ \xrightarrow{\text{GOGAT}} 2\ \text{glutamate} + \text{NAD(P)}^+$$

$$\alpha\text{-ketoglutarate} + NH_3 + \text{ATP} + \text{NAD(P)H} + H^+ \longrightarrow \text{glutamate} + \text{ADP} + P_i + \text{NAD(P)}^+$$

From glutamate, the fixed nitrogen can be assimilated into other amino acids through transamination reactions (*e.g.* Meister, 1957).

In free-living nitrogen-fixing blue-green algae (cyanobacteria) an operative GS/GOGAT pathway was also shown (Lea and Miflin, 1975). In these organisms GOGAT is not NAD(P)H-dependent but requires reduced ferredoxin as a source of reducing equivalents.

In *Rhizobium* symbioses, the ammonia formed by the nitrogenase reaction in the bacteroids is excreted into the cytoplasm of the plant cell (Brown and Dilworth, 1975; Robertson, Warburton and Farnden, 1975; Laane *et al.*, 1980) and assimilated into glutamate by the action of the GS/GOGAT pathway (Nagatini *et al.*, 1971; Sloger, 1973; Kennedy, 1973; Dunn and Klucass, 1973; Ryan and Fottrel, 1974). The nodules of most leguminous plants contain glutamine or asparagine as prominent amino acid (*e.g.* van Egeraat, 1972). These mono-amides serve as storage components for nitrogen and in addition represent the compounds in which fixed nitrogen is translocated from the nodules to other parts of the leguminous plants.

In the cyanobacterial symbiosis of a lichen belonging to the genus *Peltigera*, ammonia assimilation is reported to proceed *via* the GDH reaction (Stewart and

owell, 1977; Rai, Rowell and Stewart, 1980):

$$\alpha\text{-ketoglutarate} + NH_4^+ + NAD(P)H \rightleftarrows \text{glutamate} + NADP^+$$

Less information is available as yet with respect to the pathways of mmonia assimilation in actinomycetous root nodules. The pool of free amino cids of alder nodules was shown to differ from that of other types of root odules (Miettienen and Virtanen, 1952; Leaf, Gardner and Bond, 1958; Wheeler nd Bond, 1970). The root nodules of this non-legume contain citrulline as the ost abundant free amino acid; it contributes 20% on molar base (*i.e.* 50% on -base) to the amino acid pool, while glutamine contributes about 10%, and sparagine is not detectable. Other types of non-legume root nodules may possess ignificant quantities of specific amino acids, *e.g.* arginine , ornithine, lutamate or glutamine (Wheeler and Bond, 1970).

No information is as yet available with respect to activities of enzymes esponsible for the formation of these amino acids from NH_4^+ in alder nodules. chubert *et al.* (1981) exposed alder nodules to $[^{13}N]$-NH_4^+ and found incorporation of most of the label into glutamine and glutamate. These observations nd the results of pulse-labelling and inhibitor studies indicate that in alder odules the GS/GOGAT pathway is not operative and that GDH plays a role in the ssimilation of exogenously supplied NH_4^+. Confirmation of this hypothesis by eans of enzyme studies is required. In Chapter III of this thesis the activities of some of the ammonia-assimilating enzymes will be reported.

I.4 Aim and outline of the present study

The aim of the research reported in this thesis was to clarify some aspects f the relation of the host and the N_2-fixing microsymbiont in the *Frankia*-*lnus* symbiosis.

Chapter II deals with the free-living state of *Frankia* AvcI1, the endophyte (microsymbiont) of the root nodules of *Alnus viridis* ssp. *crispa*. The growth requirements of *Frankia* AvcI1 and some physiological research on this organism re reported.

In Chapter III the assimilation of ammonia in root nodules of *Alnus glutinosa* will be dealt with. The activities and localization of some ammonia-assimilating enzymes are reported.

Chapter IV consists of a summary and general discussion of the results reported in this thesis.

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Chapter II

THE CARBON AND NITROGEN METABOLISM OF FREE-LIVING *FRANKIA* SPP.

CHAPTER II.1

GROWTH OF *FRANKIA* AVCI1 ON MEDIA CONTAINING TWEEN 80 AS C-SOURCE

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1. INTRODUCTION

Research on the nitrogen-fixing system in actinomycete-infected nodules of non-leguminous plants has been hindered until recently by the lack of pure cultures of the microsymbiont (1). Only since 1978 the isolation and cultivation *in vitro* of endophytes of some non-legumes have been reported, *viz.* the endophytes of *Comptonia peregrina* (2,3), *Alnus rubra* (4), *Elaeagnus umbellata* (5), *A. viridis* ssp. *crispa* (5), *A. glutinosa* (6), and *Myrica pennsylvanica* (7). Until now, however, growth of these endophytes has only been achieved in complex media, containing peptone or yeast extract (2-8). To obtain a clear insight into the physiology of these endophytes, it is necessary to grow them on a well-defined medium.

Quispel (6) added a petrol-ether lipid extract from plant roots to the growth medium of the endophyte of *A. glutinosa*. Lalonde and Calvert (8) replaced this petrol-ether extract by a lipid supplement containing soybean lecithin. In the present paper it is shown that the complex lipid supplement can be replaced by Tween 80 (polyoxyethylene (20) sorbitan monooleate). It appeared that *Frankia* AvcI1, the nodule endophyte of *A. viridis* ssp. *crispa*, is able to grow on a medium containing Tween 80 as sole carbon source and either NH_4^+ or glutamic acid as sole nitrogen source. The growth rate and growth yield of AvcI1 on various media are reported.

2. MATERIALS AND METHODS

2.1. Organism and growth conditions

Frankia AvcI1, isolated from nodules of *Alnus viridis* ssp. *crispa* was obtained from D. Baker and J.G. Torrey (Harvard University, Petersham, MA 01366). It was grown aerobically at 25°C in non-shaking 100-ml Erlenmeyer flasks containing 40 ml of culture medium. In one experiment anaerobic growth was obtained by placing the Erlenmeyer flasks in an exsiccator containing a GasPak hydrogen + carbondioxide generator envelope (BBL, Cockeysville, MD 21030, USA). The following culture media were used: *Frankia broth* (1), containing yeast extract, glucose, Casamino acids, vitamin B12 and salts. *QMOD medium* (8), containing yeast extract, peptone, glucose, salts and lipid supplement.

QMOD/Tween medium. From the QMOD medium, the lipid supplement is omitted and Tween 80 (Sigma P-1754) is added to a final concentration of 2 g/l.

Tween/cas medium. In the QMOD medium (8) the lipid supplement, glucose, peptone and yeast extract are replaced by Tween 80 (varying concentration), Casamino acids (Difco 479744, varying concentration) and a vitamin mixture containing biotin, folic acid, nicotinic acid, Ca-pantothenate, pyridoxine-HCl, riboflavin and thiaminiumdichloride (final concentration of each vitamin 0.1 mg/l).
Tween/glu medium. In the Tween/cas medium the Casamino acids are replaced by L-glutamic acid (10 mg/l). The final concentration of Tween 80 is 2 g/l.
Tween/NH_4^+ medium. In the Tween/glu medium the glutamic acid is replaced by NH_4Cl (20 mg/l).

2.2. Methods

2.2.1. Inoculation

A 2-week-old culture of *Frankia* AvcI1 was used as inoculum.

Erlenmeyer flasks containing 40 ml of culture medium were inoculated with 2 ml of a washed *Frankia* culture, containing 50 or 100 µg(TOC)cells. The cells were washed 4 times by centrifugation (5 min 1100xg) with sterile phosphate buffer (50 mM K-phosphate, pH 7.0).

2.2.2. Determination of the growth yield

Cells were harvested by centrifugation (5 min 1100xg) and washed 4 times with phosphate buffer (50 mM K-phosphate, pH 7.0). The growth yield of the cells was determined by assaying the content of base-soluble proteins (9) and/or the total organic carbon content, using a Beckman 915A total organic carbon analyzer. Cell suspensions were homogenized by sonication before TOC analysis.

2.2.3. Analysis of the culture supernatants

The amino acid and ammonia contents of culture supernatants were determined by using a Biotronik LC6000E amino acid analyzer. The Tween 80-oleate content of the culture supernatants was determined according to the method of Slijkhuis (to be published). To a proper amount of culture supernatant NaOH was added to a final concentration of 1N. The suspension was heated for 10 min in a boiling water bath to hydrolyze the ester bonds between the sorbitol- and the oleate-residues of the Tween 80 molecules. The suspension was cooled to room temperature and acidified by adding H_2SO_4 to a final concentration of 2N. The E_{620} of the suspension is proportional to the content of free oleate up to 150 mg/l.

2.2.4. Examination of the organism

During and after the experiments the strain we used was examined for known *Frankia*-characteristics in three ways: (1) Macroscopically: the organism grows

in flocks, leaving the culture medium clear (1). (2) Microscopically: the organism forms typical sporangia (1,10). (3) By infectivity tests: after inoculating *A. glutinosa* seedlings with the cultures used in the experiments mentioned in Chapter 3, nitrogen-fixing nodules were formed (quantitative data to be published elsewhere). So there seems little doubt that the organism has maintained its *Frankia* characteristics during the experiments. The cultures were tested for contamination in three ways: (1) Macroscopically (a contaminant will usually cause turbidity of the medium). (2) Microscopically. (3) By plating on yeast-glucose agar.

3. RESULTS

3.1. Course of growth of Frankia *AvcI1*

Erlenmeyer flasks containing 40 ml QMOD/Tween medium were inoculated with 10 µg (TOC) cells. The cells were cultivated at 25°C either aerobically or anaerobically. At different periods of growth, the yield in 4 flasks was determined (Fig. 1).

Growth was observed to continue for 12 days. Aerobic conditions were required for growth.

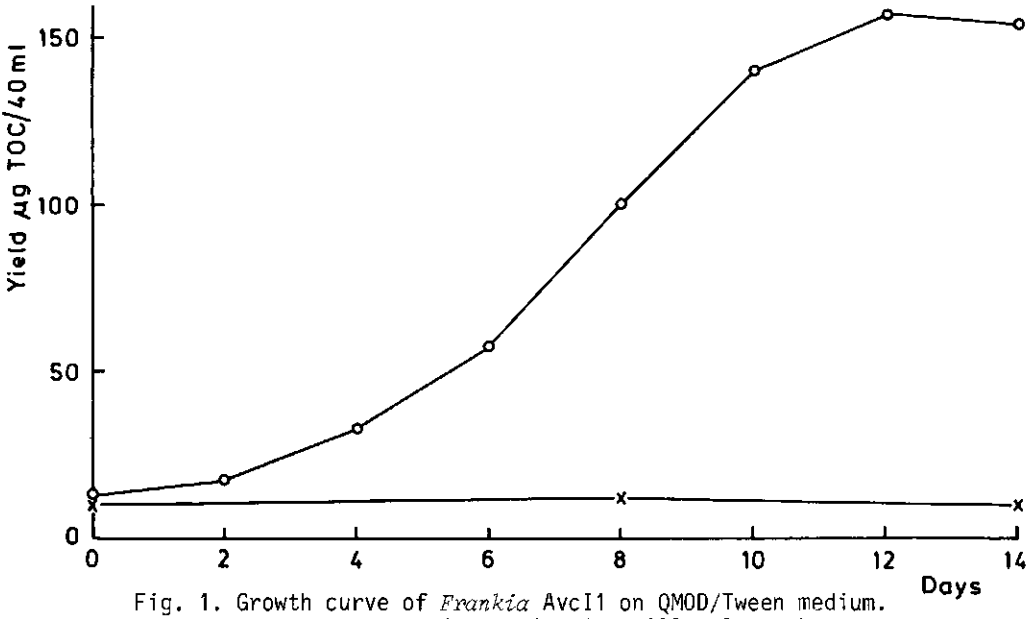

Fig. 1. Growth curve of *Frankia* AvcI1 on QMOD/Tween medium. o-o Aerobic, x-x anaerobic cultivation. All values shown are the averages of 4 determinations.

3.2. Comparison of growth yield on various media

Fifty µg (TOC) *Frankia* AvcI1 cells were used to inoculate Erlenmeyer flasks containing 40 ml of the following media: Frankia broth, QMOD, QMOD/Tween, Tween/cas (concentration of both Tween 80 and Casamino acids 1 g/l), Tween/glu and Tween/NH_4^+.

After cultivation the growth yield (protein and TOC content) of the cells was determined. Since after 12 days of cultivation no further growth was observed (by TOC analysis of the QMOD/Tween medium,visually of the other media), the differences in yield after 14 days of cultivation have not been due to differences in growing time in some media.

In the culture supernatant of cells cultivated on Tween/cas, Tween/glu and Tween/NH_4^+ media, the amount of oleate utilized during growth was determined. In the culture supernatant of cells grown on Tween/glu and Tween/NH_4^+ media, the amount of glutamic acid or NH_4^+ remaining after growth was measured. The results are shown in Table 1.

3.3. Growth of Frankia *AvcI1 on Tween/cas medium with varying concentrations of Tween 80 and Casamino acids*

Erlenmeyer flasks containing 40 ml Tween/cas medium with varying concentrations of Tween 80 and Casamino acids were inoculated with 100 µg (TOC) cells. After cultivation for 14 days at 25°C the total organic carbon content of the cells was measured. In the culture supernatants the amounts of oleate, glutamic acid and aspartic acid utilized during growth were determined. The results are shown in Table 2.

The amino acid content of the media before and after growth was determined. The results are shown in Table 3 for growth on a medium containing 0.2 g Tween 80/l and 0.2 g Casamino acids/l. Similar results were obtained with other combinations of Tween 80 and Casamino acids (not shown).

3.4. Carbon sources not used by Frankia *AvcI1*

We have not observed any growth of *Frankia* AvcI1 on media containing the same components (except Tween 80) as the Tween/cas medium with 1 g/l Casamino acids, after addition of one of the following compounds as C-source: glucose (10 g/l or 1 g/l), malate (1 g/l), succinate (1 g/l), acetate (1 g/l), sorbitol (1 g/l), polyethylene glycol (1 g/l) or K-oleate (1 g/l).

Table 1. Yield of *Frankia* AvcI1 after growth for 14 days on various media[1)]

Medium	C-source (mg/l)		N-source (mg/l)		Yield TOC (mg/l)	Yield protein (mg/l)	Oleate utilized (mg/l)	Glutamic acid or NH_4^+ left (mg/l)
Frankia broth	yeast extract glucose	5000 10000	yeast extract Casamino acids	5000 5000	10.0	-	-	-
QMOD	yeast extract peptone glucose lipid supplement	500 5000 10000	yeast extract peptone	500 5000	11.8	3.8	-	-
QMOD/Tween	yeast extract peptone glucose Tween 80[2)]	500 5000 10000 2000	yeast extract peptone	500 5000	23.5	5.8	-	-
Tween/cas	Tween 80[2)]	1000	Casamino acids	1000	42.5	-	175	
Tween/glu	Tween 80[2)]	2000	glutamic acid	10	5.0	0.7	40	0[3)] (glu)
Tween/NH_4^+	Tween 80[2)]	2000	NH_4Cl	20	9.8	2.8	45	1.1 (NH_4^+)

[1)] All values shown are the averages of 3 determinations. See text for details.

[2)] Tween 80 contains 0.21 g oleate/g Tween 80.

[3)] Detection level of assay 0.015 mg/l.

-, not determined.

Table 2. Growth of *Frankia* AvcI1 on Tween/cas medium with varying concentrations of Tween 80 and Casamino acids[1)]

Concentration in medium		Yield TOC	Oleate utilized	Glutamic acid utilized	Aspartic acid utilized
Tween 80[2)] (mg/l)	Casamino acids[3)] (mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)
0	0	3.3	ND	ND	ND
0	200	5.5	ND	1.9	1.0
0	1000	4.8	ND	11.0	4.5
200	0	7.5	40	ND	ND
200	200	17.0	120	29	11
200	1000	21.8	140	64	28
1000	0	9.0	60	ND	ND
1000	200	21.0	125	-	-
1000	1000	42.5	175	74	36

1) All values shown are the averages of 3 determinations.

2) Tween 80 contains 0,21 g oleate/g Tween 80.

3) Casamino acids contain 152 mg glu/g Casamino acids and 61 mg asp/g Casamino acids.

ND, not detected, -, not determined.

For details see text.

Table 3. Amino acid content of Tween/cas medium (0.2 g Tween 80/l and 0.2 g Casamino acids/l) before and after growth for 14 days of *Frankia* AvcI1

Amino acid	Concentration before growth (nmol/ml)	Concentration after growth (nmol/ml)
Glu	200	3
Lys	98	102
Leu	95	91
Asp	89	5
Val	84	91
Ser	80	77
Ala	74	72
Thr	59	59
Ile	52	51
Gly	47	44
Arg	36	36
Phe	35	35
His	25	26
Met	16	21
Tyr	6	6
Sum	996	719

DISCUSSION

From the growth curve presented in section 3.1 it can be concluded that the endophyte grows on QMOD/Tween medium with a doubling time of about 2 days, which is slow, even for an actinomycete. It must be pointed out, however, that growth of the endophyte in the alder nodule is slow as well (van Ham, personal communication), so that it is likely that the slow growth is a feature of the organism itself, rather than that it is due to a suboptimal medium.

Until now, growth yield of the isolated microsymbiont of actinomycete-infected nodules has only been measured indirectly, *i.e.* visually (1-5,7,8) or based on the infective capacity of endophyte suspensions (6,10). The experiments reported in the present paper give a direct measurement of the growth yield, based on the total organic carbon yield of the endophyte. Using this method one can compare the growth on various media quantitatively.

Growth of the endophyte has until now only been brought about on poorly defined media, *i.e.* media containing yeast extract or peptone. The growth yield reported here on a well-defined medium like the Tween/cas medium or the Tween/NH_4^+ medium is not outdistanced by the growth yield on a poorly-defined medium like *Frankia* broth or QMOD (section 3.2).

The poor growth on the Tween/glu medium is due to nitrogen limitation, as can be easily concluded from comparing the yield on Tween/glu and Tween/cas media (sections 3.2 and 3.2). It must be pointed out that after this nitrogen-limited growth the ratio of the protein yield to the TOC yield is low compared to this ratio after growth on other media (Table 1).

From the results presented here it can be concluded that free-living *Frankia* AvcI1 can use NH_4^+ as well as glutamic acid as sole nitrogen source. The uptake of NH_4^+ by *Frankia* AvcI1, growing on the Tween/NH_4^+ medium was confirmed in an additional experiment (to be published), in which ^{15}N-enrichment of the cells was observed after cultivation in a medium containing ^{15}N-NH_4Cl.

During growth on the Tween/cas medium (section 3.3) the endophyte uses not only oleate, but glutamic acid and aspartic acid as well. The possibility cannot be ruled out that these amino acids are used as carbon source, although this is less likely since *Frankia* AvcI1 is able to grow on a medium containing Tween 80 as sole carbon source and NH_4^+ as sole nitrogen source. Comparison of the yield on this medium with the yield on the Tween/cas medium shows that the yield in both media is proportional to the quantity of oleate utilized during growth (Table 1).

It can be concluded from Table 3 that *Frankia* AvcI1 growing on the Tween/ca- medium, of all the available amino acids, utilizes only glutamic acid and aspartic acid, leaving the concentrations of the other detectable amino acids unchanged.

Since it is clear from the experiments reported here that *Frankia* AvcI1 is able to grow on a medium containing Tween 80 as sole carbon source, the question arises, which part of the Tween 80 molecule is degraded by this organism. Although there is a significant drop in the amount of oleate residues in the medium during growth, it cannot be excluded that other parts of the molecule (*i.e.* the polyethyleneglycol or the sorbitan residues) are degraded as well. This is not likely, however, since we have not been able to observe any growth on media containing polyethyleneglycol or sorbitan as sole carbon source (section 3.4). The failure to observe any growth on a medium containing K-oleate as sole carbon source (section 3.4) might be due to a poisoning effect of free oleate (to be published elsewhere).

It remains an open question what is the carbon source for the endophyte growing in the nodule. Based on the results presented in this paper it seems opportune to look for this compound in the class of lipids.

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HAPTER II.2

TILIZATION OF FATTY ACIDS AND NH_4^+ BY *FRANKIA* AVCI1

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1. INTRODUCTION

In a previous communication (1), a description was given of the *in vitro* ultivation of the nodule endophyte (*Frankia* AvcI1) of *Alnus viridis* spp. *crispa* n a well-defined medium. It was shown that *Frankia* AvcI1 is able to grow on a edium containing Tween 80 as sole carbon source and either Casamino acids, lutamic acid or NH_4Cl as sole nitrogen source. It was not clear, however, which art of the Tween 80 molecule was utilized by *Frankia* AvcI1. In the present paper t is shown that *Frankia* AvcI1 can use other Tweens and fatty acids. The uptake y the cells of $^{15}NH_4^+$ from the medium is demonstrated, and the dependence of the rowth yield on the concentration of NH_4Cl in the medium is shown.

2. MATERIALS AND METHODS

The *Frankia* strain AvcI1, was obtained from D. Baker and J.G. Torrey (Harvard niversity, Petersham, MA 01366). The organism was grown in a medium containing arbon and nitrogen sources as indicated, and salts, trace elements and vitamins s described earlier (1).

Inoculation of the media, cultivation of the organism, washing and collecting of the cells and determination of the total organic carbon and protein contents were described previously (1).

^{15}N-enrichment of the cells was determined according to Akkermans (2); calculations were performed according to Ferraris and Proksch (3). For determination of the total-nitrogen content the cells were digested according to the Kjeldahl method, followed by distillation of the NH_4^+ formed and determination of ammonia with Nessler's reagent.

3. RESULTS

3.1. Comparison of growth yield of Frankia *AvcI1 on media containing various carbon sources*

Fifty µg (TOC) *Frankia* AvcI1 cells were used to inoculate Erlenmeyer flasks containing 40 ml of culture medium with various carbon sources and NH_4Cl (60 mg/l) as nitrogen source. After 20 days of cultivation the TOC and protein contents of the cells were determined (Table 1). No more growth was observed after cultivation for more than 20 days (not shown).

Table 1. Growth yield of *Frankia* cells after cultivation for 20 days on media containing various carbon sources [1]

C source		mg/l	Yield	
			TOC (mg/l)	Protein (mg/l)
Na-acetate	C_2	200	5.1 - 6.1	2.3 - 2.8
Na-propionate	C_3	200	12.5 - 16.2	7.0 - 9.9
Na-butyrate	C_4	200	8.8 - 9.5	3.9 - 5.6
Valeric acid	C_5	200	3.0 - 4.2	1.0 - 2.1
Caproic acid	C_6	200	4.9 - 5.9	2.6 - 3.0
Caprylic acid	C_8	200	13.1 - 14.4	9.5 - 10.5
Capric acid	C_{10}	200	7.5 - 9.0	4.9 - 5.9
Palmitic acid	C_{16}	200	+ [2]	+ [2]
Stearic acid	C_{18}	200	+ [2]	+ [2]
Oleic acid	C_{18-9}	200	0	0
Tween 20 [3]		1000	2.9 - 4.0	1.5 - 2.3
Tween 40 [3]		1000	14.0 - 15.9	10.8 - 12.9
Tween 60 [3]		1000	7.6 - 8.8	5.0 - 5.9
Tween 80 [3]		1000	10.3 - 11.9	5.8 - 6.6
Tween 85 [3]		1000	11.6 - 12.6	9.9 - 10.6

[1] Values shown are the extremes of 4 determinations

[2] +: Growth observed visually. See discussion

[3] Tween 20, 40, 60 and 80 are the polyethyleneglycol sorbitan esters of lauric, palmitic, stearic and oleic acids, respectively. Tween 85 consists of 3 oleic acid residues esterified with polyethyleneglycol sorbitan.

NH_4Cl (60 mg/l) was used as nitrogen source.

3.2. Carbon sources not utilized by Frankia *AvcI1*

No growth of *Frankia* AvcI1 was observed on media containing NH_4Cl (60 mg/l) as nitrogen source, and the following compounds as carbon source: methyl-oleate (200 mg/l), tributyrin (500 mg/l), tricaprin (500 mg/l), triundecylin (500 mg/l), tritridecylin (500 mg/l), gallic acid (250 mg/l), protocatechuic acid (250 mg/l), vanillic acid (250 mg/l), cholesterol (1000 mg/l) and tannin (1000 mg/l).

3.3. Uptake of ^{15}N-enriched NH_4^+ by Frankia *AvcI1*

Three Erlenmeyer flasks containing 250 ml of culture medium with Tween 80 (1000 mg/l) and NH_4Cl (10 mg/l, ^{15}N-enrichment 51 atom - % excess) were inoculated with a suspension of *Frankia* AvcI1 cells (50 µg TOC, 7 µg total nitrogen). After cultivation for 3 weeks the cells were collected and washed 5 times with phosphate buffer (50 mM K-phosphate; pH 7.0). The total organic carbon content, the total nitrogen content, and the ^{15}N-enrichment of the cells were determined

(Table 2). No more growth was observed after cultivation for more than 3 weeks (not shown).

Table 2. Uptake of ^{15}N-NH_4 by *Frankia* cells during growth for 3 weeks

No of batch	Yield (TOC) (mg/l)	Total N content of cells(mg/l)	Atom-% ^{15}N excess
1	5.8	0.480	35.1
2	7.0	0.680	38.9
3	6.2	0.630	36.3

Tween 80 (1 g/l) was used as carbon source

3.4. Comparison of growth yield of Frankia *AvcI1 on media varying in NH_4Cl concentration*

Fifty µg (TOC) *Frankia* AvcI1 cells were used to inoculate Erlenmeyer flasks containing 40 ml of culture medium with Tween 80 (1000 mg/l) and NH_4Cl (varying concentrations). After 20 days of cultivation the cells were collected and washed, and the total organic carbon and the total nitrogen contents of the cells were determined (Table 3). The pH of the media did not decrease below 6.5 during growth. No more growth was observed after cultivation for more than 20 days (not shown).

Table 3. Dependence of yield of *Frankia* on the NH_4^+ concentration of the culture medium after cultivation for 20 days [1)]

NH_4Cl concentration of culture medium (mg/l)	Yield		C/N ratio of cells
	TOC (mg/l)	Total N (mg/l)	
0	5.3	0.6	9
10	6.8	0.7	10
20	9.6	1.1	9
50	10.6	1.7	6
100	15.6	2.3	7
200	18.1	2.7	7
500	13.4	2.3	6
1000	10.9	1.4	8

[1)] The values shown are the averages of 4 determinations

Tween 80 (1 g/l) was used as carbon source.

4. DISCUSSION

From the data shown in Table 1, it can be concluded that *Frankia* can use a number of free fatty acids and some Tweens as sole carbon source. This conclusion is in agreement with the assumption (1) that *Frankia* can use the oleate residues of Tween 80 molecules as sole carbon source. Obviously, valeric acid and Tween 20 are very poor carbon sources, whereas Na-propionate, caproic acid and Tween 40 are relatively good carbon sources. The differences in yield on these media indicate differences in transport and/or metabolism of the carbon sources.

In all media, the TOC yield of the cells is small compared to the amount of carbon offered in the medium. This indicates that some other component of the medium is present in limiting concentration.

The yield on media containing palmitic acid and stearic acid was not determined quantitatively because of the poor solubility of these compounds. However, growth could be observed visually. No growth was observed on media containing oleic acid. This might be due to a poisoning effect of free oleate (1).

By comparing Table 1 with Chapter 3.2 it appears that *Frankia* can use simple fatty acids esterified with polyoxyethylene sorbitan as sole carbon source, but no complex organic acids (like gallic acid, protocatechuic acid and vanillic acid) or fatty acids esterified with glycerol (like tributyrin, tricaprin, triundecylin and tritridecylin).

It is not clear which carbon source *Frankia*, when living in the nodule, obtains from the plant. If the requirements of the endophyte are comparable to those of *Frankia* in pure culture, this carbon source might be a fatty acid, either free or esterified.

From the data shown in Table 2 it can be concluded that NH_4^+ is taken up by *Frankia* cells. That the enrichment of the nitrogen in the cells does not reach 51%, can in part be ascribed to dilution of the label by the nitrogen of the inoculum (28 µg N/l) and by the nitrogen in the vitamins of the medium (700 µg vitamins/l, approx. 12% nitrogen).

From the data shown in Table 3, it can be concluded that the yield of *Frankia* is dependent on the concentration of the NH_4Cl in the medium. There is an optimum concentration between 100 and 500 mg NH_4Cl/l. If the NH_4Cl concentration exceeds this optimum, the yield decreases, probably due to some toxic effect of excess NH_4^+. The C/N ratio of the cells seems to be little affected by the C/N ratio of the medium.

The amount of NH_4^+ taken up is small compared to the amount of NH_4^+ offered in the medium. This suggests that some other component of the medium is present in limiting concentration. The nature of this component remains to be clarified.

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CHAPTER II.3

METABOLIC PATHWAYS FOR GLUCONEOGENESIS AND ENERGY GENERATION IN *FRANKIA* AVCI1

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INTRODUCTION

In previous communications (1,2), a description was given of the cultivation on defined media of *Frankia* AvcI1, an actinomycete giving rise to the formation of nitrogen-fixing nodules on roots of *Alnus viridis* spp. *crispa*. It was shown that *Frankia* AvcI1 can use some free fatty acids and the fatty acid residues of some Tweens as sole source of carbon and energy, and NH_4Cl as sole source of nitrogen (1,2). No growth was observed on media containing glucose as sole carbon source (1). However, it is not excluded that in the QMOD/Tween medium, which contains both glucose (10 g/l) and Tween 80 (1 g/l), *Frankia* does utilize glucose. In the present investigation the uptake of glucose from a medium containing Tween 80 and glucose has been studied.

No quantitative information is yet available about the metabolic pathways for biosynthesis and energy generation used by *Frankia* when growing in pure culture. In this paper, studies of the metabolism of *Frankia* on the enzyme level will be reported.

MATERIALS AND METHODS

The *Frankia* strain AvcI1, was obtained from D. Baker and J.G. Torrey (Harvard University, Petersham MA 01366). The organism was cultivated for 10 days on either QMOD/Tween or Tween/NH_4^+ (60 mg NH_4Cl/l medium)at 25°C (1). The *Arthrobacter simplex* strain AC4 was obtained from J. Antheunisse (this laboratory) and grown on yeast/glucose medium, containing 7 g yeast extract and 10 g glucose/l. [^{14}C]-glucose (uniformly labelled, 336 mCi/mmole) was purchased from the Radiochemical Centre, Amersham, England. Cell-free extracts of *Frankia* cells were prepared by washing cells 3 times with an appropriate buffer, followed by sonication for 2 min (Branson sonifier B-12), and centrifugation for 10 min at 10,000 x g to remove the cell debris. In the cell-free extracts activities of the following enzymes were assayed: isocitrate lyase EC 4.1.3.1 (3); malate synthase EC 4.1.3.2 (3); malic enzyme EC 1.1.1.40 (4); pyruvate orthophosphate dikinase EC 2.7.9.1 (5); phosphoglycerate kinase EC 2.7.2.3 (6); 3-phosphoglyceraldehyde dehydrogenase EC 1.2.1.12 (7); isocitrate dehydrogenase EC 1.1.1.42 (8); α-ketoglutarate dehydrogenase analogous to other dehydrogenase assays (9) modified as follows: The assay mixture contained 150 µmoles K-phosphate pH 7.1, 0.9 µmoles phenazine metasulphate, 15 µmoles MTT (3-(4,5-dimethyl thiazolyl-2)-2,4-diphenyltetrazolium bromide),0.9 µmoles NAD, 0.5 µmoles thiamine

pyrophosphate, 10 µmoles $MgCl_2$, 150 µmoles α-ketoglutarate, cell-free extract and water to a final volume of 3 ml. After incubation, the formazan formed was precipitated by centrigugation (5 min 3000 x g) and dissolved in 3 ml dimethylformamide. The extinction at 550 nm of this solution is proportional to the amount of formazan formed (ε_{550} 1.6 x 10^{-4} $M^{-1}.cm^{-1}$). Succinyl-CoA synthetase EC 6.2.1.5 (modified from Cha, 10). The assay mixture contained 25 µmoles Tris HCl,pH 8.0, 12.5 µmoles $MgCl_2$, 1 µmole CoASH, 4 µmoles ATP, 50 µmoles K-succinate, cell-free extract and water to a final volume of 1 ml. After incubation the free CoASH was determined according to Ellman (11). Succinate dehydrogenase EC 1.3.99.1 (ref. 12, spectrophotometric adaptation with DCPIP); fumarase EC 4.2.1.2 (13); malate dehydrogenase EC 1.1.1.37 (14). Protein content of cell-free extracts was determined according to Tsuyoshi Ohnishi and Barr (15).

RESULTS

Comparison of glucose uptake by Frankia *AvcI1 and* Arthrobacter simplex. Fifty mg (total organic carbon) of cells of *Frankia* AvcI1 grown on QMOD/Tween medium and 10 mg(TOC)of cells of *Arthrobacter simplex* AC4 were incubated separately in 10 ml Tween/NH_4^+ medium to which [^{14}C]-glucose was added (final concentration of glucose 1 mM). After incubation for 6 h the suspensions were centrifuged for 20 min at 10,000 x g, and the radioactivity in the supernatants was determined (Table 1).

Table 1. Comparison of glucose uptake by *Frankia* AvcI1 cells and *Arthrobacter simplex* cells

Organism	Label added to incubation mixture (cpm) [1]	Label recovered in supernatant after incubation for 6 h (cpm) [1]
Frankia AvcI1	1,66 x 10^6	1,65 x 10^6
Arthrobacter simplex strain AC4	1,33 x 10^6	0,08 x 10^6

[1] Values denoted are the averages of 5 determinations which did not deviate more than 1%.

Activities of biosynthetic enzymes in cell-free extracts of Frankia *AvcI1.* Cell-free extracts were made of *Frankia* cells grown on QMOD/Tween and Tween/NH_4^+ media, respectively. These extracts, adjusted to 1.0 mg protein/ml, were tested for the activities of isocitrate lyase, malate synthase, malic enzyme, pyruvate

orthophosphate dikinase, phosphoglycerate kinase and 3-phosphoglyceraldehyde dehydrogenase (Table 2)

Table 2. Activities of enzymes involved in gluconeogenesis of *Frankia* AvcI1, assayed in cell-free extracts of this organism, grown on QMOD/Tween and Tween/NH_4^+ media[1)]

Enzyme \ Growth medium	QMOD/Tween (containing both glucose and Tween 80)	Tween/NH_4^+ (containing Tween 80 as sole C-source)
Isocitrate lyase	70	100
Malate synthase	70	45
Malic enzyme	250	250
Pyruvate orthophosphate dikinase	10	15
Phosphoglycerate kinase	80	50
3-Phosphoglyceraldehyde dehydrogenase	50	30

1) Values, expressed as nmoles.min^{-1}.mg $protein^{-1}$, are averages of at least 3 determinations.

Activities of citric acid cycle enzymes in cell-free extracts of Frankia *AvcI1.* Cell-free extracts were made of *Frankia* AvcI1 cells grown on QMOD/Tween and Tween/NH_4^+ media. These cell-free extracts, adjusted to 1.0 mg protein/ml, were tested for the activities of citric acid cycle enzymes (Table 3).

Table 3. Activities of citric acid cycle enzymes in cell-free extracts of *Frankia* AvcI1 grown on QMOD/Tween and Tween/NH_4^+ media[1)]

Enzyme \ Growth medium	QMOD/Tween (containing both glucose and Tween 80)	Tween/NH_4^+ (containing Tween 80 as sole C-source)
Isocitrate dehydrogenase	195	210
α-Ketoglutarate dehydrogenase	20	15
Succinyl-CoA synthetase	1	1
Succinate dehydrogenase	20	20
Fumarase	700	650
Malate dehydrogenase	2500	3000

1) Values, expressed as nmoles.min^{-1}.mg $protein^{-1}$, are averages of at least 3 determinations.

DISCUSSION

From the data shown in Table 1 it is clear that glucose uptake by *Frankia* cells is less than the detection level of the assay, while the glucose uptake by *Arthrobacter* cells demonstrates that this is not due to a technical failure. If the detection level of the counting is 1%, and the doubling time of *Frankia* AvcI1 is 48 hours (1), it appears that *Frankia* AvcI1 forms less than 0.1% of its cell material from glucose. It may thus be concluded that the uptake of glucose from the QMOD/Tween medium by *Frankia* AvcI1 can be neglected.

In agreement with this conclusion, no activity could be detected of the glycolytic enzymes hexokinase, pyruvate kinase and pyruvate dehydrogenase in cell-free extracts of *Frankia* cells grown on QMOD/Tween medium (not shown). Furthermore, it was found that the activities of isocitrate lyase and malate synthase, being enzymes involved in gluconeogenesis, are of the same order of magnitude in cells grown on QMOD/Tween medium as in cells grown on Tween/NH_4^+ medium (Table 2). In most organisms studied so far, the expression of isocitrate lyase and malate synthase is inhibited by the presence of glucose in the growth medium (16,17). It is not surprising that this effect did not occur in *Frankia* (Table 2) since no glucose uptake by *Frankia* was found (Table 1).

A similar preference of a fatty acid over glucose was shown to occur in the fungus *Aspergillus nidulans* (18). This organism selectively uses acetate when growing on a medium containing both this fatty acid and glucose. When *A. nidulans* was grown on a medium containing acetate as sole carbon source, the isocitrate lyase activity of the fungus was reported to be equal to that of cells grown on a medium containing both acetate and glucose (19). Most of the known bacteria of the genus *Acinetobacter* also can utilize fatty acids, but not glucose (20).

Organisms growing on fatty acids, and degrading these compounds to acetyl-CoA, start gluconeogenesis with the action of the glyoxylate cycle (21). *Frankia* AvcI1 contains the two key enzymes of this cycle, *i.e.* isocitrate lyase and malate synthase (Table 2). The succinate formed in the glyoxylate cycle can be converted to pyruvate by the subsequent action of succinate dehydrogenase, fumarase and malic enzyme. Pyruvate can be converted to phosphoenolpyruvate by the action of pyruvate orthophosphate dikinase. The enzymes phosphoglycerate kinase and 3-phosphoglyceraldehyde dehydrogenase are thought to have a biosynthetic function, since glucose is not taken up by *Frankia* (Table 1).

For energy generation, *Frankia* can oxidize acetyl-CoA, formed from fatty acids, in the citric acid cycle (Table 3). Of the enzymes of this cycle only

citrate synthase and aconitase have not been determined in cell-free extracts of *Frankia*, but these two enzymes are assumed to be functioning in an organism containing isocitrate lyase and isocitrate dehydrogenase. The activity of succinyl-CoA synthetase is low as compared to the activity of the other citric acid cycle enzymes.

The metabolic activities of *Frankia* spp. growing in pure culture are likely to differ from the activities of *Frankia* spp. occurring as vesicle clusters in root nodules of alder. In the former case, much biosynthetic activity is required, whereas in the latter case there is almost no growth, but energy generation is required, among others, for nitrogen fixation. In accordance with this assumption, Akkermans *et al.* detected activity of citric acid cycle enzymes in the endophyte fraction (*i.e.* the vesicle clusters) of alder root nodules (22), but no activity of glyoxylate cycle enzymes (23).

The nature of the carbon source(s) that the endophyte obtains from the plant still needs to be clarified. Based on the results presented in this paper, it is unlikely that glucose is one of these carbon sources.

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CHAPTER II.4

CARBON AND NITROGEN SOURCE REQUIREMENTS OF *FRANKIA* STRAINS

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INTRODUCTION

The isolation and *in vitro* cultivation of a *Frankia* sp. *viz. Frankia* CpI1, an actinomycete that gives rise to the formation of nitrogen-fixing root nodules on *Comptonia peregrina* was reported by Callaham, Torrey and del Tredici in 1978 (1). Since that time a number of other *Frankia* strains has been isolated from root nodules of various non-leguminous plants, *viz. Alnus glutinosa* (2,3), *Alnus rubra* (4), *Alnus viridis* ssp. *crispa* (5,6), *Myrica penssylvanica* (7), *Elaeagnus umbellata* (8,9), *Casuarina equisetifolia* (10), *Hippophaë* spp. (11) and *Shepherdia* spp. (11). Some research has been done in this laboratory on the C- and N-metabolism of *Frankia* AvcI1, isolated from root nodules of *Alnus viridis* ssp. *crispa* (12,13,14). It was shown that *Frankia* AvcI1 can utilize a number of fatty acids and the fatty acid residues of some Tweens as sole carbon source (13, 12, respectively). No growth of *Frankia* AvcI1 was observed on media containing glucose as sole carbon source, while the organism does not take up glucose from a medium containing both Tween 80 and glucose (12,14). In the present contribution it was investigated whether other *Frankia* strains behave similarly to *Frankia* AvcI1 regarding their nutrional demands.

Further investigations concerning some of the above-mentioned *Frankia* strains include the ability of these actinomycetes to form root nodules on *Alnus glutinosa*.

Earlier it was shown (12) that *Frankia* AvcI1 cannot utilize succinate as sole carbon source. This phenomenon is due to the fact that no uptake of this compound occurs as shown in the present paper. Also some additional work has been done on the nitrogen metabolism of *Frankia* AvcI1. In earlier papers it was shown that free-living *Frankia* AvcI1 can utilize either NH_4^+ (13) or Casamino acids (12) as N-source. Glutamic acid and aspartic acid were found to be preferably taken up from a mixture of amino acids, whereas the concentrations of the other amino acids remained unchanged during the growth of the organism (12). Some more details on the utilization of amino acids by *Frankia* AvcI1 are reported in the present work.

MATERIALS AND METHODS

The *Frankia* strains AvcI1(isolated from root nodules of *Alnus viridis* ssp. *crispa*)(5,6) and CpI1 (isolated from root nodules of *Comptonia peregrina*)(1) were obtained from D. Baker and J.G. Torrey (Petersham MA 01366). The *Frankia* strain AgSp+1 (isolated from spore(+)-type nodules of *Alnus glutinosa*)(15) was

obtained from A.J.P. Burggraaf (Leiden, the Netherlands). The media for cultivation of the strains contained C- and N-sources as indicated and the following basal nutrients (mg/l) K_2HPO_4, 300; NaH_2PO_4, 200; KCl, 200; $MgSO_4.7H_2O$, 200; $CaCO_3$, 100; Fe-citrate, 10; $ZnSO_4$, 1; riboflavin, 0.1; thiaminium dichloride, 0.1; biotin, 0.1; nicotinic acid, 0.1; pyridoxine HCl, 0.1; Ca-pantothenate, 0.1; folic acid, 0.1; H_3BO_3, 1.5; $MnSO_4.7H_2O$, 0.8; $CuSO_4.7H_2O$, 0.1; $(NH_4)_6MoO_{24}$, 0.2; $CoSO_4.7H_2O$, 0.01. The cells were grown and the yield was determined as described earlier (12,13). Infectivity of the strains was tested by counting the nodules formed on the roots of *Alnus glutinosa* 4 weeks after inoculation by immersing the roots for 1 min in a *Frankia* culture (20 mg TOC cells/l). Roots of control plants were immersed in sterile water. Plants were grown in perlite (Houwers, Roelofsen and Akkermans, in preparation).

Succinate and acetate were determined gaschromatographically as described in (16) and (17), respectively. Cell-free extracts were prepared as reported in (10). Isocitrate lyase (ICL, EC 4.1.3.1) and malate synthase (MS, EC 4.1.3.2) activities were determined according to (18); isocitrate dehydrogenase (IDH, EC 1.1.1.42) according to (19), and malate dehydrogenase (MDH, EC 1.1.1.37) according to (20). The amino acid content of the growth media before and after growth was determined as described previously (12).

RESULTS

Growth yield of some Frankia *strains on media containing various C- and N-sources*

Media containing various C- and N-sources were inoculated with three *Frankia* strains. The inoculation density was 0.8 mg TOC cells/l. After 20 days of cultivation the cells were harvested and the TOC content of the cells was determined (Table 1). No further growth was observed after cultivation for more than 20 days (not shown).

Infectivity of various Frankia *strains on* Alnus glutinosa *plants*

The infectivity of *Frankia* AvcI1, *Frankia* CpI1 and *Frankia* AgSp+1 on *Alnus glutinosa* plants was tested by inoculating fifteen plants with each *Frankia* strain. Four weeks after inoculation the root nodules on the plants were counted (Table 2).

The effect of acetate, propionate and succinate on Frankia *AvcI1 cells*

Media containing either acetate (60 mg/l), propionate (60 mg/l), succinate (50 mg/l) or acetate plus succinate as C-sources and NH_4Cl (60 mg/l) as N-source

Table 1. Growth yield of *Frankia* AvcI1, *Frankia* CpI1, and *Frankia* AgSp+1 after a 20-day cultivation period on media with various C- and N-sources[1)]

C-source (mg/l)		N-source (mg/l)		Yield (mg TOC/l)		
				Frankia AvcI1	*Frankia* CpI1	*Frankia* AgSp+1
Tween 80	1000	NH_4Cl	100	13.5 - 17.0	18.2 - 21.2	3.0 - 5.2
Glucose	200	NH_4Cl	100	0.2 - 0.9	0.2 - 0.5	0.3 - 0.4
Na-acetate	200	NH_4Cl	100	4.0 - 5.0	6.9 - 7.2	2.9 - 3.2
Na-lactate	200	NH_4Cl	100	0.1 - 0.5	0.0 - 0.5	0.2 - 0.4
Na-succinate	200	NH_4Cl	100	0.2 - 0.6	0.3 - 0.6	0.1 - 0.7
Ethanol	200	NH_4Cl	100	-0.3 - 0.2	-0.1 - 0.3	0.2 - 0.4
Tween 80	1000	Casamino acids	100	13.5 - 15.0	22.2 - 25.0	3.9 - 4.5
Tween 80	1000	aspartic acid	100	19.5 - 21.2	21.2 - 23.0	3.7 - 4.5
Tween 80	1000	alanine	100	13.0 - 13.9	12.7 - 13.5	6.0 - 6.9

1) Values denoted are the extremes of 4 determinations.

Table 2. Infectivity of *Frankia* AvcI1, *Frankia* CpI1 and *Frankia* AgSp+1 on *Alnus glutinosa* plants grown in perlite

	Number of nodules per 15 plants
Frankia AvcI1	225
Frankia CpI1	248
Frankia Ag + 1	2
Control	0

were inoculated with *Frankia* AvcI1 cells. Inoculation density was 0.5 mg TOC cells/l. After cultivation for 12 days, cell yields were determined and acetate and succinate assayed in the used media. Cell-free extracts of the cells were used for measuring the activities of ICL, MS, IDH and MDH (Table 3).

Amino acids as nitrogen source for Frankia *AvcI1*

Media containing Tween 80 (1 g/l) as C-source and various amino acids as N-source were inoculated with *Frankia* AvcI1 cells. Inoculation density was 1 mg TOC cells/l. After cultivation for 20 days cell yields and amino acid contents of the used media were determined (Table 4).

Table 3. Comparison of *Frankia* AvcI1 cells grown with either acetate, propionate, succinate or acetate plus succinate after cultivation for 12 days

C-source of medium (mg/l)	Yield[1] (mg TOC/l)	Acetate concentration[1] of used medium (mg/l)	Succinate concentration[1] of used medium (mg/l)	Enzyme activities in cell-free extracts (nmol.min^{-1} mg protein^{-1})			
				ICL	MS	IDH	MDH
Acetate 60	3.0 - 3.9	30 - 33	–	72	60	170	2800
Propionate 60	4.0 - 5.0	–	–	<5	<5	230	2100
Succinate 50	0 - 0.2	–	47 - 50	–	–	–	–
Acetate + succinate 60 + 50	3.6 - 4.2	30 - 32	49 - 52	68	75	210	2500

[1] Values denoted are the extremes of 4 determinations. NH_4Cl (60 mg/l) was used as N-source.

Table 4. Cell yields of *Frankia* AvcI1 after a 20-day cultivation period on media with Tween 80 (1 g/l) as C-source and various amino acids as N-source

N-source of medium	Amino acids (µmol/l)		Yield (mg TOC/l)[2]
	before growth[1]	after growth[1]	
Alanine	400	260	12.4 - 13.4
γ-Amino-butyrate	400	300	8.9 - 9.4
Aspartic acid	400	100	22.3 - 26.0
Glycine	400	325	10.2 - 10.6
Glutamic acid	400	100	22.9 - 24.1
Leucine	400	320	12.6 - 13.9
Phenylalanine	400	230	19.0 - 20.3
Serine	400	340	9.0 - 11.0
Threonine	400	300	11.5 - 12.2
Tyrosine	400	340	13.6 - 14.5
Valine	400	330	10.0 - 11.2

[1] Averages of values of 4 samples.

[2] Values denoted are the extremes of 4 determinations.

DISCUSSION

From the data presented in Table 1 it can be concluded that neither one of the three *Frankia* strains tested is able to utilize glucose, lactate, succinate or ethanol as sole carbon source. The inability of the AvcI1 strain and the CpI1 strain to grow with glucose as sole C-source is a confirmation of the results of Baker and Torrey (6) and Blom and Harkink (14). All three strains can grow on media containing either Tween 80 or acetate as C-source and NH_4^+, casamino acids, aspartic acid or alanine as N-source. Apart from the similarity in C- and N-source requirement, a significant difference in growth yield exists between the AgSp+1 strain on the one hand, and the AvcI1 and CpI1 strains on the other. The reason for this difference is not clear. Since no data have as yet been published on growth yields of other *Frankia* strains, it is not possible to determine whether or not the strains tested in this study behave like normal *Frankia* strains.

From the data shown in Table 2, it is clear that the AvcI1 and CpI1 strains are infective on *Alnus glutinosa*, whereas no infectivity of the Ag + 1 strain was observed. This might be due to the fact that the plants were grown in perlite, since *Frankia* AgSp+1 is able to infect sterile *A. glutinosa* plants grown in agar (15; A.J.P. Burggraaf, personal communication). Because the three strains tested thus belong to the same cross-inoculation group (5,9), the marked similarity in C- and N-source requirement (Table 1) is not surprising.

The data given in Table 3 show that the failure of the *Frankia* strains tested to grow with succinate as sole carbon source is due to the fact that no uptake of this compound occurs. This is also true of media containing acetate and succinate. Under such conditions, the glyoxylate cycle enzymes isocitrate lyase and malate synthase are not repressed as it is the case in cells grown with propionate (cf Table 3). The repressing effect of propionate on the glyoxylate cycle enzymes suggests that in *Frankia* AvcI1 propionate is metabolized *via* succinate as it was found to be the case in *E. coli* (21).

The activities of the citric acid cycle enzymes isocitrate dehydrogenase and malate dehydrogenase seem not to be affected by the nature of the carbon sources tested in this experiment.

Vesicle clusters of *Frankia* spp.as they occur in nitrogen-fixing root nodules of *Alnus glutinosa*, are able to respire succinate (Akkermans and Roelofsen, personal communication). This suggests an interesting alteration of the capacity to take up succinate during the transition of *Frankia* spp.from the

free-living to the symbiotic state. In this respect *Frankia* spp. show marked differences with *Rhizobium* spp., since the latter were shown to take up succinate in both the free-living and the symbiotic (bacteroid) state (22).

In an earlier study it was shown (12) that *Frankia* AvcI1 selectively takes up aspartic acid and glutamic acid from a mixture of amino acids, leaving the concentrations of the other amino acids unchanged during growth. From the data shown in Table 4 it can be concluded that *Frankia* AvcI1 can utilize other amino acids as sole nitrogen source as well. The mechanism of selection of aspartic acid and glutamic acid from a mixture of amino acids is not yet clear.

In cell-free extracts of *Frankia* AvcI1 grown with either NH_4^+, glutamate or Casamino acids as N-source, activity was observed of glutamine synthetase and glutamate-oxaloacetate transaminase, but not of glutamate dehydrogenase, glutamate synthase, L-amino acid oxidase and branched-chain amino acid amino-transferase (Blom, unpublished results). The pathways of assimilation of nitrogen in *Frankia* spp. still remain obscure.

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Chapter III

ASSIMILATION OF AMMONIA IN ROOT NODULES OF *ALNUS GLUTINOSA*

CHAPTER III.1

ASSIMILATION OF NITROGEN IN ROOT NODULES OF ALDER (*ALNUS GLUTINOSA*)

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SUMMARY

Citrulline is shown to be the predominant free amino acid in alder root nodules, while serine also occurs in relatively large amounts. Citrulline and glutamic acid are prominent free amino acids in alder xylem tissue. The activities of N_2-ase, GS, GDH and OCT in root nodule homogenates are reported. From the Km values of GDH for NH_4^+ (16 mM) and glutamate (0.9 mM) and the concentrations in the nodule of NH_4^+ (1.5 mM) and glutamate (0.5 mM) it is concluded that GDH in alder nodules is responsible for the deamination of glutamate. The important function of GDH in the nitrogen metabolism of alder nodules is confirmed by the much higher activity of this enzyme in homogenates of nodules as compared to that in homogenates of root-tips and leaves.

The vesicle clusters, which contain the N_2-ase activity, do not show activity of GS, GDH and OCT. The cytoplasm of the host cells was shown to possess the GS activity, while GDH and OCT probably are localized in the organelles of the host cells.

Abbreviations: C fraction, plant cytoplasm; E fraction, endophyte vesicle clusters; GDH, glutamate dehydrogenase EC 1.4.1.3; GOGAT, glutamine: α-ketoglutarate aminotransferase (NAD(P)H-oxidizing) EC 2.6.1.53; GS, glutamine synthetase EC 6.3.1.2; N_2-ase, nitrogenase; OCT, ornithine carbamyl transferase EC 2.1.3.3; O fraction, plant organelles; P fraction, plant fraction; TN fraction, total nodule homogenate.

INTRODUCTION

At least two pathways for assimilation of ammonia occur in nitrogen-fixing organisms. The first, the reductive amination of α-ketoglutarate, is catalyzed by GDH.

$$\alpha\text{-ketoglutarate} + NH_4^+ + NADH \rightleftharpoons \text{glutamate} + NAD^+ + H_2O$$

The second pathway includes the combined action of GS and GOGAT

$$\text{glutamate} + NH_3 + ATP \rightarrow \text{glutamine} + ADP + P_i$$
$$\text{glutamine} + \alpha\text{-ketoglutarate} + NAD(P)H + H^+ \rightarrow 2\ \text{glutamate} + NAD(P)^+.$$

Recent studies (reviewed by Scott, 1978) have demonstrated that the second pathway functions in many free-living nitrogen-fixing microorganisms and in *Rhizobium*-legume symbioses, whereas the former is predominant in the *Nostoc*-containing lichen of the genus *Peltigera* (Stewart and Rowell, 1977; Rai, Rowell and Stewart, 1980). No information has been reported so far on the ammonia assimilation in actinomycete root nodule symbioses.

It has been shown (Miettienen and Virtanen, 1952; Leaf, Gardner and Bond, 1958; Wheeler and Bond, 1970) that the pool of free amino acids of *Alnus*-type root nodules differs from that of other types of root nodules. In root nodules of *Alnus glutinosa*, citrulline is the most prominent free amino acid; it contributed about 20% (on molar base, *i.e.* about 50% on N-base) to the amino acid pool, whereas asparagine was not detectable and glutamine contributed about 10%. Different types of non-legume root nodules may possess significant quantities of specific other amino acids, *e.g.* arginine and ornithine.

In contrast, root nodules of most leguminous plants contain glutamine and asparagine as dominant amino acids (van Egeraat, 1972).

In the present paper the composition of the pool of free amino acids in alder root nodules is shown. Activities of enzymes concerned with the assimilation of ammonia and with the production of citrulline in the nodules will be reported with special attention to the distribution of these enzymes between different fractions of the nodule.

MATERIALS AND METHODS

Nodules, root-tips, stems and leaves were collected from 0.5-year-old greenhouse-grown *A. glutinosa* plants, which had been inoculated with nodule brei (final concentration 750 µg nodule fresh weigt/l) 4 weeks after germination. Per plant 20-50 nodules developed. Samples of 2-10 g fresh weight of nodules were obtained from each plant and assayed for acetylene-reducing activity (Akkermans, van Straten and Roelofsen, 1977), and those which produced 500-1500 nmoles $C_2H_4.g^{-1}$ nodule fresh weight.h^{-1} (assayed after detaching) were used for homogenization and fractionation. They were homogenized in a Virtis mixer (45-Hi-speed) for 2 min at 5,000 rpm in the following buffer: K-phosphate (50 mM), sucrose (300 mM), dithioerythritol (2.5 mM), $MgCl_2$ (1 mM), polyvinyl pyrrolidone (4%), pH 7.6.

The homogenate was filtered through a 100-µm filter to remove large plant cell debris. The filtrate, denoted as total nodule (TN) fraction.

as filtered through a 20 µm filter, and the residue was taken up in homo-
enization buffer. The 20-µm residue (E fraction) contained intact vesicle
lusters of the endophyte (Akkermans, 1978), whereas the 20-µm filtrate
P fraction) contained plant cell material, distorted vesicle clusters and
yphae. The P fraction was centrifuged for 15 min at 10,000 x g. The pellet
O fraction) contained plant cell organelles and distorted vesicle clusters
nd hyphae, while the supernatant (C fraction) contained the plant cytoplasm
nd the contents of distorted vesicles. All fractions were sonicated for 1 min
t 40 W.

Root-tips and leaves were homogenized in the same way as the nodules, and
he homogenates were filtered through a 100-µm filter. Amino acid and ammonium
nalyses of 80%-ethanolic extracts of mortar-homogenized nodules and xylem
issue of stems were made using a Biotronic LC6000E amino acid analyzer.

For the determination of the nitrogenase activity (Akkermans *et al.*, 1977)
he homogenization and fractionation of the nodules were carried out anaer-
bically by flushing the buffer with argon and adding sodium dithionite to a
inal concentration of 10 mM.

GS activity was determined using the transferase assay (Shapiro and Stadtman,
970). The biosynthetic assay could not be used because of the colour of the
odule fractions.

GDH activity was determined according to Fahien and Cohen (1970). The Km
alues of GDH for NH_4^+ and glutamate were determined according to Ahmad, Larker,
hodes and Stewart (1979); KCN (0.1 mM) was added to prevent NADH oxidase
ctivity in assaying GDH activity in the direction of NH_4^+ formation.

The activity of OCT was determined by incubating the fractions with ornithine
5 mM) and carbamyl phosphate (5 mM) at 30°C and assaying the amount of
itrulline formed using a Biotronic LC6000E amino acid analayzer. A colorimetric
ssay could not be used due to the intense colour of the nodule fractions.

RESULTS AND DISCUSSION

Free amino acids in alder nodules and stems

Root nodules and the xylem tissue of the stems of 4 plants of *A. glutinosa*
were homogenized in 80% ethanol to extract the free amino acids. The results of
he amino acid analyses (Table 1) confirm the observation (Miettienen and
irtanen, 1952) that citrulline is the predominant free amino acid in root
odules of *A. glutinosa*. Serine is also found in relatively high amounts in the

Table 1. The free-amino acid content of root nodules and the xylem tissue of stems of *Alnus glutinosa* (µmoles of amino acid/g of tissue, dry weight)

Amino acid	Nodules	Xylem
Citrulline	13.5	10.2
Serine	13.2	4.8
Glycine	7.6	2.1
Aspartic acid	5.5	3.4
Alanine	5.0	2.0
Ornithine	3.3	1.0
γ-Amino butyric acid	3.1	0.8
Threonine	3.1	1.4
Arginine	2.9	0.5
Valine	2.0	0.9
Glutamic acid	1.9	8.1
Leucine	1.6	0.6
Glutamine	1.5	2.1
Histidine	1.2	0.3
Isoleucine	1.2	0.5
Tyrosine	1.1	1.0
Proline	1.1	0
Phenylalanine	1.0	1.3
Lysine	0.9	0.5
Tryptophan	0.9	0
Asparagine	0.6	0
Cystine	0.6	0.4
Methionine	0.2	0.1

nodules, whereas the other amino acids occur in relatively small quantities. Comparison of the amino acid content of nodules and xylem tissue supports the hypothesis that citrulline is the main transport vehicle of fixed nitrogen in alder (Miettienen and Virtanen, 1952; Leaf *et al.*; Wheeler and Bond, 1970).

Activities of some nitrogen-assimilating enzymes in the total nodule fraction

The activities of N_2-ase, GS, GDH and OCT in the total nodule fraction of nodules of 4 plants of *A. glutinosa* were determined (Table 2).

Table 2. Activities of some nitrogen-assimilating enzymes in the total nodule fraction of alder[1)]

	µmoles.min^{-1}.g nodule dry weight^{-1}
N_2-ase	0.09 - 0.25
GS	100 - 130
GDH	8.8 - 10.0
OCT	0.11 - 0.12

[1)] The activities of N_2-ase, GS and GDH have been determined four times, that of OCT twice. The values reported in the Table are the extremes.

The relatively high GS activity may be ascribed to the fact that the transferase assay (which in the presence of Mn^{2+} does not require deadenylation of the enzyme) gives higher values for the enzyme activity than the biosynthetic assay, so that the GS activity *in vivo* is likely not as high as suggested by the transferase assay (Shapiro and Stadtman, 1970).

We have not been able to detect any activity of GOGAT, using the assay method described by Meers, Tempest and Brown (1979). In this method NAD(P)H is used as donor of reduction equivalents.

The absence of GOGAT activity may be explained in two ways: either the concentration of the enzyme in alder nodules is too low to be observed, or GOGAT in these nodules does not function with NADH or NADPH, but requires reduced ferredoxin as donor of reduction equivalents. This is the case with GOGAT in *e.g.* pea chloroplasts (Lea and Miflin, 1974) and *Vicia faba* leaves (Wallsgrove, Harel, Lea and Miflin, 1977). The latter possibility is being studied at the moment.

GDH activity was dependent on NADH as cofactor; NADPH gave no activity.

The direction of the reaction catalyzed by GDH

The Km of GDH for NH_4^+ (assayed in the TN fraction) was found to be 16 mM, while the NH_4^+ concentration in the nodule was only 1.5 mM. Since toxic effects of NH_4^+ in plant tissue at concentrations of 1 mM have been reported (Krogman, Jagendorf and Avron, 1959; Chibnal, 1939; Prianishnikov, 1951), the existence of sub-cellular pools of higher NH_4^+ concentrations is unlikely. The Km of GDH for glutamate (assayed in the TN fracton) was found to be 0.9 mM, while the glutamate concentration in the nodule was 0.5 mM.

These data suggest that GDH in alder nodules catalyzes the deamination rather than the synthesis of glutamate. A possible function of GDH might be the production of NH_4^+ required for the formation of carbamyl-phosphate, which is in turn needed for the formation of citrulline.

GDH activities in homogenates of nodules, root-tips and leaves

The activity of GDH in a homogenate of alder nodules is about 5-10 times higher than that in homogenates of alder root-tips and leaves (activities based on tissue dry weight((Table 3). This is the more significant since nodules contain more woody material than leaves or root-tips. The relatively high GDH activity in nodules suggests that this enzyme plays an important role in the nitrogen metabolism of nodules.

Table 3. Activity of GDH in homogenates of nodules, root-tips and leaves of alder[1])

	$\mu moles.min^{-1}.g$ tissue dry $weight^{-1}$
Nodules	8.8 - 10.0
Root-tips	1.1 - 1.5
Leaves	1.1 - 2.1

1)The values reported are the extremes of four determinations.

Localization of nitrogen-assimilating enzymes within the nodule

The distribution of N_2-ase, GS, GDH and OCT activities over the different fractions of the nodule is shown in Table 4. The distribution of N_2-ase activity over the E fraction and the P fraction is in accordance with earlier investigations (Akkermans, 1978; Akkermans, Roelofsen and Blom, 1979) in which diaminopimelic acid was used as an endophyte cell wall marker. The large proportion of N_2-ase in the P fraction depends on the presence of small and broken vesicle clusters. Therefore, it is concluded that N_2-ase is an endophyte vesicle cluster enzyme.

Table 4. Distribution of some nitrogen-assimilating enzymes over different fractions of the nodule

Fraction	N_2-ase	GS	GDH	OCT
TN (total nodule)	100%	100%	100%	100%
E (endophyte vesicle clusters)	20%	1%	0%	0%
P (plant fraction)	70%	102%	91%	100%
O (plant organelles)	ND 1)	3%	46%	70%
C (plant cytoplasm)	ND 1)	98%	63%	20%

1)ND, not determined.

From the distribution of GS activity it is concluded that GS is a plant cytoplasm enzyme. The distribution of GDH activity over the different fractions suggests that GDH is localized in plant cell organelles. This observation, which is in contrast to earlier results (Akkermans *et al.*, 1979), has further been supported by the absence of GDH activity in cell-free extracts of *Frankia* AvcI1 (the nodule endophyte of alder), grown in pure culture (Blom, in preparation).

The activity of OCT also seems to be associated with plant cell organelles. The difference in distribution of GDH and OCT suggests a different degree of

disintegration of plant cell organelles during homogenization of the nodules (GDH and OCT activities were determined in different samples).

The nature of these organelles remains to be clarified, although GDH is generally accepted as a marker enzyme for plant mitochondria (*e.g.* Lehninger, 1975; Nauen and Hartmann,1980). Gardner (1976), using a cytochemical assay, demonstrated OCT in alder nodules in the mitochondria and the endoplasmic reticulum of the host cell; after longer incubation periods staining was also observed in the endophyte.

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CHAPTER III.2

INHIBITION OF GOGAT ACTIVITY IN LUPIN NODULE HOMOGENATES BY ALDER NODULE HOMOGENATES

INTRODUCTION

In a previous contribution (1), the activities of some enzymes concerned with the assimilation of nitrogen in root nodules of alder (*Alnus glutinosa*) were reported. It was shown that nitrogenase (N_2-ase) is localized in the vesicle clusters of the endophyte, whereas glutamine synthetase (GS) is found in the cytoplasm of the host cells, and glutamate dehydrogenase (GDH) and ornithine carbamyl transferase (OCT) are localized in the organelles of the host cells. No activity of glutamate synthase (GOGAT) was observed.

Two pathways are known for the assimilation of ammonia in nitrogen-fixing organisms. The first, the reductive amination of α-ketoglutarate catalyzed by GDH, is found in the *Nostoc*-containing lichen of the genus *Peltigera* (2,3). The second, the combined action of GS and GOGAT, occurs in many free-living nitrogen-fixing organisms and in *Rhizobium*-legume symbioses (reviewed in ref. 4).

The K_m of GDH for NH_4^+ in alder nodules was found to be 16 mM and the concentration of NH_4^+ to be 1.5 mM, whereas the K_m of GDH for glutamate was 0.9 mM and the concentration of glutamate 0.5 mM (1). These data render it unlikely that in root nodules of alder the GDH pathway for assimilation of ammonia is prominent. They suggest that ammonia is assimilated *via* the GS/GOGAT pathway.

In the present contribution it will be shown that our failure to detect any GOGAT activity in alder nodule homogenates might be ascribed to the presence of an inhibiting compound in this homogenate.

MATERIALS AND METHODS

Lupin nodules were collected from non-inoculated field-grown *Lupinus luteus* (yellow lupin). The crude bacteroid fraction was obtained from the nodules as described by Robertson *et al.* (5). This fraction was sonicated for 2 min at 40 W and centrifuged for 10 min at 20,000 g yielding a cell-free preparation containing GOGAT activity in which alder nodules were homogenized. Alder nodules were collected as described earlier (1). Varying amounts of nodules were homogenized in a Virtis mixer (45-Hi-speed) for 2 min at 5000 $r.min^{-1}$ in 30 ml of the GOGAT-containing lupin extract. The homogenate was filtered through a 100 μm filter to remove large plant cell debris.

NADH-dependent GOGAT activity was determined in this fraction according to Planqué *et al.* (6).

RESULTS AND DISCUSSION

The effect of added alder nodule homogenate on the GOGAT activity of the lupin nodule extract is shown in Table 1. From the data shown in this table it can be concluded that during the homogenization of alder nodules some

Table 1. Inhibitory effect of homogenized alder nodules on the GOGAT activity of lupin nodule extract

Concentration of homogenized alder nodules in the lupin nodule extract (mg/ml)	Inhibition of NADH-dependent GOGAT activity (%)
0	0[1]
200	35
250	40
400[2]	80[3]
∞	-

[1] The GOGAT activity varied between 50 and 100 nmol.min^{-1}.g lupin nodule fresh weight^{-1}.

[2] Alder nodules homogenized in buffer containing no lupin nodule extract (ref. 1)

[3] No GOGAT activity detected

compound is released which is responsible for the inhibition of GOGAT activity in the lupin nodule extract. This may explain why no GOGAT activity was observed in homogenates of alder nodules (1). Therefore the failure to detect any GOGAT activity in homogenates does not exclude the operation of the GS/GOGAT pathway in the assimilation of ammonia in alder root nodules *in vivo*.

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Chapter IV

GENERAL DISCUSSION AND SUMMARY

The research reported in this thesis deals with the symbiosis of *Frankia* spp. and *Alnus glutinosa*. *Frankia* spp. are actinomycetes giving rise to the formation of nitrogen-fixing nodules on the roots of a number of non-leguminous plants. In these nodules *Frankia* spp. live within the plant cells and obtain all sources of carbon and energy from the plant, giving fixed nitrogen in exchange.

To answer the question what compounds *Frankia* spp. obtain from the plant, insight into the metabolism of this microorganism is required. To obtain this insight, researches have been made with the free-living *Frankia* AvcI1, isolated from root nodules of *Alnus viridis* ssp *crispa* by Baker *et al.* (1979). This organism has been isolated and cultivated on complex media. In order to obtain insight into the C- and N-source requirements of *Frankia* AvcI1, a simple and well-defined growth medium is needed. The composition of this medium and the C- and N-metabolism of *Frankia* AvcI1 are the subjects of Chapter II of this thesis.

In Chapter II.1 it is shown that *Frankia* AvcI1 is able to grow on a medium containing Tween 80 (an oleate ester of polyethyleneglycol sorbitan) as sole C-source and either glutamic acid or NH_4Cl as sole N-source. The growth yield of *Frankia* AvcI1 on various media is reported. It is shown that the doubling time of *Frankia* AvcI1 growing on QMOD/Tween medium is about 2 days, which is slow, even for an actinomycete. When growing with Casamino acids as nitrogen source, *Frankia* AvcI1 selectively takes up glutamic acid and aspartic acid, leaving the concentrations of the other detectable amino acids unchanged. The mechanism of this selection is still unclear.

Chapter II.2 contains further data on the C-sources utilized by *Frankia* AvcI1. It is shown that *Frankia* AvcI1 can utilize as C-source also other Tweens, *viz.* Tweens 20, 40, 60 and 85, and in addition several fatty acids, *viz.* acetic, propionic, butyric, valeric, caproic, caprylic, capric, palmitic and stearic acids. No growth of *Frankia* AvcI1 was observed on media containing triglycerides as C-sources.

The dependence of the growth yield on the nature of the carbon source and the concentration of NH_4^+ in the media is shown. Utilization of NH_4^+ as nitrogen source of *Frankia* AvcI1 growing in the Tween/NH_4^+ medium is confirmed by incorporation experiments with ^{15}N-NH_4Cl.

The results reported in Chapter II.3 show that *Frankia* AvcI1 does not take up glucose from a medium containing both glucose and Tween 80. In agreement with this observation, it is demonstrated that the activities of isocitrate lyase and malate synthase in cells grown on the QMOD/Tween medium (containing both glucose and Tween 80) are of the same order of magnitude as in cells grown on the Tween/NH_4^+ medium (containing Tween 80 as sole C-source).

Organisms growing on fatty acids, and degrading these compounds to acetyl-CoA, start gluconeogenesis with the action of the glyoxylate cycle (Kornberg and Krebs, 1957) leading to the conversion of 2 acetyl-CoA molecules to 1 molecule of succinate. The presence of the glyoxylate cycle enzymes isocitrate lyase and malate synthase in *Frankia* AvcI1 cells grown with Tween 80 as C-source is therefore not surprising. The succinate formed in the glyoxylate cycle can be converted to phosphoenolpyruvate by the subsequent action of the enzymes succinate dehydrogenase, fumarase, malic enzyme and pyruvate orthophosphate dikinase, which are found in cell-free extracts of *Frankia* AvcI1. From phosphoenolpyruvate, gluconeogenesis can continue with the action of phosphoglycerate kinase and 3-phosphoglyceraldehyde dehydrogenase.

For energy generation, *Frankia* AvcI1 can oxidize the acetyl-CoA derived from fatty acid breakdown in the citric acid cycle, although the low activity of the enzyme succinyl-CoA synthetase leaves room for the presumption that the citric acid cycle is not very operative in *Frankia* AvcI1. This is not impossible since the oxidation of fatty acids yields many reducing equivalents.

The data contained in Chapter II.4 show that *Frankia* AvcI1 does not take up succinate from a medium containing acetate plus succinate or succinate alone. In accordance, no repressing effect of succinate on the activities of the glyoxylate cycle enzymes was observed, whereas in cells grown on propionate these enzymes were not found, indicating that they are not constitutive in *Frankia* AvcI1.

Frankia AvcI1 is able to utilize several amino acids as sole nitrogen source, *viz.* alanine, γ-aminobutyric acid, aspartic acid, glutamic acid, glycine, leucine, phenylalanine, serine, threonine, tyrosine and valine.

No differences in C- and N-source requirements were observed for the three *Frankia* strains AvcI1, CpI1 (isolated by Callaham *et al.*, 1978, from *Comptonia peregrina* root nodules) and AgSp+1 (isolated by Quispel and Burggraaf, 1981, from *Alnus glutinosa* spore-(+) type root nodules). These three strains were shown to utilize either Tween 80 or acetate, but no ethanol, lactate, glucose or succinate as sole C-source, and either NH_4Cl, casamino acids, aspartic acid or alanine as N-source.

From the data given in Chapter II it will be clear that *Frankia* AvcI1 is able to grow on a well-defined medium, which is a prerequisite for obtaining a clear insight into the physiology of the organism. The alcoholic root extract (Quispel and Tak, 1978) or the soybean lecithin (Lalonde and Calvert, 1979), added to the medium as growth factors, can be replaced by Tween 80 or fatty acids which are utilized as carbon source, while NH_4Cl or amino acids can be utilized as nitrogen source.

It is unknown whether *Frankia* spp. grow with the same growth rate on media with different constituents. The growth yields presented in Chapter II are usually small as compared to the amount of carbon present in the media. This suggests that either an additional component of the medium is present in limiting concentrations or that *Frankia* spp. can only grow for a limited time after inoculation.

The only group of compounds known so far to be utilized as C-source by free-living *Frankia* AvcI1 are fatty acids, either free or esterified. The question what carbon source *Frankia* spp. living symbiotically in the nodule obtain from the plant, still remains to be answered. Based on the data obtained for free-living *Frankia* spp. it is not unlikely that some fatty acid functions as C-source under such conditions. Other possible candidates for this function are plant lipids like the alcoholic root extract (Quispel and Tak, 1978) or the soybean lecithin (Lalonde and Calvert, 1979). It is unlikely that sugars like glucose or dicarboxylic acids like succinate are playing this role, unless the ability of *Frankia* spp. to take up one or more of these compounds should alter during the transition from the free-living to the symbiotic stage.

The cultivation of free-living *Frankia* spp. is a powerful tool in discovering symbiotic interactions of the endophyte and the host. In Chapter II.4 of this thesis it is shown that by replacing acetate as C-source of the medium by propionate, the activities of the glyoxylate

cycle enzymes are repressed. Other authors (Tjepkema, Ormerod and Torrey, 1980 and 1981; Gauthier, Diem and Dommergues, 1981) reported a medium in which free-living *Frankia* spp. show vesicle formation and N_2-ase activity. The present knowledge thus enables one to influence regulation in free-living *Frankia* spp., which is important in studying the symbiotic interactions mentioned above.

In Chapter III attention is paid to the assimilation of the ammonia produced by the endophyte living symbiotically in the root nodules of *Alnus glutinosa* grown in the greenhouse from seeds collected in Wageningen. In Chapter III.1 the composition of the pool of free amino acids in root nodules and the xylem tissue of stems is reported. It is shown that citrulline is the predominant free amino acid in nodules, while serine also occurs in relatively large amounts. In xylem tissue citrulline and glutamic acid are prominent.

The activities of N_2-ase, GS, GDH and OCT in root nodule homogenates are reported. From the K_m values of GDH for NH_4^+ (16 mM) and glutamate (0.9 mM) and the concentrations in the nodule of NH_4^+ (1.5 mM) and glutamate (0.5 mM) it is concluded that GDH in alder nodules probably is responsible for the deamination of glutamate and not for the synthesis of this key amino acid. The important function of GDH in the nitrogen metabolism of alder nodules is confirmed by the much higher activity of this enzyme in homogenates of nodules as compared to that in homogenates of root-tips and leaves.

The vesicle clusters, which contain the N_2-ase activity, did not show activity of GS, GDH and OCT. The cytoplasm of the host cells was shown to possess the GS activity, while GDH and OCT are localized in the organelles of the host cells. No activity of NADH-dependent GOGAT was observed.

In Chapter III.2 it is shown that the activity of NADH-dependent GOGAT from root nodules of lupins is inhibited by some compound in the homogenate of alder nodules.

Simultaneously with and independently of the present research, Schubert *et al.* (1981) analyzed the composition of the pool of free amino acids in nodules and the xylem tissue of *Alnus glutinosa* grown in the field in East Lansing, Michigan. The only difference with respect to these amino

acid pools between the American alders and the European alders studied in the present research, is the relatively high amount of serine in nodules of the latter, whereas in nodules of the former this amino acid is not found. The results of both Schubert *et al.* (1981) and the present research confirm the hypothesis that in alder,citrulline is the main transport vehicle of fixed nitrogen (Miettienen and Virtanen, 1952; Leaf, Gardner and Bond, 1958; Wheeler and Bond, 1970).

From the results shown in Chapter III.2 it can be concluded that our failure to find any GOGAT activity may be ascribed to the presence of an inhibiting compound in the homogenate of alder nodules, so that it is not excluded that GOGAT is active in alder nodules *in vivo*.

The data reported in Chapter III are in accordance with the following model: The nitrogen fixed as ammonia in the vesicle clusters of the endophyte, is assimilated in the cytoplasm of the host cell into glutamate by the action of GS and presumably GOGAT. Glutamate is in part deaminated in the plant organelles by the action of GDH to supply the NH_4^+ required for the synthesis of carbamyl phosphate. Another part of the glutamate is converted to ornithine, which in the organelles of the host cell reacts with carbamyl phosphate to form citrulline according to the OCT reaction. Citrulline is excreted from the nodule and serves as nitrogen source for the plant.

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SAMENVATTING

Het onderwerp van dit proefschrift is de symbiose van *Frankia* spp. en *Alnus glutinosa*. *Frankia* spp. zijn actinomyceten die de vorming van N_2-bindende wortelknollen induceren bij een aantal niet-vlinderbloemige planten, waaronder de els. In deze wortelknollen bevinden de *Frankia* cellen zich in de cellen van de plant. *Frankia* spp. ontvangen voor hun energievoorziening een of meer koolstofverbindingen van de plant. Zij binden molekulaire stikstof die als ammonia wordt uitgescheiden in de gastheercel. Hier vindt vorming van organische N-verbindingen plaats die dienen voor de groei van de plant.

Om de vraag te kunnen beantwoorden, welke koolstofverbindingen *Frankia* spp. van de plant ontvangen, is inzicht in het metabolisme van dit micro-organisme vereist. Voor het verkrijgen van dit inzicht is onderzoek gedaan met de vrijlevende *Frankia* AvcI1, geïsoleerd uit wortelknollen van *Alnus viridis* ssp. *crispa* door Baker en medewerkers (1979). Isolatie en kweek van dit organisme vonden tot het begin van het hier beschreven onderzoek plaats in complexe media. Om inzicht te verkrijgen in de C- en N-stofwisseling van *Frankia* AvcI1, is het nodig deze actinomyceet te kweken in een goed gedefinieerd medium. In hoofdstuk II van dit proefschrift worden de samenstelling van een dergelijk medium en het C- en N-metabolisme van enkele *Frankia* spp. behandeld.

In hoofdstuk II.1 wordt aangetoond dat *Frankia* AvcI1 kan groeien in een medium met Tween 80 (een oleaat-ester van polyethyleenglycol-sorbitan) als koolstofbron en NH_4Cl of glutaminezuur als stikstofbron. De opbrengsten aan celmateriaal in verschillende media, en de groeisnelheid in het QMOD/Tween medium worden vermeld. Bij groei van *Frankia* AvcI1 met Casaminozuren als stikstofbron worden glutaminezuur en asparaginezuur selectief opgenomen en benut, terwijl de concentratie van de andere aminozuren in het voedingsmedium tijdens de groei ongewijzigd blijft.

In hoofdstuk II.2 wordt aangetoond dat *Frankia* AvcI1 als koolstofbron,

behalve Tween 80, ook de Tweens 20, 40, 60 en 85 kan gebruiken, alsmede een aantal vetzuren, nl. azijn-, propion-, boter-, valeriaan-, capron-, capryl-, caprine-, palmitine- en stearinezuur. In media met triglyceriden als koolstofbron werd geen groei waargenomen. De celopbrengsten zijn afhankelijk van de aard van de koolstofbron en van de concentratie van NH_4Cl in het groeimedium. Gebruik van NH_4^+ als N-bron door *Frankia* AvcI1 bij groei in het Tween/NH_4^+ medium werd bevestigd in een experiment met ^{15}N-NH_4Cl.

Uit de experimenten beschreven in hoofdstuk II.3 blijkt dat *Frankia* AvcI1 geen glucose opneemt uit een medium dat zowel Tween 80 als glucose bevat. In cellen gekweekt in het QMOD/Tween medium (dat zowel Tween 80 als glucose bevat) is de aktiviteit van de glyoxylaat-cyclus enzymen isocitraatlyase en malaat-synthase dan ook van dezelfde orde van grootte als in cellen gekweekt in het Tween/NH_4^+ medium (met Tween 80 als enige C-bron). Het produkt van de glyoxylaat-cyclus, succinaat, kan worden omgezet in fosfoenolpyruvaat door de enzymen succinaat-dehydrogenase, fumarase, "malic enzyme"en pyruvaat-orthofosfaat-dikinase, die allen in celvrije extracten van *Frankia* AvcI1 werden aangetroffen. Ook werd aktiviteit van de enzymen fosfoglyceraat-kinase en 3-fosfoglyceraldehyde-dehydrogenase gevonden. Deze enzymen spelen waarschijnlijk een rol bij de biosynthese van glucose en andere suikers (gluconeogenese) uit fosfoenolpyruvaat.

Acetyl-CoA, verkregen bij de afbraak van vetzuren, kan voor de generering van energie in *Frankia* AvcI1 waarschijnlijk worden geoxideerd in de citroenzuur-cyclus, hoewel de lage aktiviteit van het enzym succinyl-CoA-synthetase in celvrije extracten van *Frankia* AvcI1 ruimte laat voor de veronderstelling dat de citroenzuur-cyclus in *Frankia* AvcI1 niet zeer aktief is. Dit is mogelijk omdat bij de afbraak van vetzuren tot acetyl-CoA reeds veel reduktie-equivalenten vrijkomen.

In hoofdstuk II.4 wordt aangetoond dat *Frankia* AvcI1 geen succinaat opneemt uit een medium dat als koolstofbron alleen succinaat of succinaat plus acetaat bevat. In overeenstemming hiermee werd geen represserend effekt van succinaat op de aktiviteit van de glyoxylaat-cyclus enzymen waargenomen, terwijl propionaat wel een dergelijk effekt bleek te hebben.

Frankia AvcI1 kan als enige stikstofbron een aantal aminozuren gebruiken, nl. alanine, γ-aminoboterzuur, asparaginezuur, glutaminezuur, glycine, leucine, fenylalanine, serine threonine, tyrosine of valine.

De stammen *Frankia* AvcI1, CpI1 (geïsoleerd door Callaham en medewerkers, 1978, uit wortelknollen van *Comptonia peregrina*) en AgSp+1 (geïsoleerd door Quispel en Burggraaf, 1981, uit spore-positieve wortelknollen van *Alnus glutinosa*) vertoonden geen verschillen wat betreft hun C- en N-bron behoeften. Alle drie de stammen kunnen Tween 80 en acetaat, maar geen ethanol, lactaat, glucose of succinaat, als C-bron gebruiken. Als N-bron kunnen alle drie de stammen NH_4Cl, Casaminozuren, asparaginezuur en alanine benutten.

Uit de resultaten beschreven in hoofdstuk II blijkt dat *Frankia* AvcI1 kan groeien in een gedefinieerd medium. Het wortel-extract (Quispel en Tak, 1978) en de soja-lecithine (Lalonde en Calvert, 1979) die aan het medium werden toegevoegd als groei-faktor, kunnen worden vervangen door Tween 80 of vetzuren die worden gebruikt als koolstofbron, terwijl NH_4Cl of sommige aminozuren geschikt zijn als stikstofbron.

Het is niet bekend of *Frankia* AvcI1 in alle in hoofdstuk II beschreven media met dezelfde snelheid groeit. De opbrengsten aan celmateriaal van *Frankia* AvcI1 zijn gewoonlijk klein vergeleken bij de hoeveelheid koolstof die in het medium wordt aangeboden. Dit wijst erop dat een andere component in het medium beperkend is, of dat *Frankia* AvcI1 slechts gedurende een beperkte tijd na enting kan groeien.

De enige groep verbindingen waarvan tot nu toe bekend is dat ze als koolstofbron door vrijlevende *Frankia* spp. gebruikt kunnen worden, zijn de vetzuren, hetzij vrij, hetzij veresterd. Het is nog niet duidelijk welke koolstofverbinding symbiontische *Frankia* spp. in de wortelknol van de plant ontvangen. Gelet op de resultaten die verkregen zijn met vrijlevende *Frankia* spp. is het niet onwaarschijnlijk dat deze koolstofbron een vetzuur is. Ook lipiden van planten zoals wortelextract (Quispel en Tak, 1978) of soja-lecithine (Lalonde en Calvert, 1979) zouden deze funktie kunnen vervullen. Het is niet waarschijnlijk dat suikers zoals glucose of dicarbonzuren zoals succinaat geschikt zijn als koolstofbron, tenzij het vermogen van *Frankia* spp. om deze verbindingen op te nemen verandert tijdens de overgang van de vrijlevende in de symbiontische fase.

Het onderwerp van hoofdstuk III betreft de assimilatie van de stikstof die als ammoniak in de blaasjes-"clusters" van de endofyt (de symbiontische fase van de *Frankia* spp.) in wortelknollen van de els wordt gebon-

den. Het onderzoek is verricht met *Alnus glutinosa* opgekweekt in de kas uit zaden verzameld in Wageningen.

In hoofdstuk III.1 wordt de samenstelling van de "pool" van vrije aminozuren in wortelknollen en in het xyleem van de stam van deze planten vermeld. In knollen blijkt citrulline het meest voorkomende aminozuur te zijn, terwijl ook serine in relatief grote hoeveelheden voorkomt. In het xyleem van de stam worden citrulline en glutaminezuur in grote hoeveelheden aangetroffen.

De aktiviteiten van de enzymen N_2-ase, GS, GDH en OCT in homogenaten van wortelknollen worden vermeld. Uit de K_m van GDH voor NH_4^+ (16 mM) en voor glutamaat (0.9 mM), en de concentratie in de knol van NH_4^+ (1.5 mM) en glutamaat (0.5 mM) wordt afgeleid dat GDH in elzeknollen waarschijnlijk verantwoordelijk is voor de deaminering van glutamaat. Dat GDH een belangrijke funktie vervuld in elzeknollen blijkt uit de hoge aktiviteit van dit enzym in knolhomogenaten vergeleken bij die in homogenaten van bladeren en wortelpunten.

N_2-ase aktiviteit wordt aangetroffen in de blaasjes-"clusters", terwijl GS zich in het cytoplasma van de plantecellen bevindt. GDH en OCT zijn gelokaliseerd in de organellen van de gastheercellen. Geen aktiviteit van NADH-afhankelijk GOGAT kon in elzeknollen worden waargenomen.

In hoofdstuk III.2 wordt aangetoond dat de aktiviteit van NADH-afhankelijk GOGAT uit wortelknollen van lupine wordt geremd door een component van het homogenaat van elzeknollen.

Gelijktijdig met, maar onafhankelijk van, het in dit proefschrift beschreven onderzoek, onderzochten Schubert en medewerkers (1981) de samenstelling van de "pool" van vrije aminozuren in wortelknollen en het xyleem van de stam van *A. glutinosa* in East Lansing, Michigan. Het enige verschil tussen de elzen uit Michigan en die uit Nederland, met betrekking tot deze aminozuren, is gelegen in de relatief grote hoeveelheid serine in wortelknollen van de Nederlandse elzen, terwijl de elzen uit Michigan dit aminozuur niet bevatten. De resultaten van zowel Schubert en medewerkers (1981) als die van het in dit proefschrift beschreven onderzoek, bevestigen de hypothese dat in elzen gebonden stikstof voornamelijk in de vorm van citrulline wordt getransporteerd (Miettienen en Virtanen, 1952; Leaf, Gardner en Bond, 1958; Wheeler en

Bond, 1970).

Uit de resultaten die in hoofdstuk III.2 worden vermeld kan worden geconcludeerd dat in homogenaten van elzeknollen een verbinding voorkomt die de aktiviteit van GOGAT remt. Uit het feit dat tot nu toe in elzeknolhomogenaten geen GOGAT-aktiviteit werd aangetroffen, mag dus niet worden geconcludeerd dat GOGAT in elzeknollen *in vivo* niet aktief is.

De resultaten van hoofdstuk III zijn in overeenstemming met het volgende model: De stikstof die in de blaasjes-"clusters" van de endofyt wordt gebonden, wordt in het cytoplasma van de gastheercel geassimileerd tot glutamaat door GS en waarschijnlijk GOGAT. Een gedeelte van het aldus gevormde glutamaat wordt in organellen van de gastheercel gedeamineerd door GDH, waarbij de voor de vorming van carbamyl-fosfaat vereiste NH_4^+ vrijkomt. Een ander gedeelte van het glutamaat wordt omgezet in ornithine, dat in de organellen van de gastheercel met carbamylfosfaat reageert tot citrulline in de OCT-reaktie. Citrulline wordt door deze cellen uitgescheiden en dient als stikstofbron voor de plant.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 24 augustus 1952 te Rotterdam. In 1970 behaalde hij het gymnasium-β diploma aan het Marnix gymnasium aldaar, en begon met de studie biologie aan de Vrije Universiteit te Amsterdam. In 1974 werd het kandidaats examen B 4 (biologie en scheikunde met natuurkunde en wiskunde) afgelegd. In 1977 volgde het doktoraal examen (hoofdvak biochemie, bijvakken radiobiofysica en moleculaire genetica). In 1978 werd begonnen met het in dit proefschrift beschreven onderzoek.