The involvement of the *fixABCX* genes and the respiratory chain in electron transport to nitrogenase in *Azotobacter vinelandii.*

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René Wientjens

The involvement of the *fixABCX* genes and the respiratory chain in the electron transport to nitrogenase in *Azotobacter vinelandii*.

Aan Petra

Stellingen

1. De paralellen die getrokken worden tussen de koppeling van het elektronen transport en de ATP hydrolyse door nitrogenase en de signaal transductie door G-eiwitten berust op een over-interpretatie van structuurgegevens.

    Dana Wolle, Dennis R. Dean and James Bryant Howard *(1992) Science,* **258:** 992-995.

2. Het door Thorneley en medewerkers gepresenteerde kinetische mechanisme voor de ATPase activiteit van nitrogenase is in tegenspraak met hun eigen waarnemingen.

    Roger N.F. Thorneley, G. Ashby, J.V. Howarth, Neil C. Millar and H. Gutfreund (1989) *Biochem. J.,* **264:**657-661.

3. Het verschil in reactiviteit van het mitochondriale NADH:Q oxidoreductase met NADH en NADPH zoals waargenomen en geïnterpreteerd als bewijs voor een functioneel dimeer model door van Belzen en medewerkers, kan ook verklaard worden door het aannemen van twee verschillende enzymsystemen voor de pyridine nucleotide oxidatie.

    R. van Belzen, M.C.M. van Gaalen, P.A. Cuypers en S.P.J. Albracht (1990) *Biochem. Biophys. Acta,* **1017:**152-159.

    Dit proefschrift hoofdstuk 4.

4. De studie van Mattia en medewerkers bewijst dat studies met dierproefmodellen niet zonder meer toepasbaar zijn op andere organismen, zoals de mens.

    Mattia *et al.,* (1993) Drug Development Research **28:**176-182

5.   Er dient een goede standaard te komen voor het melden van bijwerkingen in een geneesmiddelenonderzoek, zodat het verschil tussen spontaan gemelde en gevraagde bijwerkingen duidelijk wordt.

Coßman and Wilsmann, (1987) *Münch. Med. Wochenschrift* **129:** 851-854.

6.   De stellingname van staatsecretaris Simons van W.V.C. in zijn brief van 22 april 1993, nr. 62, waarin hij stelt dat slechts nieuwe medicijnen volledig vergoed zouden moeten worden, leidt tot een verarming van het onderzoek naar nieuwe toepassingen van bestaande medicijnen en is dientengevolge nadelig voor de volksgezondheid.

7.   De Visual Analog Scale (VAS), zoals deze gebruikt wordt in studies ter vaststelling van de pijnintensiteit, kan, indien deze gekoppeld wordt aan een analoge schaal waarop de psychologische toestand van de patiënt vermeld staat, leiden tot een betere interpretatie van de pijn die de patiënt ondervindt.

8.   Arbeidsongeschiktheid, welke veroorzaakt is in de privésfeer van de werknemer, mag niet tot een boete voor de werkgever leiden.

9.   Motorrijders rijden niemand dood, zij worden doodgereden

Stellingen behorende bij het proefschrift

"The involvement of the *fixABCX* genes and the respiratory chain in the electron transport to nitrogenase in *Azotobacter vinelandii*"

Wageningen, 30 juni 1993   René Wientjens

Voorwoord

Dit proefschrift is niet alleen door mij in elkaar gezet, alhoewel de voorkant anders doet vermoeden. Er zijn een hele hoop mensen bij betrokken geweest die ik wil bedanken voor hun bijdrage aan dit proefschrift. Veel promovendi eindigen hun voorwoord met het bedanken van hun familieleden en bekenden. Ik niet, ik begin ermee omdat zij mijns inziens de grootste input hebben gehad.

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De studenten Harm Deckers, Michiel van Hoof, René Geurts, Marjolein Schalk, Vincent van der Velden en Jos Brouwers ben ik erkentelijk voor hun bijdrage aan het onderzoek en met name vanwege het feit dat er door hun aanwezigheid altijd wel iets op het lab gebeurde, of dat nu ging om het verorberen van weer een taart of om het verleiden van secretaresses.

Magda Safran and Andrey Sergeev, I wish to thank you for your interest in the work presented in this thesis and for the nice atmosphere present on lab III/IV. I wish you both lots of succes in the future in Poland and Russia.

Met een aantal mensen heb ik nauw samengewerkt en ik wil hen dan ook niet vergeten in dit dankwoord: Axel Berg, Marc Verhagen, Ronnie Wolbert, Antonio Pierik, Fred Hagen, Adrie Westphal, Jacques Benen, Jack Stokkermans, Laura Ausma, Michel Eppink en Willy van den Berg.

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CHAPTER 1.

GENERAL INTRODUCTION

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1.1. Introduction

Nitrogen is one of the most vital elements in nature, mainly because in all forms of life nitrogen is required. The only way inert atmospheric nitrogen can enter biological systems, is when it is "fixed". In Fig. 1 the pathway is shown, by which nitrogen can enter the various biological systems.
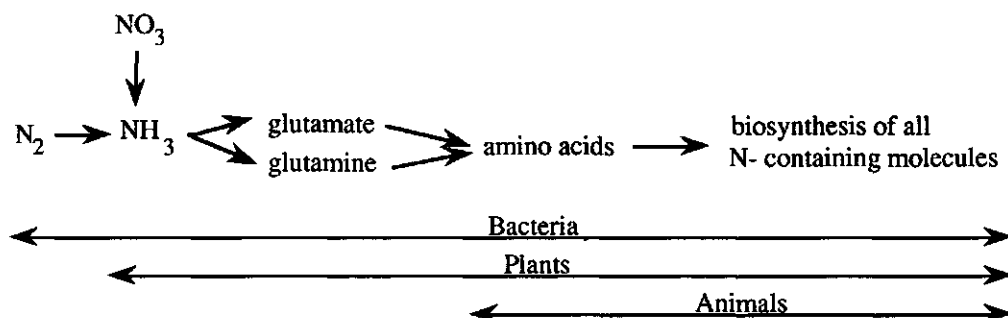
Figure 1. Nitrogen in the biological system of various organisms.

Nitrate, ammonia, glutamate, glutamine, other amino acids and N-containing biomolecules can serve as N-sources for both plants and bacteria, whereas animals are only capable of uptake of nitrogen in the form of amino acids (not being glutamate and glutamine), which are then converted into other N-containing biomolecules. It is important to realise that in biosynthesis of N-containing molecules from inert nitrogen gas, nitrate or nitrite, ammonia is an obligatory intermediate, which places ammonia in a key role in nitrogen metabolism of all live on earth.

It is calculated that only 1/400,000 of the total amount of nitrogen present in the atmosphere is incorporated in biological structures of plant and animal kingdoms [Delwiche, 1977; Garrels *et al.*, 1975]. Therefore the amount of dinitrogen in the atmosphere certainly is not the limiting factor for life on earth, but the conversion into adequate supplies of fixed nitrogen is the bottleneck.

Nitrogen fixation can be accomplished either industrial or biological. Industrial nitrogen fixation is carried out according the Haber-Bosch process, which requires high temperatures and pressure. The reaction is shown in equation 1. Hydrogen gas, used in the reaction, is produced from methane in an energy consuming process and, with respect to the depletion of fossil fuels, chemical nitrogen fixation will become a money consuming process, so biological nitrogen fixation is an important alternative for the production of fixed nitrogen sources.
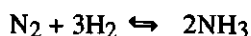
$N_2 + 3H_2 \leftrightharpoons 2NH_3$ $T \geq 400°C, P \geq 10^4 kPa$

Equation 1. Nitrogen fixation by the Haber-Bosch process

Another way of fixing dinitrogen is carried out by a relatively small number of microorganisms, which are able to fix dinitrogen biologically using the enzyme nitrogenase (see eq. 2.).

$N_2 + 8H^+ + 8e^- + 16\,MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i$ $T \approx 30°C, P = 10^2 kPa$
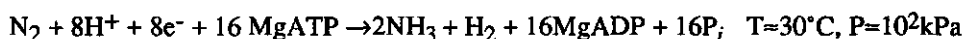
Equation 2. Nitrogen fixation by diazotrophic microorganisms.

Until now, no higher organisms have been discovered that are able to perform this reaction, although it is known that an increasing number of higher organisms live in symbiotic associations with nitrogen-fixing bacteria.

1.2.1. Biological nitrogen fixation.

As mentioned above, biological nitrogen fixation plays an important role in the nitrogen cycle of this planet. Therefore, research into the secrets of biological nitrogen fixation could help to solve the worlds nutritional problem. Although western society deals with an excess of ammonia in the soil that is used for agricultural purposes, third world countries are still lacking a soil that has sufficiently fertility to produce a crop that can feed its people.

Although the process of dinitrogen fixation is reserved to a relatively few genera of microorganisms, the list of N-fixing organisms covers a wide range of microbial types. Among them are obligate aerobic, microaerobic and anaerobic bacteria, either living on their own or in symbiosis with higher organisms. An overview is given in table 1.

		Genus or type	Species (examples only)
Free-living	anaerobic	*Clostridium*	*C.pasteurianum*
		Desulfovibrio	*D.vulgaris*
	facultative aerobic	*Klebsiella*	*K.pneumoniae*
		Bacillus	*B. polymyxa*
	aerobic	*Azotobacter*	*A. vinelandii*
		Azotococcus	*A.agilis*
	cyanobacteria	*Anabaena*	*A.variabilis*
		Plectonema	*Plectonema boryanum*
	photosynthetic	*Rhodobacter*	*Rhodobacter sphaeroides*
Symbiotic	microaerobic	*Rhizobia*	*Rhizobium leguminosarum*
		Azorhizobium	*A. caulinodans*
		Bradyrhizobium	*B. japonicum*
	aerobic	*Anabaena*	*Anabaena azollae*

Table 1. Some nitrogen fixing microorganisms.

The process of nitrogen fixation has been known now for more than 100 years and during this era, the process has been elucidated. Since the discovery of the process of biological nitrogen fixation by Hellriegel and Willfarth [1888], the enzyme complex involved in this process, nitrogenase, has been isolated and extensively studied. The enzyme complex is present in all nitrogen fixing organisms known and the polypeptide composition of the different enzymes is highly conserved amongst them. In *Azotobacter vinelandii* and *Azotobacter chroococcum*, nitrogenase systems were found, containing other metals. These alternative nitrogenase systems, which have a different subunit structure, are discussed later. The nitrogenase complex, present in all nitrogen fixing organisms, comprises two components, a molybdenum-iron containing protein (subunit structure $\alpha_2\beta_2$), and an iron containing protein (subunit structure γ_2) [Bulen and LeCompte, 1966]. Both components are extremely oxygen sensitive.

1.2.2. The molybdenum-iron protein

The larger component of the nitrogenase complex, its properties have been extensively reviewed by Burgess [1985], Lowe [1985] and Smith and Eady [1992], is the molybdenum-iron protein (MoFe-protein), which has a molecular weight of 220,000 Dalton. It is composed of two types of subunits, the α-subunit with a molecular weight of approximately 50 kDa and the β-subunit, with a molecular weight of 60 kDa. Two molybdenum atoms are present in one MoFe protein, together with 24-32 atoms of iron and 24-30 acid labile sulphur atoms, which are distributed over two types of clusters: the FeMo-cofactor (FeMoco) and the P-clusters.

FeMoco from the MoFe protein can be extracted from the holo-protein, but the composition [Shah and Brill, 1977; McLean *et al.*, 1988; Wink *et al.*, 1989] and electrochemical properties [Newton *et al.*, 1989] of the cofactor depend on the extraction method used. When the extracted FeMo-cofactor is added to MoFe protein of *Azotobacter vinelandii* or *Klebsiella pneumoniae*, which have been genetically altered so that they lack the cofactor, catalytic activity is restored [Shah and Brill, 1977]. Elemental analyses revealed that the FeMo cofactor contained one Mo-, 6-8 Fe- and 4-8 S-atoms. Homocitrate has also been found to be a component of FeMoco [Shah *et al.*, 1990]. The results from side-directed mutagenesis studies indicate that the FeMo-cofactor is associated with the α-subunit [Scott *et al., 1990*; Kent *et al.*, 1989].

No univocal decision is present about the remaining iron atoms, not involved in FeMoco. They are located in either four special forms of [4Fe-4S] clusters [Zimmermann *et al., 1978*] or two [8Fe-8S] superclusters [Pierik *et al.*, 1992]. The role of these clusters, together with their distribution over the protein, is still unknown, but a function in the intramolecular transfer of electrons has been proposed. McLean *et al.* [1988] have proposed

different functions: the P clusters may function as 1) an electron sink, a temporary deposit for electrons used for reduction of the substrate, which requires 8 electrons; 2) an acceptor for electrons donated from the Fe-protein; 3) a delocalisation system for electrons from FeMoco; 4) the substrate binding site or 5) the site were hydrogen atoms are reduced to form hydride.

1.2.3. The iron protein

The Fe-protein is a dimer composed of two identical subunits with a molecular mass of ± 30 kDA each and is generally believed to contain one [4Fe-4S] cluster [Burgess, 1985; Lowe, 1985], although Braaksma et al. [1983] found the iron and sulphur content could be higher. This [4Fe-4S] cluster determines the biophysical properties of the Fe-protein. The cluster is located between the two subunits of the Fe-protein and ligated to cysteines 97 and 132 [Hausinger and Howard, 1983], which was also confirmed by site directed mutagenesis studies [Howard et al., 1989] and crystallographic studies [Georgiadis et al., 1990; Georgiadis et al., 1992]. A schematic representation of the structure of the Fe protein is shown in Figure 2.
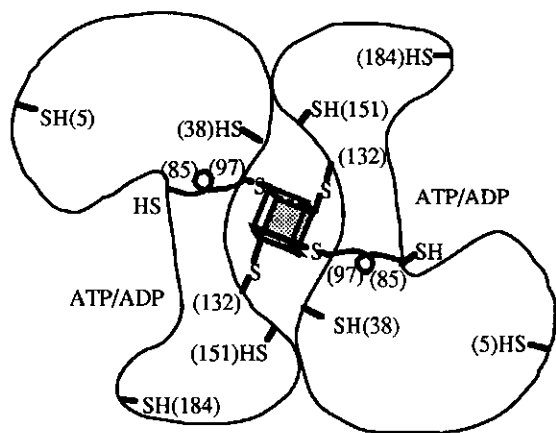

Figure 2. Schematic representation of the Fe protein of nitrogenase. From Hausinger and Howard [1983].

In 1936, Bortels had already discovered that vanadium could replace molybdenum as a requirement in medium for nitrogen fixation by Azotobacters [Bortels, 1936], but this observation was not further investigated. In 1980, the idea of the existence of an alternative, non-molybdenum nitrogenase system was proposed by Bishop *et al.*, which was proven six years later, indicating that more than one nitrogenase system is present [Bishop *et al.*, 1986a; Joerger *et al.*, 1986].

Until now, three nitrogenase systems are known, for which both biochemical and genetical evidence is present. Extensive research has been performed on the molybdenum nitrogenase, described above. The two alternative nitrogenases, the vanadium nitrogenase and the all-Fe nitrogenase, have been discovered recently. The vanadium nitrogenase is present in both *A. vinelandii* and *A. chroococcum* when molybdenum is lacking from the medium and vanadium is present [Eady *et al.*, 1988; Robson *et al.*, 1986a; Smith *et al.*, 1988]. In both organisms, the genes encoding the vanadium nitrogenase, the *vnfHDGK* genes, have been cloned, sequenced and mutagenised [Robson *et al.*, 1986b; Robson *et al.*, 1989; Raina *et al.*, 1988; Joerger *et al.*, 1990]. The genes do not form one operon, but the *vnfH* gene, encoding the Fe-protein of the vanadium nitrogenase system, is separated from the other three genes, that encode the VFe-protein.

The V-nitrogenase complex consists of two components, the VFe protein and the Fe-protein-2. The VFe-protein is thought to be a hexamer (M_r= 240 kDa) of two dissimilar pairs of large subunits (α and β, encoded by *vnfD* and *vnfK*) and a pair of small subunits (δ, encoded by *vnfG*) [Robson *et al.*, 1989; Joerger *et al.*, 1990]. The VFe protein has been found to contain a cofactor similar to the FeMo-cofactor of the MoFe protein, in which Mo is substituted by V [Smith *et al.*, 1988].

The Fe-protein-2 of the vanadium nitrogenase system is a dimer of two identical subunits (γ, encoded by *vnfH*), containing four Fe atoms and four acid-labile sulphide groups [Hales *et al.*, 1986; Eady *et al.*, 1988].

A. vinelandii was found to contain a third nitrogenase system, synthesised under Mo- and V- deficient conditions [Chisnell *et al.*, 1988], a nitrogenase system, which was also found in *Rhodobacter capsulatus* [Schneider *et al.*, 1991]. The genes encoding this nitrogenase system in *A. vinelandii* (*anfHDGK*) were cloned, sequenced and mutagenised by Joerger and coworkers [1989a]. The third nitrogenase is composed of two components, of which the Fe protein-3 is a homodimer with a molecular mass of 65000 Dalton. The second component of the alternative nitrogenase, containing only iron, resembles the MoFe and VFe proteins and is a tetramer (M_r = 216 kDa) of two subunits (α and β, encoded by *anfD* and *anfK*). However, it can not be excluded that this protein is a hexamer, since the genetic organisation of the structural genes reveals the presence of an open reading frame,

of which the predicted product is similar to the δ-subunit of the VFe-protein [Joerger *et al.*, 1989a].

When the genes for both the Mo-nitrogenase- and the V-nitrogenase were deleted in *A. chroococcum*, the double-mutated strain was unable to grow under any nitrogen fixing conditions, indicating that *A. chroococcum* does not contain the third nitrogenase system, as does *A. vinelandii* [Robson *et al.*, 1989].

1.3. The nitrogenase reaction

The stoichiometry of the reaction catalysed by nitrogenase under optimal conditions is shown is equation 2. As can be seen from the reaction equation, 8 electrons are required to reduce one molecule of atmospheric dinitrogen. Since the Fe protein is capable of donating only one electron at a time, which is stored in the MoFe protein, the electron-transfer reaction has to cycle eight times before two molecules of NH_3 and one molecule of H_2 are formed.

$$N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i$$
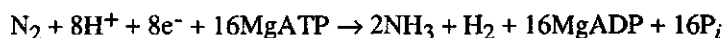
Equation 2. Nitrogen fixation by diazotrophic microorganisms.

The reduction of atmospheric dinitrogen is actually taking place at the MoFe protein, whereas ATP-hydrolysis is considered to take place at the Fe-protein. Detailed information concerning the mechanism of this complex enzyme system is given by Mensink [1992]. It should be emphasised that the reaction, depicted in equation 2, is the reaction under optimal conditions; under less optimal conditions, the amount of MgATP hydrolysed and H_2 per molecule of NH_3 formed, is higher. The production of hydrogen is concomitant with the formation of ammonia. The hydrogen formed is used as substrate by a hydrogenase, present in both Azotobacters and some Rhizobia, to (a) produce ATP (b) increase respiratory protection and (c) prevent H_2 from inhibiting nitrogen fixation [Yates, 1988].

Not only atmospheric dinitrogen is used as substrate for nitrogenase, several other molecules containing triple bonds, like acetylene (C_2H_2), N_2O, N_3^-, CN^- and H^+, can be reduced by nitrogenase [Postgate, 1982]. The acetylene reduction assay is used to determine nitrogenase activity by gaschromatography.

1.4. Influence of oxygen on the nitrogenase system

Nitrogen fixation can take place only under anaerobic conditions. Oxygen is a disturbing agent in the nitrogen fixing environment, since (a) the electron carriers that

donate electrons to nitrogenase are rapidly oxidised (b) the nitrogenase enzyme complex is rapidly oxidised and (c) the nitrogenase is inactivated when exposed to relative high oxygen concentrations. Anaerobic nitrogen fixers do not have the problem of oxygen being in the way, but (micro)aerobic nitrogen fixing bacteria have to protect the nitrogenase proteins against oxygen, because nitrogenase is extremely sensitive to oxygen [Bergesen, 1984].

They do so by physical barriers (Rhizobia), high respiratory rates (Azotobacters) and special proteins that bind to nitrogenase (Azotobacters) to protect it against oxygen damage, after a sudden exposure to oxygen. When the respiration rate can not keep up with the entrance of O_2, a second protection mechanism is activated. Binding of a [2Fe-2S] protective protein (also called Fe/S II or "pink protein") to nitrogenase inhibits O_2 to damage the nitrogenase proteins [Haaker and Veeger, 1977; Robson, 1979a; Robson 1979b; Scherings et al., 1983]. A system called respiratory protection is then activated The respiratory capacity is then increased to meet the new demand (respiratory protection). This lowers the oxygen concentration in the cell. When the cell has become anaerobic, the blockade of the nitrogenase complex by the pink protein is relieved, yielding active nitrogenase.

In various symbiotic bacteria, a two component regulatory system, analogous to the ntrB/ntrC system of K.pneumoniae, is discovered, regulating nitrogen fixation in response to oxygen. This system is formed by the gene products of the fixL and fixJ genes (Fig. 3) [David et al., 1988; Kaminski and Elmerich, 1991].
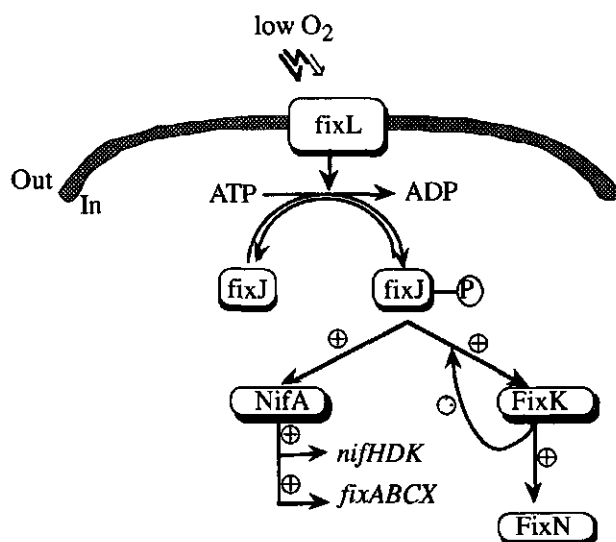
Figure 3. The regulation of nitrogen fixation in R. meliloti. After David et al., 1988.

The FixL protein recognises a microaerobic environment and triggers the FixJ protein, probably by phosphorylation. The activated FixJ protein then directs transcription of the *nifA* and the *fixK* genes. The mechanism and function of the FixLJ complex of *B. japonicum* may be different from the situation in *R. meliloti* , since aerobic expression of the *B. japonicum fixRnifA* operon was not effected by mutations in the *fixLJ* genes [Anthamatten and Hennecke, 1991]. *FixL* and/or *fixJ* -like genes have not been found in *A. vinelandii.*

The *fixK* gene product was also suggested by Batut *et al.* to be involved in regulation of *nifA*-expression in *R. meliloti* in response to oxygen [1989], but their results do not support this conclusion.

In *A. vinelandii*, cytochrome *d* was found to be involved in protection of nitrogenase against oxygen and in such the cytochrome *d* is necessary for nitrogen fixation in air, but not under microaerobic conditions [Kelly *et al.*, 1990].

1.5. Regulation of nif gene expression.

The regulation of the *nif* gene expression has been studied extensively in *K.pneumoniae*. The genetic studies of *nif* regulation in azotobacter indicate a considerable agree of similarity with the *ntr/nif* system in Klebsiella [Kennedy and Robson, 1983; Santero *et al.*, 1986; Toukdarian and Kennedy, 1986], although the response to environmental stimuli does not always resemble the situation present in *K.pneumoniae*.

The situation in Azotobacters is complicated by differential expression of the genes for the three nitrogenases, depending on the availability of metals in the medium [Bishop *et al.*, 1990]. Each nitrogenase system has been found to have their own regulatory protein, called NifA, VnfA and AnfA [Joerger *et al.*, 1989b].

The model of regulation of nitrogen fixation in *K.pneumoniae* has been proposed by Merrick [1983] and Ow and Ausubel [1983]. In this model, three genes, *rpoN* (*ntrA*), *ntrB* and *ntrC* control expression of the *nif* regulatory operon *nifLA* in response to the N-status of the cell. The model is shown in Fig. 4.
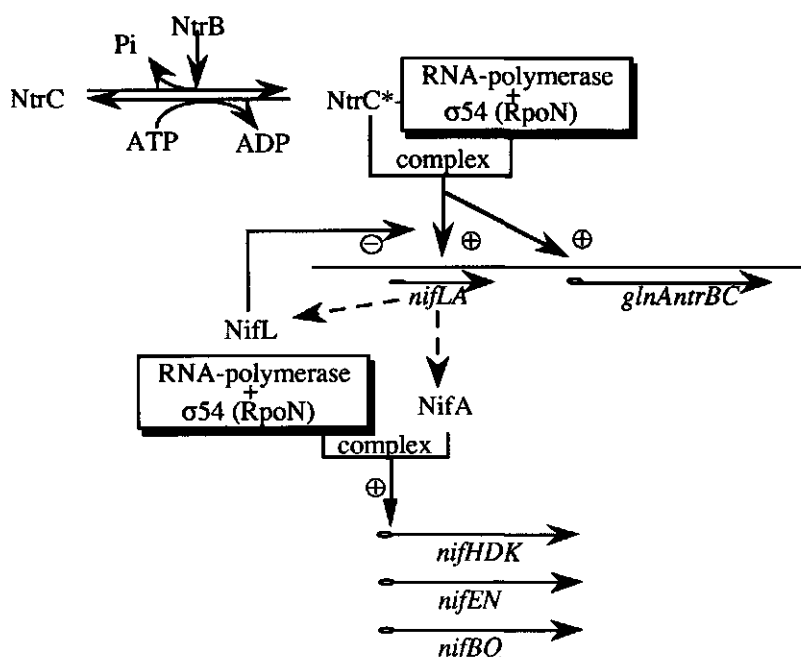
Figure 4. Regulation of nitrogen fixation in *K.pneumoniae*. ⊕ indicates activation, whereas ⊖ indicates repression. "+" indicates that both factors are required to perform the stimulation.

The *nifLA* operon is the master switch for *nif* gene expression. NifL counteracts NifA activity. NifA and NtrC are in more than one case similar: both are present in operons involved in activation and repression, and also sizes and iso-electric points do not differ much [Merrick, 1983]. NifA can replace activated NtrC in activation of the promoter of *nifLA*, but NtrC cannot replace NifA in activation of other *nif* genes [Ow and Ausubel, 1983; Merrick, 1983].

The gene products of *nifL* and *nifA* regulate, together with the *rpoN* gene product and RNA polymerase, expression of the other *nif* operons in response to both oxygen and nitrogen status in *K.pneumoniae*. The product of *rpoN* is a RNA polymerase sigma factor (σ^{54}) which directs the core RNA polymerase to recognise a distinctive consensus sequence at position -24/-12 of the transcriptional start site [Beynon *et al.*, 1983]. This -24/-12 promoter sequence has not only been found in *K.pneumoniae*, but also in other diazotrophic organisms [for review, see Gussin *et al.*, 1986].

The NifA protein binds to a sequence further upstream of the promoter, the so-called Upstream Activator Sequence (UAS). This sequence has the consensus TGT-N_{10}-ACA and is located more than 100 base pairs upstream of the transcriptional start site [Buck *et al.*, 1986]. Santero and coworkers [1990] proposed, that a special protein, the Integration

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Host Factor (IHF), binds to a region of DNA between the UAS and the -24/-12 region. Binding of the IHF causes the DNA to nick, resulting in a conformation of the DNA, in which the NifA protein, bound to the UAS, is in close contact with the RNA polymerase complexed with σ^{54}. When this complex is formed, enhanced transcription of the adjacent genes follows.

The NifL protein is a negative regulatory protein and counteracts the *nifA* gene product. In *K.pneumoniae*, high concentrations of oxygen and/or nitrogen cause the NifL protein to inhibit nitrogen fixation. The *ntrC* gene is also switched off when a high concentration of nitrogen is present in the environment. This is caused by dephosphorylation, mediated by the NtrB protein.

NifA-like and *nifL*-like genes have also been found in *A. vinelandii* [Bennet *et al.*, 1988a; Bali *et al.*, 1992] but, in contradiction to the situation in *K.pneumoniae*, activation of *nifA* is not controlled by fixed nitrogen.

The regulation of nitrogen fixation in Rhizobia is mainly dependent on the oxygen concentration of the environment and less on the availability of fixed nitrogen sources in the environment. The mechanism of regulation (by the *fixLJ* gene products) is described in the paragraph concerning the influence of oxygen on the nitrogenase system.

Recently it was proposed by Thomson that ferredoxin I (Fd I) of both *A. vinelandii* *(Av)* and *A.choococcum* *(Ac)* represent a novel class of DNA-binding proteins that regulate gene expression in response to cellular iron(II) [Thomson, 1991]. *Av* Fd I will complex iron avidly above pH 8.0. only when the ferredoxin is reduced and when cellular nucleic acid is bound. A similar mechanism has been found in eucaryotic cells, where a specific protein, the iron-responsive element binding protein (IRE-BP) controls protein expression at the level of mRNA. A similar mechanism could apply for the ferredoxins of *A. vinelandii* and *A. chroococcum*. The proposed control mechanism that is involved in this process, is depicted in figure 5.

The binding of the ferredoxin to the DNA is believed to take place in the wide groove of the DNA. The binding of the iron(II) to the ferredoxin serves two purposes. First it controls the iron(II) levels of the cell, and secondly it is the trigger, together with the redox potential of the cell, to start gene transcription. As long as the ferredoxin is bound to the DNA, no gene transcription takes place. The semi-reduced ferredoxin-DNA complex (with a [3Fe-4S] cluster charge of 0) has a high affinity for iron(II). When the semi-reduced 7Fe ferredoxin has taken up an iron(II) atom, thus becoming a 8Fe ferredoxin, the affinity of the ferredoxin for the DNA lowers and the binding is broken and transcription commences. The [3Fe-4S] cluster is the place where the eighth iron(II) atom is believed to be stored in the ferredoxin. A possible regulatory role for the ferredoxin of *A. vinelandii* in nitrogen fixation can not be ruled out, since nitrogen fixation involves a the reduction of low redox potential mediators such as ferredoxins.

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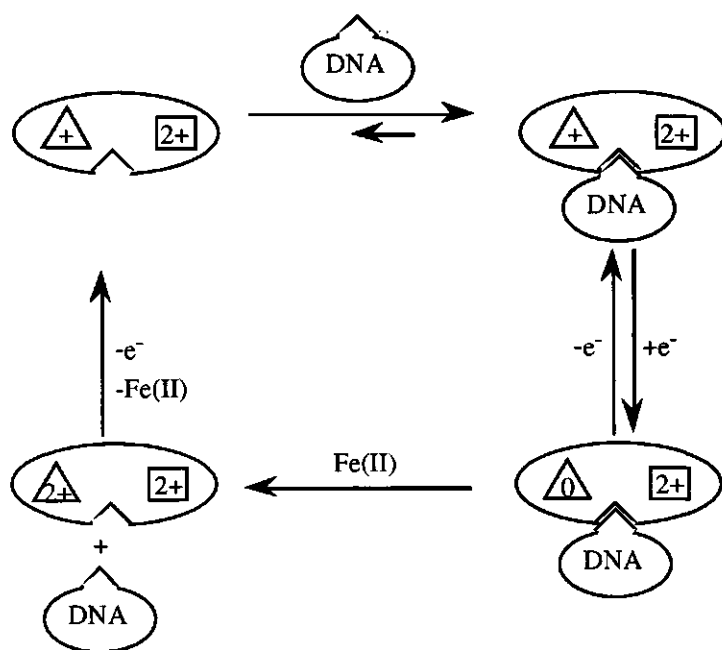
Figure 5. Proposed reaction scheme for the binding of the 7Fe form of ferredoxin I of
Azotobacter to DNA and the loss of binding following reduction of the [3Fe-
4S] cluster an uptake of iron(II) to generate the 8Fe Fd I. Recovery of the 7Fe
FdI takes places when the iron(II) level drops and when the redoxpotential
rises. No transcription of genes takes place when the 7Fe Fd I is bound to the
DNA. △ : [3Fe-4S] cluster, □ : [4Fe-4S] cluster, the numbers in the symbol
indicate the charge of the cluster.

1.6. Function of the nif and fix genes involved in nitrogen fixation

The genes involved in nitrogen fixation, the *nif* and *fix* genes, will be discussed in
this paragraph. The so-called *nod*-genes of symbiotic bacteria [Fuller *et al.*, 1983], involved
in the infection process and root nodule formation, and the genes involved in the alternative
nitrogenase systems [Bishop *et al.*, 1990] will not be discussed here.

1.6.1. The nif genes

Since *K.pneumoniae* was the one of the first organisms of which the genes involved
in nitrogen fixation were investigated, most information is known about this organism. The
genes involved in nitrogen fixation form in *K.pneumoniae* one cluster, the so-called *nif-*

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cluster. This cluster comprises 21 genes, organised in 7 transcription units, which encode all polypeptides involved in nitrogen fixation [Merrick, 1988; Arnold *et al.*, 1988].

The genes are present in the cluster in the following order: *nifJCHDKTYENXUSVWZMFLABQ*. Table 2 gives an overview of the functions and clustering of the genes in *K.pneumoniae*.

In *A. chroococcum*, a group of genes spanning 25-30 kb of DNA was characterised to hybridise to the *nifHDK* genes of *K.pneumoniae* [Jones *et al.*, 1984]. The *nif* genes *HDKENUSVPWZMF* are present in this region [Evans *et al.*, 1985; Jones *et al.*, 1984; Evans *et al.*, 1991], so there seems to be a resemblance to the *nif* cluster of *K.pneumoniae*, though not all *A. vinelandii* genes are present in the *K.pneumoniae* gene cluster. The same is also found for genes, present in Azotobacters, but absent in Klebsiella, *e.g.* the genes involved in the alternative nitrogenases. *A. vinelandii* was found to have a similar arrangement of *nif* genes [Brigle *et al.*, 1985; Kennedy *et al.*, 1986; Helfrich *et al.*, 1985].

Gene	Function	cluster
nifJ	electron transport to nitrogenase	1¶
nifC	unknown	1¶
nifH	Fe protein	2
nifD	MoFe protein α-subunit	2
nifK	MoFe protein β-subunit	2
nifT	unknown	2
nifY	unknown	2
nifE	FeMo cofactor biosynthesis	3
nifN	FeMo cofactor biosynthesis	3
nifX	unknown	4
nifU	unknown	4
nifS	unknown	4
nifV	FeMo cofactor biosynthesis	4
nifW	unknown	4
nifZ	MoFe protein processing?	4
nifM	Fe protein processing	4
nifF	electron transport to nitrogenase	5¶
nifL	repression	6
nifA	activation	6
nifB	Mo processing	7
nifQ	Mo processing	7

Table 2. Functions of the *K.pneumoniae nif* gene cluster. For detailed information see text. The numbers indicate the gene cluster to which the particular gene belongs, ¶ indicates that the direction of the transcription unit is reverse, compared to the other clusters.

The nifH, nifD and nifK genes

The genes involved in the biosynthesis of the structural proteins of the nitrogenase complex are *nifH*, *nifD* and *nifK*. The *NifH* gene encodes for the two identical subunits of

the Fe-protein (AV$_2$) [Brigle *et al.*, 1985] and is also required for insertion of the FeMo-cofactor (FeMoco) in the MoFe-protein, both in *Azotobacter* and *Klebsiella*. Extracts of a Δ*nifH*-strain, containing FeMoco-less MoFe protein can be activated with purified Fe-protein, FeMoco, dithionite and ATP in *K.pneumoniae* and *A. vinelandii* [Filler *et al.*, 1986; Robinson *et al.*, 1987; Robinson *et al.*, 1989]. *NifD* encodes the α-subunit of the MoFe-protein, whereas *nifK* encodes the β-subunit of the MoFe-protein [Brigle *et al.*, 1985]. The gene products of the *A. vinelandii* and *A. chroococcum* structural genes resemble those of *K.pneumoniae* [Brigle *et al.*, 1985; Robson *et al.*, 1985]. The acetylene-reducing activity of mutants in the structural genes of *A. vinelandii* and *K.pneumoniae* can be restored by plasmids containing the *A. chroococcum nifHDK* genes [Jones *et al.*, 1984; Kennedy *et al.*, 1986], so the nitrogenase polypeptides from one organism can substitute for the identical proteins of other organisms.

In *A. vinelandii*, two other regions distinct from *nifHDK* hybridise to *nifH* [Bishop *et al.*, 1986b; Jacobson *et al.*, 1986]. These regions encode the structural proteins of the alternative nitrogenase systems.

The nifQ and nifB genes

Biosynthesis of the FeMo-cofactor requires the *nifQ* and *nifB* gene products. The NifQ protein of *K.pneumoniae*, also found in *A. vinelandii* [Joerger and Bishop, 1988], is involved in early steps of Mo-processing for FeMoco synthesis. [Imperial *et al.*, 1984]. The NifB protein of *A. vinelandii* is also involved in FeMoco biosynthesis, similar to the *nifE* gene product [Tal *et al.*, 1991].

The nifE and nifN genes

NifE encodes a polypeptide that forms a α$_2$ß$_2$ heterodimer together with the *nifN* gene product. The NifN and NifE proteins of *K.pneumoniae, A. vinelandii* and *B. japonicum* are required for transfer of the FeMo-cofactor to the MoFe-protein of nitrogenase: Δ*nifE* (and Δ*nifN*) mutants do not synthesise active MoFe-protein [Roberts *et al.*, 1978; Brigle *et al.*, 1987; Aguilar *et al.*, 1990]. This FeMo-cofactor-less MoFe protein cannot be re-activated to wild type levels with Fe-protein and ATP. *NifE* has homology with *nifD*, the gene for the α-subunit of the MoFe-protein [Dean and Brigle, 1985], whereas *nifN* has homology with *nifK*, the gene encoding the ß-subunit of the MoFe-protein [Brigle *et al.*, 1987; Tal *et al.*, 1991; Paustian *et al.*, 1989; Roberts *et al.*, 1978].

The nifU gene

NifU encodes a protein, essential for diazotrophic growth in Azotobacters: Δ*nifU*-mutants do not grow under nitrogen-fixing conditions [Kennedy and Dean, 1992]. Furthermore, these mutants produce both MoFe-protein and Fe-protein with a low activity in the acetylene reduction assay [Jacobson *et al.*, 1989b]. The activity of the Fe-protein and

MoFe-protein in the double mutant ΔnifUS is still further reduced, compared to both single mutants. The low activity of the MoFe-protein cannot be restored with purified FeMo-cofactor and Fe-protein. The nifU-product might be involved in activation or stabilisation of the Fe-protein. NifU is dispensable in Klebsiella [Roberts & Brill, 1980], but is required for all three nitrogenase systems in A. vinelandii [Kennedy and Dean, 1992].

The nifS gene

The NifS protein is also required for optimal diazotrophic growth of Azotobacter: ΔnifS-mutants grow very slowly under nitrogen-fixing conditions, and produce MoFe-protein with a low activity in the acetylene reduction assay. Identical to ΔnifU mutants, the Fe-protein has hardly any activity. The low activity of the MoFe-protein cannot be restored with purified MoFe-cofactor and Fe-protein. The nifS-product might be involved in activation or stabilisation of the Fe-protein, as is the nifU gene product [Jacobson et al., 1989b; Kennedy and Dean, 1992]. NifS is required for all three nitrogenase systems in A. vinelandii [Kennedy and Dean, 1992].

The nifV gene

NifV encodes homocitrate synthase. Homocitrate is part of the MoFe-cluster and probably forms a scaffold around which the cluster is made. ΔnifV-mutants have low diazotrophic growth rates and produce MoFe-protein with an altered FeMo-cofactor. In vitro reconstitution experiments with extracts of ΔnifV-mutants leads to an altered substrate reduction properties [Jacobson et al., 1989b; Hawkes et al., 1984; Hoover et al., 1986; Hoover et al., 1988; Hoover et al., 1989].

NifV is required for all three nitrogenase systems in A. vinelandii [Kennedy and Dean, 1992].

The nifW gene

The NifW gene product of A. vinelandii and K.pneumoniae is required for optimal diazotrophic growth and activity of the MoFe-protein. ΔnifW-mutants have a twofold reduced growth rate under diazotrophic conditions and a twofold reduced activity of the MoFe-protein. The activity of the Fe-protein is not altered [Jacobson et al., 1989b; Paul and Merrick, 1989]. The nifW gene product is required fro the full expression, stability or processing of the MoFe protein.

The nifM gene

The nifM gene encodes for a protein, essential for diazotrophic growth and activity of the Fe-protein. ΔnifM-mutants do not grow under nitrogen fixing conditions, contain no active Fe-protein and have a three-fold lower activity of the MoFe-protein. A ΔnifZM double mutant shows no MoFe-protein activity.

Active *K.pneumoniae* Fe-protein can be made in *Escherichia coli* by co-expression of the *nifH* and *nifM* genes, suggesting that these two genes are sufficient for biosynthesis of the Fe-protein. The function of *nifM* might be related with biosynthesis of the [FeS]-cluster in this protein [Jacobson *et al.*, 1989b; Howard *et al.*, 1986; Howard *et al.*, 1989].

The nifZ gene

The gene product of *nifZ* is necessary for active MoFe protein in *A. vinelandii*. When a mutant in *nifZ* was made by insertion of Tn5, a Nif⁻ phenotype was found [Beynon *et al.*, 1988]. No Nif⁻ phenotype was found in the case of a deletion mutant of *nifZ* in the same organism, but instead a reduced MoFe-protein activity, together with a twofold reduced growth rate was observed [Jacobson *et al.*, 1989b]. It was believed that insertion of Tn5 in *nifZ* also inactivated *nifM*, located directly downstream of *nifZ*.

The *nifZ* gene is also not essential in *K.pneumoniae*, although a delayed derepression of nitrogen fixation and a reduced MoFe protein activity is observed in mutants lacking a functional *nifZ* gene [Paul and Merrcik, 1988; Paul and Merrick, 1989].

The nifT gene

NifT might encode a protein of unknown function, that is not required for diazotrophic growth in *A. vinelandii* and *K.pneumoniae*. There is no direct evidence, that the *nifT*-gene is really transcribed in *A. vinelandii* [Jacobson *et al.*, 1989a].

The nifP gene

The *nifP* gene product in *A. chroococcum* displays a serine acetyltransferase activity, known to be required for cysteine biosynthesis in *E.coli*.[Evans *et al.*, 1991]. The *nifP* gene is not present in the *K.pneumoniae nif* gene cluster [Evans *et al.*, 1991]. It was suggested that *nifP* gene product may be required to boost rates of synthesis or intracellular concentrations of cysteine or methionine, required for biosynthesis of nitrogenase polypeptides. A Δ*nifP* mutant displays a decreased diazotrophic growth rate, whereas growth on ammonium was normal.

The nifJ and nifF genes

The electron transport to nitrogenase in *K.pneumoniae* is carried out by the products of the *nifJ* and *nifF* genes [Hill and Kavanagh, 1980]. *NifF* encodes a flavodoxin involved in transport of electrons from pyruvate to the Fe-protein of nitrogenase in *K.pneumoniae* (but in Azotobacters also ferredoxins may have this function) and *nifJ* encodes a pyruvate-flavodoxin oxidoreductase [Shah *et al.*, 1983]. *NifF* is dispensable in Azotobacters [Bennet *et al.*, 1988b; Shah *et al.*, 1983].

A *nifJ*-like gene has not been found in Azotobacters [Shah *et al.*, 1983]. Electron transport to nitrogenase in Azotobacters as discussed later, has not been elucidated yet.

The nifX and nifY genes

The *nifX* and *nifY* genes of *K.pneumoniae* were reported not to be essential for nitrogen fixation, although mutations in these genes caused a reduced growth rate under diazotrophic conditions [Gosink *et al.*, 1988]. Later studies by the same authors led to the hypothesis that *nifX* gene product is a negative regulator of nitrogen fixation [Gosink *et al.*, 1990].

In *Azotobacter*, *ΔnifY* or *ΔnifX* mutants have normal diazotrophic growth. *NifX* has some homology with *nifY*. [Brigle *et al.*, 1987; Jacobson *et al.*, 1989a].

The nifL gene

The *nifL* gene encodes a transcriptional regulator of *nif*-gene expression. NifL forms with NifA a two-component regulatory system. The NifL protein is a sensor for *e.g.* the presence of ammonia and/or oxygen and makes the positive regulator *nifA* inactive in the presence of (one of) these compounds. The mechanism by which *nifLA* regulates transcription and the stimuli required for activation are probably different in different organisms [Drummond and Wooton, 1987; Morett *et al.*, 1990; Morett *et al.*, 1991]

The nifA gene

The *nifA* gene product is a transcriptional activator for *nif*-genes. NifA forms with NifL a two-component regulatory system. NifA binds to an upstream activator sequence (UAS): TGT-N_{10}-ACA. Binding enhances transcription of genes containing the *nif* promoter-consensus, These genes are transcribed by RNA-polymerase, complexed with sigma factor (σ^{54}) [Buchanan-Wollaston *et al.*, 1981; Beynon *et al.*, 1983; Gussin *et al.*, 1986; Bennet *et al.*, 1988a]. The regulation of *nif* gene expression by the NifA protein is described above.

DNA intergenic region in nifH, nifD and nifK.

The intergenic regions between *nifH* and *nifD*, and between *nifD* and *nifK* in *A. vinelandii* and *A. chroococcum* contain a potential secondary structure [Brigle *et al.*, 1985; Robson *et al.*, 1985]. These regions are 20-40 bp sequences, which are capable of formation of single stem and loop structures that may influence gene expression or mRNA stability. During derepression of *A. chroococcum* cells, three mRNA's are formed, having sizes indicating that termination of each mRNA occurs at the intergenic region [Jones *et al.*, 1984; Krol *et al.*, 1982]. The consequences for nitrogen fixation are not known, but it could be a way to regulate the relative amounts of the nitrogenase components in different growth conditions. Since these special intergenic regions are not present in *K.pneumoniae* [Holland *et al.*, 1987], it is uncertain whether these DNA fragments are involved in regulation of transcription or stabilisation of mRNA [Kennedy and Toukdarian, 1987].

A number of genes have been found to be involved in nitrogen fixation in Rhizobia, for which no homologous genes could be identified in *K.pneumoniae*. By definition, these genes are called *fix* genes.

Mutagenesis of these genes in Rhizobia led to a phenotype, in which normal nodules were formed, but from which no nitrogenase activity could be observed. Most of these genes have been found in bacteria that are able to fix nitrogen in symbiosis with higher plants [Earl *et al.*, 1987; Fuhrmann *et al.*, 1986; Kaminski *et al.*, 1988], but also their presence is assumed in free-living diazotrophic bacteria such as *A. vinelandii* and *A. chroococcum* [Gubler et al, 1986; Evans *et al.*, 1988].

For a number of these *fix* genes, no function has been assigned yet, but the function of some *fix* genes has already been assigned, *e.g.* the *fixL* and *fixJ* gene products are involved in the regulation of *nif* gene expression in response to oxygen [Batut *et al.*, 1989; Hertig *et al.*, 1989] and the *fixGHIS* operon of *R. meliloti* probably encodes a membrane bound redox complex, absent in *A. chroococcum* [Kahn *et al.*, 1989]. The *fixABCX* genes have been suggested to be involved in electron transport to nitrogenase in *B. japonicum* [Gubler and Hennecke, 1986].

The *fix* genes, found so far, and their proposed functions are summarised in table 3.

fix	*Species*	*Function proposed/Homologies*	*Reference*
A	*B. japonicum*	Electron transport to nitrogenase	Gubler and Hennecke, 1986
	R. meliloti	Not known	Earl *et al.*, 1987
	A. caulinodans	Not electron transport to nitrogenase	Kaminski *et al.*, 1988
	R. leguminosarum	Not known	Hontelez *et al.*, 1989
	A. caulinodans	Not known	Arigoni *et al.*, 1991
	A. vinelandii	Not known	This thesis
B	*A. caulinodans*	Homology to α-subunit of Electron Transfer Flavoprotein	Arigoni *et al.*, 1991
	R. meliloti	Not known	Earl *et al.*, 1987
	B. japonicum	Not known	Gubler and Hennecke, 1986
	A. vinelandii	Not known	This thesis
C	*R. meliloti*	Not known	Earl *et al.*, 1987
	R. trifolii	Not known	Iismaa and Watson, 1987.
	R. leguminosarum	Not known	Grönger *et al.*, 1987
	B. japonicum	Not known	Gubler and Hennecke, 1986
	A. caulinodans	Contains FAD bindingsite, involved in maturation of Fe protein	Arigoni *et al.*, 1991
	A. vinelandii	Not known	This thesis
D	*R. meliloti*	Regulatory protein, *nifA* like protein	Weber *et al.*, 1985
F	*R. meliloti*	Unknown	Aguilar *et al.*, 1985

fix	Species	Function proposed/Homologies	Reference
G	R. meliloti	FixG protein contains two cysteine clusters of the type found in bacterial ferredoxins. and is localised in cytoplasmic membrane. *fixGHI* and *fixS* form a membrane bound complex, involved in redox process catalysed by FixG, in which the FixI product acts as an cation ATPase	Kahn *et al.*, 1989
H	R. meliloti	See *fixG*	Kahn *et al.*, 1989
I	R. meliloti	the FixI protein is a cation pump. See also *fixG*:	Kahn *et al.*, 1989
J	R. meliloti	FixJ is a positive regulatory protein FixJ is activated by the FixL-protein	David *et al.*, 1988
	B. japonicum	Regulation of symbiotic nitrogen fixation, different from *R. meliloti* regulation	Anthamatten and Hennecke, 1991
	A. caulinodans	Regulatory protein, activated by FixL protein	Kaminski and Elmerich, 1991
K	R. meliloti	Negative <u>and</u> positive regulatory protein of nitrogen fixation. It belongs to the CRP/FNR family.	Batut *et al.*, 1989
	A. caulinodans	Positive regulator of nifA protein	Kaminski and Elmerich, 1991
L	R. meliloti	FixL recognises a signal in symbiotic environment and triggers FixJ. FixL probably acts as a kinase that phosphorylates the DctA protein.	David *et al.*, 1988
	B. japonicum	Regulation of symbiotic nitrogen fixation. Different from *R. meliloti* regulation.	Anthamatten and Hennecke, 1991
	A. caulinodans	Sensor protein of two component system with FixJ, the regulatory protein.	Kaminski and Elmerich, 1991
N	R. meliloti	Unknown	Renalier *et al.*, 1987
P	A. vinelandii	7Fe-Ferredoxin like protein	This thesis
R	B. japonicum	Symbiotic regulatory operon together with *nifA*, *fixR* can encode an oxidoreductase of the insect alcohol-dehydrogenase/ribitol dehydrogenase family. It is expressed aerobically and subject to a novel, *nifA*-independent type of activation.	Thöny *et al.*, 1987

fix	Species	Function proposed/Homologies	Reference
S	R. meliloti	See fixG	Kahn et al., 1989
W	R. leguminosarum	Not known	Hontelez et al., 1989
Yfx1	R. leguminosarum	Hypothetical protein	Hontelez et al., 1989
X	R. meliloti	Ferredoxin-like protein	Earl et al., 1987
	R. meliloti	Ferredoxin-like protein	Dusha et al., 1987
	R. trifolii	Ferredoxin-like protein	Iismaa and Watson, 1987
	R. leguminosarum	Ferredoxin-like protein	Roelvink et al., 1989
	R. leguminosarum	Ferredoxin-like protein	Gronger et al., 1987
	B. japonicum	Ferredoxin-like protein	Gubler et al., 1989
	A. caulinodans	Ferredoxin-like protein	Arigoni et al., 1991
	A. vinelandii	7Fe- Ferredoxin-like protein	This thesis
Z	R. leguminosarum	NifB-like protein, cysteine rich, involved in metal binding, consistent with its function in FeMo cofactor biosynthesis.	Rossen et al., 1984

Table 3. Overview *fix* genes known in various species and their functions.

1.6.3. General genetics of Azotobacter vinelandii.

The genius Azotobacter belongs to the family Azotobacteracae, of which all members are able to fix atmospheric nitrogen. The Azotobacteracae are limited to two genera, according to *Bergey's Manual* [Tchan, 1984] namely *Azotobacter* and *Azomonas*. Azotobacters are aerobic organisms that are mainly found in the soil. Most of the strains are free-living, but some strains associate with roots of plants, as do Rhizobia, *e.g.* *Azotobacter paspali* associates with the tropical grass *Paspalum notatum* [Barea and Brown, 1974; Boddey et al., 1983].

A feature of Azotobacters is their extreme tolerance for oxygen while fixing nitrogen, using high respiratory rates, as described above.

The developments in molecular biology have facilitated the genetic analysis of nitrogen fixation and related aspects of nitrogen fixation, oxygen, and hydrogen metabolism in Azotobacters. With the exception of a number of papers on *Azotobacter beijerinckii*, most of the genetical research on Azotobacters has been carried out in *A. vinelandii* and *A. chroococcum*. Between the latter two, there is a difference in uptake of DNA: *A. vinelandii* is generally a better recipient of DNA than is *A. chroococcum*. This may be related to the fact that in most *A. chroococcum* strains indigenous plasmids (size 5.5-200 Mb) are present [Robson et al., 1984] which could inhibit DNA transfer. No plasmids have been found in laboratory strains of *A. vinelandii*.

Because of the higher transformation efficiency, most genetic research has been carried out in *A. vinelandii*. The way to induce competence in *A. vinelandii* differs from the traditional method of Ca^{2+} shock [David *et al.*, 1981] in such a way, that growth under molybdenum and iron limiting conditions suffices to induce *A. vinelandii* to become competent [Glick *et al.*, 1985; Page and Doran, 1981; Page and Sadoff, 1976; Page and vonTigerstrom, 1978; Page and vonTigerstrom, 1979]

The fact, that Azotobacters contain more DNA than most other bacteria, whereas the genome size of the DNA is typical for prokaryotes (2000 kb [Robson, 1984]), may be related to the fact that the azotobacter cell is ± 10-fold larger than other prokaryotic cells [Sadoff *et al.*, 1979]. To come to such a high DNA content per cell, the number of genome copies is high, for it was found by Robson and coworkers [1984] that *A. chroococcum* contains at least 20-25 copies per cell, whereas the copy number for *A. vinelandii* has been estimated to range from at least 40 [Sadoff, 1979] till over 80 [Punita *et al.*, 1989]. Mutagenesis of Azotobacters is hampered by this phenomenon, since the high copy number probably causes problems in chromosome segregation and selection of mutants. When one copy of the wild type gene is maintained in the cell a phenotype not reflecting the effect of the mutation can be found. Misinterpretation of the function of certain genes can be the consequence. It has been found that certain essential genes, *e.g. glnA*, are not exchanged in all copies of the genome, since this would have lethal consequences for the cell. One or more copies of the wild type gene are maintained in the bacterial cell to ensure survival [Kennedy and Toukdarian, 1987].

1.7. Electron transport to nitrogenase

The elucidation of the metabolic processes generating electrons for the reduction of dinitrogen in aerobic bacteria fixing nitrogen, and the routes by which these electrons are transferred to nitrogenase, have been the subject of research throughout many years. This topic is only partially been clarified yet and in this paragraph the current knowledge about electron transport to nitrogenase in diazotrophs will be discussed. Reviews [Haaker and Klugkist, 1987; Haaker, 1988] also deal with the aspects of electron transport to nitrogenase in cyanobacteria, anaerobic and photosynthetic diazotrophs.

The source of both energy and reducing power in anaerobic nitrogen fixing organisms, such as *K.pneumoniae*, is generally believed to be the thioclastic reaction, in which the electrons are generated by the oxidation of pyruvate (eq. 3.) [Yates, 1977].

$$CH_3-\underset{\underset{O}{\|}}{C}-\underset{\underset{O}{\|}}{C}-O^- + COASH + 2Fld/Fd(ox) \longrightarrow CH_3-\underset{\underset{O}{\|}}{C}-\underset{\underset{O}{\|}}{C}-SCoA + CO_2 + 2H^+ + 2Fld/Fd(red)$$

pyruvate   acetyl-CoA
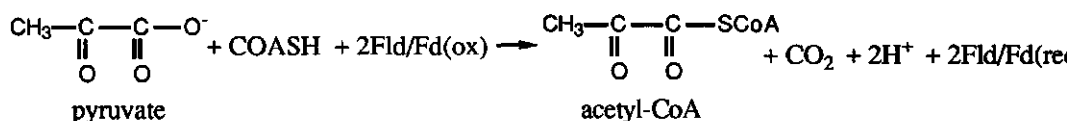
Equation 3. Thioclastic reaction, resulting in the reduction of ferredoxin (Fd) or flavodoxin (Fld).

The enzyme, catalysing this reaction is the pyruvate:ferredoxin (or flavodoxin) oxidoreductase, encoded by the *nifJ* gene (as mentioned above).

The acetyl-CoA formed is further used to generate one molecule of ATP, which explains why in anaerobic nitrogen fixing organisms, the synthesis of ATP is the limiting factor for nitrogen fixation and not the production of reductant, since equation 2 shows that reduction of one molecule of atmospheric dinitrogen requires 8 electrons from reduced ferredoxin, but twice the amount of ATP.

K.pneumoniae is the only organism of which the electron transfer pathway is elucidated both biochemically and genetically. The *nifJ* and *nifF* gene products are the two proteins involved in the transfer of electrons from pyruvate to nitrogenase [Hill and Kavanagh, 1980]. Electrons are transferred from pyruvate to flavodoxin (*nifF* gene product) by pyruvate:flavodoxin-oxidoreductase (the *nifJ* gene product). Electrons are subsequently donated by the flavodoxin to the Fe protein of nitrogenase (*nifH* gene product), which transfers the electrons to the MoFe protein (*nifD* and *nifK* gene products). The MoFe protein finally reduces dinitrogen to yield ammonia. The electron flow to nitrogenase in *K.pneumoniae* is depicted in equation 4.

$$\text{pyruvate} \rightarrow \text{ferredoxin/flavodoxin} \rightarrow Kp.\ \text{Fe protein} \rightarrow Kp.\ \text{MoFe protein} \rightarrow \overset{N\equiv N}{\underset{NH_3}{\Big\downarrow}}$$
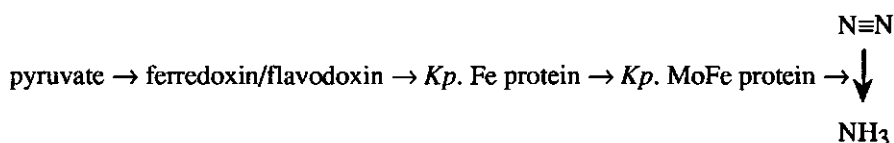
Equation 4. Electron flow to nitrogenase in *K.pneumoniae*. *Stoichiometry* is not according to equation 2. *Kp* Fe protein is *K.pneumoniae* Fe protein; *Kp* MoFe protein is *K.pneumoniae* MoFe protein

Recently it was found that *K.pneumoniae* flavodoxin was posttranslationally modified by covalent attachment of coenzyme A, when flavodoxin was overexpressed in *Escherichia coli*. This attachment prevents electron transfer from the *nifJ* gene product to nitrogenase and could possibly thus be a regulator for nitrogenase activity *in vivo* to the level of dissolved oxygen and the carbon status of the cell [Thorneley *et al.*, 1992]. A regulation in *A. vinelandii*, similar to the proposed regulation in *K.pneumoniae* can not be ruled out yet.

No pyruvate:flavodoxin (ferredoxin) oxidoreductase activity has been detected in aerobic nitrogen fixing organisms, nor has a gene, homologous to the *nifJ* gene been found by interspecies hybridisation. A thioclastic reaction, as depicted in equation 4, is not present in aerobic nitrogen fixing organisms. The major source of reducing equivalents in aerobic nitrogen fixing organisms is NADH or NADPH [Haaker and Klugkist, 1987], which are mainly generated during glycolysis and in the TCA cycle. Another source of NADPH and NADH might be a reversed electron flow from succinate and H_2 to site I of the respiratory chain. The contribution of the reversed electron transport to dinitrogen fixation in Azotobacters will be not significantly, since the relative efficiency of this reaction is low [Häger *et al.*, 1988]. Reversed electron transport is of importance in photosynthetic bacteria to generate reducing agents for nitrogenase. The transhydrogenase, present in *A. vinelandii*, could also generate

Benemann and Valentine [1972] suggested a linear electron transport from NADPH to nitrogenase via ferredoxin and/or flavodoxin. When the NADPH/NADP+ ratio in cell free extracts of *A. vinelandii* was maintained high (approximately 20), using a NADPH regenerating system, electron transfer from NADPH to nitrogenase was observed. However, Haaker and coworkers [1974] discovered that such a high NADPH/NADP+ ratio is not present in intact *A. vinelandii* cells. They found a ratio of 0.4 under diazotrophic conditions, corresponding with a midpoint potential of -330 mV [Klugkist, 1985]. This potential is too high to reduce the Fe protein of nitrogenase *in vivo*, which acts at a midpoint potential of approximately -500 mV when bound to MgADP [Thornley *et al., 1988*]. A second criticism of Benemann and Valentine's hypothesis is, that high NAD(P)H/NAD(P) ratios could inhibit several key enzymes in Azotobacters [Yates, 1977]. It seems therefore not possible that, under physiological conditions, NADPH donates its electrons directly to nitrogenase.

Haaker *et al.* [1974] reported that nitrogen fixation in *A. vinelandii* is dependent on a high energy level of the cytoplasmic membrane. This proton motive force ($\Delta\mu$) was composed of a difference in proton concentration and a difference in charge ($\Delta\psi$) (equation 5)

$$\Delta\mu = \Delta\psi - 59\Delta pH \quad (mV)$$

Equation 5. The proton motive force is composed of two components, a difference in charge ($\Delta\psi$) , and a difference in proton concentration (ΔpH).

The proton motive force is used to synthesise ATP. Addition of small amounts of uncoupler of the respiratory chain, thereby lowering the $\Delta\mu$, caused an immediate decline in nitrogenase activity, but left the respiration of the cell and the ATP/ADP ratio unchanged.

This was the evidence that the proton motif force had a direct role in the electron transport to nitrogenase. Three years later, Haaker and Veeger presented results, indicating that the cytoplasmic membrane of *A. vinelandii* was involved in nitrogen fixation [Haaker and Veeger, 1977]. A model was proposed in which electrons are transferred from reduced pyridine dinucleotides (NADPH and/or NADH) to flavodoxin via a membrane bound NAD(P)H dehydrogenase . The influx of protons, depending on $\Delta\mu$, was held responsible for a local pH drop of more than 2 pH units, which would increase the midpoint potential of the flavodoxin to -380 mV, a potential, high enough for the NAD(P)H:flavodoxin dehydrogenase to reduce the flavodoxin semiquinon to the hydroquinon state. The fully reduced flavodoxin would then dissociate from the dehydrogenase, and diffuse into the cytosol, where, because the pH of the cytosol is higher than 7.0, the midpoint potential would become -490 mV [Barman and Tollin, 1972], low enough to reduce nitrogenase.

This model was further investigated by Laane *et al.* [1979], who showed that the effect of $\Delta\mu$ on nitrogenase activity was caused by $\Delta\psi$, rather than by ΔpH. They showed that, when the $\Delta\psi$ decreased by addition of ionophores, the nitrogenase activity dropped. $\Delta\mu$ may have a function in keeping the nitrogenase enzyme complex in a conformation, in which reduction by NAD(P)H is possible, rather than in the generation of reducing equivalents.

This model was supported by the results of Howard *et al.* [1985], who showed that nitrogenase, though being a soluble protein, might have structural associations with the cytoplasmic membrane. However, using NAD(P)H, flavodoxin and Azotobacter membranes, coreconstituted with bacteriorhodopsin membrane vesicles, no nitrogenase activity could be observed [Haaker, unpublished].

Klugkist and coworkers showed the presence of a linear relationship between the rate of electron transport to oxygen and nitrogenase activity, thereby presenting further evidence that the cytoplasmic membrane is involved in nitrogen fixation in aerobic diazotrophic bacteria [Klugkist *et al.*, 1986]. Their results led to a model, in which two electrons derived from NAD(P)H with a midpoint potential of -320 mV are used to reduce a membrane bound NAD(P)H-dehydrogenase (figure. 6].
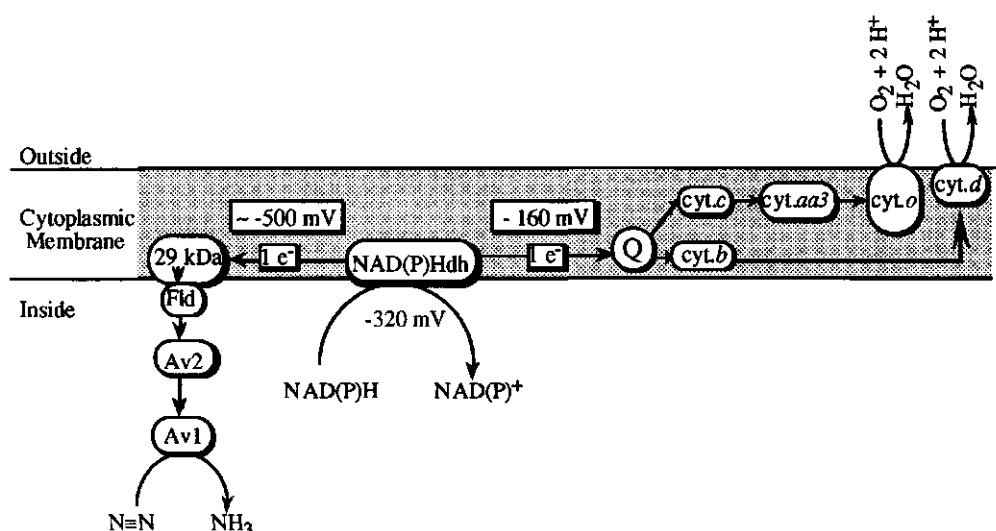
Figure 6. Electron transport mechanism according to Klugkist [1985].

This membrane bound NAD(P)H dehydrogenase, which is expressed during derepression of nitrogen fixation, donates one of the electrons to the respiratory chain at the level of ubiquinone Q ($E_m \sim -140$ mV), thereby transferring the remaining energy of the departing electron to the other electron. This electron now has an E_m of -320 mV + -180 mV = $\sim$ -500 mV, which is sufficient for the reduction of flavodoxin of ferredoxin. This model explains the observed correlation between the respiration rate and nitrogenase activity, since for every electron donated to nitrogenase, one electron has to flow through the respiratory chain to oxygen. All conditions required for nitrogenase activity, a low E_m and the role of $\Delta\psi$, can be explained by this model, since the $\Delta\psi$ is required to hold together a membrane bound complex, consisting of a NAD(P)H dehydrogenase, flavodoxin or ferredoxin and nitrogenase. This model has not been proven yet.

1.9. Outline of this thesis

This thesis deals with the electron transport to nitrogenase in the free-living, obligate aerobic, nitrogen fixing organism *Azotobacter vinelandii*. Little is known about how the flavodoxins and/or ferredoxins, probably the electron donors for nitrogenase, are reduced.

As mentioned, it was suggested that the *fixABCX* genes of various Rhizobia are involved in electron transport to nitrogenase in both symbiotic and free-living state, and that the *fixABCX* genes might also be present in *A. vinelandii* and *A. chroococcum*. The

hypothesis of the involvement of the *fixABCX* genes in electron transport to nitrogenase was contradicted by other groups.

The research described in this thesis deals with the isolation, molecular cloning and sequence analysis of the *fixABCX* genes of *A. vinelandii*. In order to determine whether the *fixABCX* gene products are involved in electron transport to nitrogenase in *A. vinelandii*, or in another process essential for nitrogen fixation, mutagenesis of the *A. vinelandii fixABCX* genes was performed, and the effect of these mutations on diazotrophic growth was investigated. The expression of the *fixABCX* genes was also investigated.

The second part of this thesis deals with the model of electron pair splitting, as described by Klugkist in his thesis [Klugkist, 1985]. The model, described in this introduction, is very attractive, but is not supported by many experimental data. This thesis deals with experimental work, designed to investigate the validity of this model.

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CHAPTER 2.

ISOLATION OF THE *FIXABCX* GENES OF *AZOTOBACTER VINELANDII* AND IDENTIFICATION OF A NEW GENE, *FIXP*, UPSTREAM OF *FIXA*.

ISOLATION OF THE *FIXABCX* GENES OF *AZOTOBACTER VINELANDII* AND IDENTIFICATION OF A NEW GENE, *FIXP*, UPSTREAM OF *FIXA*.

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The nucleotide sequence data reported in this paper have been submitted to Genbank and have been assigned the accession codes X63772 and X65515.

SUMMARY

The nucleotide sequence of a 4.4 kb SmaI-EcoRI fragment of *Azotobacter vinelandii* has been determined. Five open reading frames and the beginning of a sixth one were found. Homology with the *fixA, fixB, fixC* and *fixX* genes of *Rhizobium meliloti*, *Azorhizobium caulinodans* ORS571 and with partial sequences of *Bradyrhizobium japonicum* and *Rhizobium leguminosarum fix* genes was found.

Homology searches revealed that the gene product of *fixB* has a homology with the protein sequence of the α–subunit of the Electron Transfer Flavoprotein (ETF) of both human and rat origin. The N-terminus of the *fixC* gene product contains a sequence homologous with the consensus sequence for an ADP binding site, as found in NAD^+ or FAD dependent enzymes, identical to the *Azorhizobium caulinodans fixC* gene product.

The protein encoded by *fixX* has a high degree of similarity with the FixX proteins of various *Rhizobia*, coding for ferredoxin-like proteins. FixX contains one Cys-X_2-Cys-X_2-Cys-X_3-Cys motif, indicative for a binding site of a [4Fe-4S] cluster. A second Cys-motif (Cys-X_7-Cys-X_3-Cys) was found exclusively found in the *A. vinelandii* FixX protein; the FixX proteins from *rhizobia* lacked this second cluster. The second motif is also present in ferredoxin I of *A. vinelandii*, where it is involved in ligation of a [3Fe-4S] cluster.

In contrast to the rhizobial *fixABCX* genes, upstream of the *fixA* gene, an open reading frame encoding a second 7Fe-ferredoxin was found. This open reading frame was called *fixP*. Based on the position of the [3Fe-4S] and the [4Fe-4S] cluster binding domains in the proteins, the different 7Fe-ferredoxins of *A. vinelandii* can be devided into two groups. FixX and ferredoxin I form the first group, whereas FixP, FdN (the ferredoxin in the *nif* gene cluster) and FdV (the ferredoxin in the *vnf* gene cluster) form the second group.

Downstream of the *fixX* gene, the start of a sixth open reading frame was found, but the N-terminal sequence did not show any homology with other proteins in the database.

A sequence motif with high homology with the promoter consensus for RNA polymerase complexed with sigma factor 54 (σ^{54}) was found 63 bp upstream of the start codon of the *fixP* gene. A putative binding site for the regulatory NifA protein (TGT-N_9-ACA) was found 164 bp upstream of the start codon .

No terminator sequence could be found downstream of the stop codon of any of the genes described in this paper.

INTRODUCTION.

It is known that a number of genes involved in nitrogen fixation do not exhibit homology with any of the *Klebsiella pneumoniae nif* genes. By general agreement these genes are called *fix* genes [Gubler and Hennecke, 1986]. The first genes of this class that were discovered were the *fixABC* genes of *Rhizobium meliloti* [Ruvkun *et al.*, 1982; Corbin *et al.*, 1983; Pühler *et al.*, 1984; Earl *et al.*, 1987].

Since the identification of the *fixABCX* genes in *R. meliloti* [Earl *et al.*, 1987], one or more of the genes have also been found in *Bradyrhizobium japonicum* [Fuhrmann *et al.*, 1985], *Rhizobium leguminosarum* [Schetgens *et al.*, 1985; Grönger *et al.*, 1987], *Rhizobium trifolii* [Iismaa and Watson, 1987] and *Azorhizobium caulinodans* ORS571 [Kaminski *et al.*, 1988, Arigoni *et al.*, *1991*]. It was inferred from Southern blot analysis that homologous genes might also be present in the free-living nitrogen fixing species *Azotobacter vinelandii* and *Azotobacter chroococcum* [Gubler and Hennecke, 1986; Evans *et al.*, 1988]. We have demonstrated previously that the *fixA* gene indeed is present in *A. vinelandii* [Wientjens *et al.*, 1990].

The genetic organisation of the *fixABCX* genes varies between the species. In *R. meliloti*, *R. leguminosarum* and *A. caulinodans*, the *fixABCX* genes are organised as a single operon, whereas in *B. japonicum* the *fixA* gene is separated from the *fixBCX* genes.

Recently, the *fixABCX* genes have also been found in *Escherichia coli* [Yura *et al.*, 1992], which could be an indication that these genes are not only involved in nitrogen fixation, but also in other processes.

Here we report the cloning and determination of the nucleotide sequence of the *fixABCX* genes of *A. vinelandii.* and the identification of a new open reading frame upstream of the *fixA* gene, *fixP*. The nucleic acid derived protein sequence of *fixP* shows characteristics of a bacterial 7Fe-ferredoxin. A homology search was carried out to determine a possible function of the genes. Physiological analysis of the *fixABCX* genes is the subject of the accompanying paper.

METHODS.

Bacterial strains, vectors and growth conditions.

A. vinelandii (strain ATCC 478) was cultured on Burks nitrogen-free basic salts medium with sucrose as sole carbon source, as described earlier [Newton *et al.*, 1953].

E. coli TG2 [Gibson, 1984], a recA⁻ version of TG1 [Δ(*lac-pro*) *thi supE*, [Res⁻ Mod⁻ (k)] F'(*traD36 proA⁺B⁺*, *lacIᑫZΔM15*)] was used as a host for recombinant plasmids and for the propagation of recombinant DNA in M13-derived vectors. *E. coli* was grown at 37°C in TY medium or on TY plates containing 1.5% agar [Miller, 1972]. Ampicillin was added, when appropriate, in a final concentration of 100 μg.ml^{-1}.

Vectors used for cloning were pUC9 [Vieira and Messing, 1982] and pUC18 [Yanisch-Perron *et al.*, 1985]. M13mp18 and M13mp19 were used as vectors for nucleotide sequencing [Norrander *et al.*, 1983].

Construction of a partial library of *A. vinelandii* DNA in *E. coli* and isolation of the *fixABCX* gene cluster.

A. vinelandii DNA was isolated according to Westphal and De Kok [1988]. The DNA was digested with *Eco*RI and 6-9 kb *Eco*RI fragments were isolated after size fractionation of the digested DNA in a 0.7% (w/v) low-gelling-temperature agarose gel as described by Maniatis *et al.* [1982]. These fragments were ligated into *Eco*RI digested and alkaline phosphatase treated pUC9 and the recombinant plasmids were introduced into *E. coli* TG2. As a probe for screening the library, the *fixA* gene of *R. leguminosarum* was used. Therefore, plasmid pRleH21 [Hontelez *et al.*, 1989] was digested with *Hind*III and *Sal*I and a 2.0 kb fragment, containing the C-terminal domain of *fixW* and the *fixA* gene was isolated. Plasmid DNA was isolated according to the method developed by Birnboim and Doly [1979]. All other molecular biological techniques used were as described by Ausubel *et al.* [1987].

Hybridisation conditions

Genomic *A. vinelandii* DNA was digested with *Eco*RI. The DNA was size-fractionated in a 0.6% (w/v) high-gelling-temperature agarose gel and transferred to nitrocellulose according to the method developed by Southern [1975].

The isolated *fixA* gene of *R. leguminosarum* was labelled with [α-^{32}P]dATP by nick-translation [Rigby *et al.*, 1977] and used as a probe for hybridisation with Southern blots containing *A. vinelandii* DNA or with colony-blots of *E. coli* clones. Hybridisation was carried out in 5 x SSC, 5 x Denhardt's solution, 100 μg.ml^{-1} herring sperm DNA and

0.1% (w/v) SDS at 65°C for 16 hours. Blots were washed 3 x 45 min in 3 x SSC, 0.1% (w/v) SDS at 65°C.

DNA sequence determination and analysis.

Several fragments from the originally constructed plasmid pRW4 (Fig. 1a) were ligated into pUC18 and M13 derived vectors. pRW41 (Fig. 1b) was constructed by deleting the 2.9 kb fragment between the *Sma*I-sites in the insert and in the polylinker of pRW4. Plasmid pRW42, containing the *fixA* gene, was made by insertion of the 1.7 kb *Sma*I/*Sst*I fragment of pRW41 into *Sma*I/*Sst*I digested pUC18. pRW43 (Fig. 1b) was constructed from the 2.6 kb *Sst*I/*Eco*RV fragment of pRW41, ligated into *Sst*I/*Hinc*II digested pUC18. For determination of the nucleotide sequence, fragments of the pRW4 subclones were ligated in M13 derived vectors.
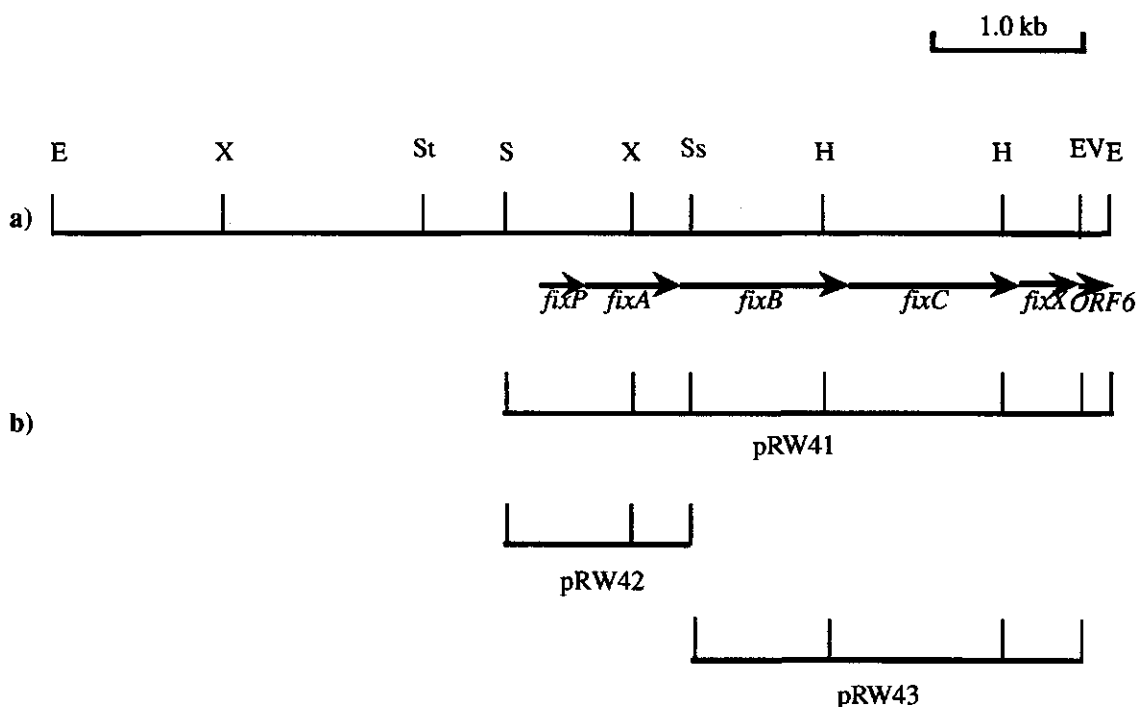
Fig. 1. (*a*) Physical map of the insert of plasmid pRW4 with the *fixPABCX* genes and the 5'-end of sequence of *ORF6* of *A.vinelandii*.
(*b*) Various subcloned fragments of pRW4. Restriction enzymes are abbreviated as follows: *Eco*RI, E; *Eco*RV, EV; *Hin*dIII, H; *Sma*I, S; *Sst*I, Ss; *Stu*I, St; *Xho*I, X.

The M13mp18 and M13mp19 derived vectors, containing the *Azotobacter* DNA, were introduced into *E. coli* TG2 by transformation and single-stranded DNA was isolated according standard techniques [Ausubel *et al.*, 1987]. Sequencing was by the dideoxy-chain-termination method as developed by Sanger *et al.* [1977] using the universal M13 primer. The sequence reactions with Klenow fragment of *E. coli* DNA polymerase I were carried out at 50°C and 7-deaza-dGTP was used in all reactions to prevent compression at G+C-rich regions. Both strands were sequenced entirely. In two cases oligonucleotides were synthesised to be used as primer (for determination of the sequences near the *Sst*I site and around position 680), because of the lack of suitable restriction sites to reclone the fragment.

Sequences were analysed with computer programs developed by Staden [1982, 1984]. Homology searches were done with the FASTA program of Pearson and Lipman [1988], using the EMBL, Swiss-Prot and Brookhaven databases. The percentages of matches were calculated by this program. The sequences have been submitted to Genbank (Accession numbers : X63772 and X65515)

RESULTS AND DISCUSSION

Cloning and nucleotide sequence of the *A. vinelandii fixPABCX-ORF6* gene cluster.

Hybridisation of a Southern blot with *Eco*RI digested *A. vinelandii* DNA with the ^{32}P-labelled *R. leguminosarum fixA* gene resulted in a unique hybridising band at approximately 7 kb.

A library of 6-9 kb *Eco*RI fragments of *A. vinelandii* DNA cloned in *E. coli* contained this fragment in recombinant plasmid pRW4 (Fig. 1[a]). Within this 7.2 kb fragment, the homology with the *fixA* probe was found to be confined in the 4.4 kb *Sma*I-*Eco*RI-fragment (pRW41, Fig. 1[b]). The nucleotide sequence of this 4.4 kb fragment was determined and six open reading frames were found (Fig. 2). In the next sections, the sequences of the polypeptides encoded in the six open reading frames of pRW41 are compared with those of the Fix proteins of *R. leguminosarum* [Hontelez *et al.*, 1989; Grönger *et al.*, 1987], *A. caulinodans* [Arigoni *et al.*, 1991], *R. meliloti* [Earl *et al.*, 1987] and *Rhizobium trifolii* [Iismaa and Watson, 1987]

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GTTGGGGTGAAGGGGCCGAGGATGATGCCGGCCAGCAGGTAGCCGACCAGGGCCGGCAGCCGGAGGCGGGCGGCGAGGAC
        10        20        30        40        50        60        70        80

ACCCAGCACCAGGGCCAGGCCGAACGCAGTGGCCAGGGTGGTGATCAGGGGAAGGCTATGCGGCATGGGGATGCTCCCTG
        90       100       110       120       130       140       150       160

CCGGTCACGACGGGGAAATGGTCGGGCATACGGGCTCCTTGGATATGCGGGGCGATAAAGTGTAGCGACACCATAACGGA
       170       180       190       200       210       220       230       240

TTGATCGGTGGTTGCCTGCCCTCGCTGGCTGTGTTTCTGGTATCCCGATTTATCCGCCCGGTCCGGAAGTACGTTTTTAT
       250       260       270       280       290       300       310       320

TTGCGGCAAACACTACTAAAAGCATGTGCGCCGGGCACATCACCGCATAAAGAGAACAATTTTTATCTGAATGATTTTAA
       330       340       350       360       370       380       390       400

AGGATTTTTATATTTATTTTATATAAAAATCCTCTGTTTCGGGTGTGGTACGGCTGTTGCAGTTTCACTGTCAGCCAATT
       410       420       430       440       450       460       470       480

                    M  P  Y  K  I  N  G  S  E  C  T  A  C  A  A  C  E  S
AGCCGATAGCTCATCAGGAGATTTCCCATGCCCTACAAGATCAACGGTTCCGAATGCACTGCCTGCGCTGCCTGCGAGTC
       490       500       510       520       530       540       550       560

 E  C  P  N  D  A  I  H  E  K  N  G  V  Y  A  I  K  S  E  L  C  T  E  C  V  G
CGAGTGCCCGAACGACGCCATCCATGAAAAGAACGGCGTCTACGCCATCAAGAGCGAACTGTGCACCGAATGCGTGGGCG
       570       580       590       600       610       620       630       640

D  H  D  E  P  Q  C  V  S  N  C  P  V  D  C  V  R  I  D  K  T  T  P  R  Y  Q  A
ACCACGACGAACCCCAGTGCGTGTCGAACTGCCCGGTCGATTGCGTTCGCATCGACAAGACGGTACCGCGCTACCAAGCC
       650       660       670       680       690       700       710       720

L  *                          M  H  S  V  V  C  I  K  Q  L  P  D  S  A
CTCTGAGCCCCGTCACCCCCTTATCGGAGTGCCATCCCATGCACAGCGTCGTCTGTATCAAGCAGTTGCCGGACTCGGCC
       730       740       750       760       770       780       790       800
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    Q   I   R   V   H   P   V   T   N   T   I   M   R   Q   G   V   P   A   I   I   N   P   Y   D   L   F   A
CAGATCCGGGTTCACCCGGTCACCAACACCATCATGCGCCAGGGTGTCCCGGCGATCATCAACCCTTATGACCTGTTCGC
        810         820         830         840         850         860         870         880

    L   E   E   A   L   R   L   K   D   K   F   G   G   T   V   T   V   V   T   M   G   P   P   M   A   E
CCTGGAGGAGGCCCTGCGCCTCAAGGATAAGTTCGGCGGCACGGTCACCGTGGTCACCATGGGCCCGCCCATGGCCGAGC
        890         900         910         920         930         940         950         960

A   A   L   R   K   C   L   S   F   G   A   D   D   A   I   L   V   S   D   R   A   F   A   G   S   D   T
CGGCGCTACGCAAGTGCCTGTCCTTCGGCGCCGACGACGCCATCCTGGTGTCGGACCGGGCCTTCGCCGGCTCCGACACC
        970         980         990        1000        1010        1020        1030        1040

    L   A   T   S   Y   A   L   S   A   V   I   R   K   I   M   E   D   M   P   V   D   L   I   F   T   G   F
CTGGCCACTTCCTATGCCCTCAGCGCCGTCATCCGCAAGATCATGGAAGACATGCCGGTGGACCTGATCTTCACCGGCAA
       1050        1060        1070        1080        1090        1100        1110        1120

    Q   T   I   D   G   D   T   A   Q   V   G   P   G   I   A   K   R   L   D   Y   Q   L   L   T   Y   V
GCAGACCATCGACGGCGACACCGCCCAGGTCGGGCCGGGCATCGCCAAGCGCTTGGACTACCAGTTGCTCACCTATGTGT
       1130        1140        1150        1160        1170        1180        1190        1200

S   R   I   V   D   V   D   T   A   K   K   E   I   Q   V   E   R   R   A   E   G   G   V   Q   L   L   E
CCAGGATCGTCGACGTCGACACCGCGAAGAAGGAAATCCAGGTGGAGCGTCGCGCCGAAGGCGGCGTGCAATTGCTCGAC
       1210        1220        1230        1240        1250        1260        1270        1280

    T   S   L   P   C   L   I   T   M   L   E   G   T   N   E   M   R   F   G   D   L   D   D   L   F   R   A
ACCTCCCTTCCCTGCCTGATCACCATGCTGGAAGGCACCAATGAAATGCGTTTCGGCGATCTGGACGACCTGTTTCGCGC
       1290        1300        1310        1320        1330        1340        1350        1360

    A   R   H   E   L   K   V   W   D   R   V   A   A   G   I   D   T   V   E   M   I   G   L   K   G   S
CGCGCGCCACGAACTCAAGGTCTGGGACCGCGTGGCCGCCGGCATCGATACGGTGGAGATGATCGGCCTCAAGGGCAGC
       1370        1380        1390        1400        1410        1420        1430        1440

P   T   V   V   S   K   V   F   A   P   K   P   R   S   K   R   A   E   L   I   E   S   H   D   S   D   F
CCACGGTGGTCAGCAAGGTCTTCGCGCCCAAGCCCCGCAGCAAGCGCGCCGAACTCATCGAAAGCCACGACAGCGATCCC
       1450        1460        1470        1480        1490        1500        1510        1520

    K   N   L   A   E   A   A   L   A   K   L   F   T   Q   H   P   N   L   E   Q   E   I   A   K   R   A   V
AAGAATCTGGCCGAGGCCGCGCTGGCCAAGCTGTTCACCCAGCATCCCAACTTGGAACAGGAAATCGCCAAGCGGGCGG
       1530        1540        1550        1560        1570        1580        1590        1600

    *·····          M   S   E   Q   P   K   K   P   T   K   K   K   V   E   L   D   P   R   F   V   D   L
CTGAGGAGCGAACACATGAGCGAACAACCGAAAAAGCCGACGAAGAAGAAGGTCGAACTGGACCCGCGCTTTGTCGATG
       1610        1620        1630        1640        1650        1660        1670        1680

    R   H   V   W   V   C   I   E   S   E   R   G   V   V   H   P   V   S   W   E   L   L   G   E   G   R
CCGTCACGTCTGGGTCTGCATCGAAAGCGAGCGCGGCGTGGTGCACCCGGTTTCCTGGGAACTCCTGGGCGAGGGCCGC
       1690        1700        1710        1720        1730        1740        1750        1760

K   S   V   D   A   L   G   G   E   L   Y   G   V   V   I   C   G   P   G   E   R   G   K   E   I   C   G
AGTCGGTTGACGCCCTGGGCGGTGAGCTCTACGGTGTGGTCATCTGCGGCCCCGGCGAGCGCGGCAAGGAAATCTGCGG
       1770        1780        1790        1800        1810        1820        1830        1840

    E   P   V   Q   H   G   A   D   K   A   Y   L   L   Q   H   E   I   L   R   D   Y   R   N   E   P   Y
GAACCTGTCCAGCATGGTGCCGACAAGGCTTATCTGCTGCAGCACGAAATCCTGCGGGACTACCGCAACGAGCCCTACA
       1850        1860        1870        1880        1890        1900        1910        1920

    K   A   L   T   D   L   V   T   A   T   Q   P   E   I   L   M   L   G   A   T   T   L   G   R   D   L
CAAGGCGCTCACTGACCTGGTCACAGCTACCCAGCCGGAAATCCTCATGCTCGGCGCCACCACCCTGGGCCGCGACCTG
       1930        1940        1950        1960        1970        1980        1990        2000

A   G   S   V   A   T   T   L   G   T   G   L   V   A   D   C   T   E   L   V   I   D   T   E   T   R   N
CCGGCTCGGTGGCCACCACCCTGGGCACCGGCCTGGTGGCCGACTGCACCGAACTGGTGATCGACACGGAAACCCGCAA
       2010        2020        2030        2040        2050        2060        2070        2080
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  L   A   S   T   R   P   T   F   D   G   S   L   L   C   K   P   S   S   A   Q   R   H   R   P   Q   M   A
CTGGCCTCCACCCGTCCGACTTTCGACGGTTCCCTTCTGTGCAAGCCATCCTCAGCCCAGCGCCACCGGCCCCAGATGGC
         2090      2100      2110      2120      2130      2140      2150      2160

    T   V   R   P   R   M   A   M   P   E   P   D   A   S   R   S   G   E   I   I   E   V   P   F   S   M
CACCGTGCGGCCGCGGATGGCCATGCCGGAGCCCGACGCCAGCCGCAGCGGCGAGATCATCGAGGTGCCGTTCTCGATGA
         2170      2180      2190      2200      2210      2220      2230      2240

  I   E   T   D   I   I   T   K   V   L   E   F   I   P   D   D   T   R   D   K   P   N   L   P   F   A   D
TCGAGACCGACATCATCACCAAGGTGCTGGAGTTCATTCCCGACGATACCCGCGACAAGCCCAACCTGCCTTTCGCCGAC
         2250      2260      2270      2280      2290      2300      2310      2320

  I   I   V   A   G   G   R   G   L   R   N   Q   E   N   F   Q   L   V   W   D   L   A   K   V   L   G   A
ATCATCGTCGCCGGCGGCCGTGGGCTGCGCAACCAGGAGAATTTCCAGCTGGTCTGGGACCTGGCCAAGGTCCTCGGCGC
         2330      2340      2350      2360      2370      2380      2390      2400

    E   V   G   A   S   R   P   I   V   Q   A   G   W   A   E   L   D   R   Q   V   G   Q   S   G   K   T
CGAAGTGGGCGCCTCGCGCCCCATCGTCCAGGCCGGTTGGGCCGAGCTGGACCGCCAGGTCGGCCAGTCCGGCAAGACCG
         2410      2420      2430      2440      2450      2460      2470      2480

  V   R   P   K   L   Y   I   A   A   G   I   S   G   A   I   Q   H   R   V   G   M   D   G   A   D   V   I
TGCGGCCCAAGCTGTACATCGCCGCCGGCATCTCCGGCGCCATCCAGCACCGGGTGGGCATGGACGGGGCGGACGTGATC
         2490      2500      2510      2520      2530      2540      2550      2560

  I   A   I   N   T   D   P   N   A   P   I   F   D   F   A   H   Y   G   I   V   G   N   A   I   T   V   L
ATCGCCATCAACACCGATCCCAACGCACCCATCTTCGATTTCGCCCACTACGGCATCGTCGGTAACGCCATCACCGTCCT
         2570      2580      2590      2600      2610      2620      2630      2640

    P   A   L   T   E   A   F   K   A   R   L   G   Q   L   K   K   A   G   *  •••••        M   A   E
GCCGGCACTGACCGAAGCTTTCAAGGCCCGTCTGGGACAACTGAAAAAAGCCGGCTGACAGGAGAACATCATGGCTGAAA
         2650      2660      2670      2680      2690      2700      2710      2720

  R   F   D   V   I   V   V   G   A   G   M   A   G   N   A   A   A   Y   T   L   A   K   G   G   L   K   V
GATTCGACGTGATCGTGGTCGGCGCCGGCATGGCCGGTAACGCCGCCGCATACACCCTGGCCAAGGGGGGGCTCAAGGTG
         2730      2740      2750      2760      2770      2780      2790      2800

  L   Q   I   E   R   G   E   T   P   G   S   K   N   V   Q   G   A   I   L   Y   A   D   A   I   E   K   I
CTGCAGATCGAGCGGGGCGAAACCCCCGGTTCGAAGAACGTGCAGGGCGCCATCCTCTATGCCGACGCCATCGAGAAGAT
         2810      2820      2830      2840      2850      2860      2870      2880

    I   P   D   F   R   D   D   A   P   L   E   R   H   L   I   E   Q   R   V   W   V   M   D   D   A   S
CATTCCCGACTTCCGCGACGACGCGCCCCTGGAGCGGCACCTGATCGAGCAGCGCGTGTGGGTGATGGACGACGCTTCCT
         2890      2900      2910      2920      2930      2940      2950      2960

  Y   G   Y   H   Y   R   S   E   D   F   N   K   P   P   Y   N   R   Y   T   I   I   R   V   H   F   D   Q
ACGGGTACCACTACCGTTCGGAGGACTTCAACAAGCCGCCCTACAACCGCTACACCATCATCCGCGTGCATTTCGACCAG
         2970      2980      2990      3000      3010      3020      3030      3040

    W   F   N   K   K   A   R   E   A   G   V   L   T   I   C   E   T   R   H   D   L   L   I   E   G   G   K
TGGTTCAACAAGAAGGCCCGCGAGGCCGGCGTGCTGACCATCTGCGAAACACGTCACGATCTGCTGATCGAGGGTGGCAA
         3050      3060      3070      3080      3090      3100      3110      3120

    V   V   G   V   R   T   D   R   Q   G   G   E   V   R   A   D   A   V   I   L   A   D   G   V   N   S
GGTGGTGGGCGTGCGCACCGATCGCCAGGGCGGCGAAGTCCGTGCCGACGCGGTGATCCTGGCCGACGGGGTGAACTCGC
         3130      3140      3150      3160      3170      3180      3190      3200

  R   L   A   V   K   A   G   F   S   R   D   Q   P   E   N   W   A   L   A   V   K   E   I   H   F   L   P
GCCTGGCGGTGAAGGCCGGCTTCAGCCGAGATCAGCCCGAGAACTGGGCCCTGGCGGTGAAGGAAATCCATTTCCTGCCC
         3210      3220      3230      3240      3250      3260      3270      3280

    Q   E   T   M   E   A   R   F   N   I   G   E   E   E   A   A   I   E   M   A   G   K   I   D   A   G   M
CAGGAAACCATGGAGGCGCGCTTCAACATCGGCGAAGAGGAAGCTGCCATCGAGATGGCCGGCAAGATCGACGCCGGCAT
         3290      3300      3310      3320      3330      3340      3350      3360
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  M   G   T   G   F   L   Y   T   N   K   E   S   I   T   L   G   V   G   C   M   L   S   D   F   K   Q
GATGGGCACCGGCTTCCTCTACACCAACAAGGAGTCGATCACCCTGGGGGTCGGCTGCATGCTGTCCGACTTCAAGCAGC
     3370      3380      3390      3400      3410      3420      3430      3440

  Q   K   I   S   P   Y   E   L   L   D   R   M   K   T   H   P   S   I   A   P   L   I   A   G   S   D   M
AGAAGATCTCCCCCTATGAGCTGCTGGACCGGATGAAGACCCACCCCTCCATCGCGCCCTTGATCGCCGGCAGCGACATG
     3450      3460      3470      3480      3490      3500      3510      3520

  K   E   Y   A   A   H   L   I   P   E   G   G   Y   N   A   I   P   Q   V   Y   G   D   G   W   M   I
AAGGAGTACGCCGCCCACCTGATCCCCGAAGGCGGCTACAACGCCATTCCCCAGGTATACGGCGACGGCTGGATGATCGC
     3530      3540      3550      3560      3570      3580      3590      3600

  G   D   A   P   I   R   H   G   I   H   R   E   G   S   N   L   A   M   T   T   G   M   L   A   A   Q
CGGCGACGCGCCGATTCGTCACGGCATCCATCGCGAAGGCTCCAACCTGGCCATGACCACCGGCATGCTGGCGGCCCAGA
     3610      3620      3630      3640      3650      3660      3670      3680

  T   L   V   E   L   R   A   A   D   K   P   F   S   A   A   N   L   A   E   Y   K   K   K   L   D   D   S
CCCTGGTCGAGCTGCGGGCCGCGGACAAGCCCTTCAGCGCCGCCAACCTGGCCGAGTACAAGAAGAAGCTGGACGACAGC
     3690      3700      3710      3720      3730      3740      3750      3760

  F   V   M   K   D   L   K   K   Y   R   R   M   P   E   I   F   H   K   N   K   Q   F   F   T   T   Y
TTCGTCATGAAGGACCTGAAGAAGTACCGGCGCATGCCGGAGATCTTCCACAAGAACAAGCAGTTCTTCACCACCTATC
     3770      3780      3790      3800      3810      3820      3830      3840

  D   L   L   S   R   A   A   Q   T   L   I   R   V   D   G   V   D   K   K   T   K   E   K   E   I   K
GGACCTGCTCTCCAGGGCCGCGCAGACCCTTATCCGGGTCGATGGCGTGGACAAGAAAACCAAGGAAAAGGAAATCAAGA
     3850      3860      3870      3880      3890      3900      3910      3920

  K   S   F   I   G   L   R   S   L   F   G   L   I   G   D   A   F   K   F   W   R   A   V   E   *•••••
AAAGCTTCATCGGCCTACGTTCGCTGTTCGGGCTGATCGGCGATGCCTTCAAGTTCTGGCGTGCCGTCGAGTAAGGAGA
     3930      3940      3950      3960      3970      3980      3990      4000

      M   S   K   I   E   E   K   L   F   Q   D   R   Y   R   V   D   S   G   R   P   H   I   R   I   K
AGCCCATGTCGAAGATCGAAGAAAAGCTGTTCCAGGATCGTTATCGGGTCGACTCCGGCCGGCCGCACATCAGGATCAAG
     4010      4020      4030      4040      4050      4060      4070      4080

  D   P   D   H   C   T   E   L   S   E   K   Q   C   T   V   C   C   P   A   G   C   Y   T   R   E   T
GACCCCGACCATTGCACCGAATTGTCTGAAAAGCAGTGCACCGTCTGCTGTCCGGCCGGTTGCTACACCCGCGAAACCA
     4090      4100      4110      4120      4130      4140      4150      4160

  G   K   V   T   L   V   T   D   G   C   L   E   C   G   T   C   R   I   I   C   Q   D   S   G   N   L
CGGCAAGGTCACCCTGGTGACCGACGGCTGCCTGGAGTGCGGAACCTGCAGGATCATTTGCCAGGACAGCGGCAACCTG
     4170      4180      4190      4200      4210      4220      4230      4240

  E   W   E   W   P   R   G   G   F   G   I   L   F   K   F   G   *•••••         M   N   D   I   Q   L   F
AGTGGGAATGGCCCAGGGGCGGATTCGGAATCCTGTTCAAGTTCGGTTGAGGGATGACAATGAACGATATCCAGCTTTT
     4250      4260      4270      4280      4290      4300      4310      4320

  L   A   H   A   I   Q   L   E   K   E   S   A   R   R   Y   E   E   L   A   E   A   M   Q   S   L
CTGGCCCACGCCATCCAGTTGGAGAAGGAGTCGGCGCGCCGCTATGAGGAACTGGCCGAGGCCATGCAGAGCCT
     4330      4340      4350      4360      4370      4380      4390
```

Fig. 2. Complete nucleotide sequence of the 4394 basepairs *SmaI/EcoRI* fragment of plasmid pRW41 with the *fixP* (nucleotides 509-726), *fixA* (nucleotides 760-1605), *fixB* (nucleotides 1617-2699), *fixC* (nucleotides 2712-3995) and *fixX* (nucleotides 4007-4291) genes of *A.vinelandii*. ORF6 starts at position 4301 and reads to the end. Indicated are putative Shine-Dalgarno like sequences (•••••), a

possible Upstream Activator Sequence (━━) and the possible promoter region (▨).

<u>Sequence analysis of the coding regions.</u>

The fixA gene

ORF2 encodes a protein with a molecular mass of 30 kDa, which has 62.2% and 60.8% conserved amino acid residues compared to the FixA protein of *A. caulinodans* [Arigoni *et al.*, 1991] and *R. meliloti* [Earl *et al.*, 1987], respectively. When the N-terminal 20 amino acids of the *fixA* gene products of *R. leguminosarum* and *B. japonicum* are compared to the those of ORF2, 88.9% and 89% of the amino acids are conserved. FixA has no homology with other proteins in the databases.

The fixB gene

The protein encoded by ORF3 (molecular mass 39 kDa) has 58.9% conserved amino acids compared to the *fixB* gene product of *R. meliloti* [Earl *et al.*, 1987] and 57.6% to the gene product of *fixB* of *A. caulinodans* [Arigoni *et al.*, 1991]. The partial sequence of the *B. japonicum* FixB protein [Gubler *et al.*, 1989] showed 44.7% homology with the protein encoded by ORF3.

The FixB protein is also homologous with the α-subunits of the Electron Transfer Flavoprotein of human and rat origin [Finocchiaro *et al.*, 1988; Shinzawa *et al.*, 1988], 33.6% and 31.5% of the amino acids being conserved, respectively (Fig. 3). The homology of FixB with the α-subunit of ETF has also been reported by Arigoni and coworkers [1991]. ETF is a heterodimer consisting of an α- and β-subunit (32 and 27 kDa, respectively [Ikeda *et al.*, 1986]). ETF has been found in both prokaryotes and in eukaryotes and is involved in transfer of electrons from several dehydrogenases to ubiquinone in the respiratory chain *via* ETF-ubiquinone-oxidoreductase [Finocchiaro *et al.*, 1988].

```
                30        40        50        60        70        80
ETFrat: ASLLRFQSTLVIAEHANDSLAPITLNTITAAGRLGGEVSCLVAGTKCDKVVQDLCKVAG
                       .  |  |  |  |  |       |                     |
AvFIXB: HVWVCIESERGVVHPVSWELLGEGRKSVDALGGELYGVVICGPGER-GKEICGEPVQ-HG
                         |||  |    |         |       |    |
ETFhum: LRFQSTLVIAEHANDSLAPITLNTITAATRLGGEVSCLVAGTKCDKVAQDLCKVA----G

                90       100       110       120       130       140
ETFrat: VAKVLVAQHDAYKGLLPEELTPLILETQKQFSYTHIVAGASAFGKNLLPRVAAKLNVAPV
         |       ||         |   |                 ||   |   |   ||   |      |
AvFIXB: ADKAYLLQHEILRDYRNEPYTKALTDLVTATQPEILMLGATTLGRDLAGSVATTLGTGLV
         |       ||         |   |                 ||   |   |    ||   |
ETFhum: IAKVLVAQHDVYKGLLPEELTPLILATQKQFNYTHICAGASAFGKNLLPRVAAKLEVAPI

               150       160       170       180       190       200
ETFrat: SD----IIEIKSPD-TFVRTIYAANALCT-VKCDEKVKVFSVRGTSFEAAAASGGSASSE
         |         |    |       ||         |        |     |       |
AvFIXB: ADCTELVIDTETRNLASTRPTFDGFLLCSHPQPSATGRHDGHRAAAVMAMPEPDASRSGE
         |         |    |       ||         |        |     |       |
ETFhum: SD----IIAIKSPD-TFVRTIYAGNALCT-VKCDEKVKVFSVRGTSFDAAATSGGSASSE

               210       220       230       240       250       260
ETFrat: KAPSSSSAGISEWLDQKL-----TKSDRPELTGAKVVVSGGRGLKSGENFKLQYDLADQL
         |             |           |  |  |     |   |||||    |||    |||    |
AvFIXB: IIEVPFSMIETDIITKVLEFIPDDTRDKPNLPFADIIVAGGRGLRNQENFQLVWDLAKVL
                       |             |  |  |     |   |||||    |||    |||    |
ETFhum: KASSTSPVEISEWLDQKL-----TKSDRPELTGAKVVVSGGRGLKSGENFKLLYDLADQL

               270       280       290       300       310       320
ETFrat: HAAVGASRAAVDAGFVPNDMQVGQTGKIVAPELYIAVGISGAIQHLAG*
         |  |||||   |  ||    |  ||||  ||  |  |  ||||  ||||||||   |
AvFIXB: GAEVGASRPIVQAGWAELDRQVGQSGKTVRPKLYIAAGISGAIQHRVGMDGADVIIAINT
         |  |||||   |  ||    |  ||||  ||  |  |  ||||  ||||||||   ||        |  |||
ETFhum: HAAVGASRAAVDAGFVPNDMQVGQTGKIVAPELYIAVGISGAIQHLAGMKDSKTIVAINK

               330       340       350       360
AvFIXB: DPNAPIFDFAHYGIVGNAITVLPALTEAFKARLGQLKKAG*
         ||  ||||   |  ||||       |   |   ||    |
ETFhum: DPEAPIFQVADYGIVADLFKVVPEMTEILKKK*
```

Fig. 3. Homology between the FixB protein of *A.vinelandii* and the α-subunits of the Electron Transfer Flavoprotein (ETF) of human and rat origin. Identical amino acids are indicated by "l". AvFIXB: *Azotobacter vinelandii* FixB protein, ETFhum: ETF of human origin[Finocchiaro *et al.*, 1988] ETFrat: ETF of rat origin [Shinzawa *et al.*, 1988]. Numbers above the alignment are according to *A.vinelandii* FixB protein; Human ETF is shown from position 17; rat ETF is shown from position 25. "*" indicates a stop codon, "-" indicates insertion of one amino acid residue.

The fixC gene

ORF4 encodes a protein (molecular mass 48 kDa) that shows homology with the FixC protein of *A. caulinodans* [Arigoni *et al.*, 1991] and *R. meliloti* [Earl *et al.*, 1987] and the fragments of FixC of *R. leguminosarum* [Grönger *et al.*, 1987] and *B. japonicum* [Gubler *et al.*, 1989] (57.1%, 55.3%, 49.7% and 50.0% identical residues, respectively). The N-terminal domain of the protein contains a sequence homologous with the consensus sequence for ADP binding, as found in NAD$^+$ and FAD-dependent enzymes [Wierenga *et al.*, 1986] (Fig. 4).

In many FAD-containing enzymes (*e.g.* lipoamide dehydrogenase [Benen *et al.*, 1989] and mercuric reductase [Fox and Walsh, 1982]), the FAD-binding site is located close to the N-terminus. This suggests, that also FixC might be a FAD-containing protein. It is known, that the ß-subunit of ETF contains FAD. However, since no primary structure of this subunit has been published yet, it is not known whether FixC has homology with the ß-subunit of ETF.

Fig. 4. Homology of the amino acids 6–37 of the FixC protein with the consensus sequence for ADP binding.

Position	Av FixC	Consensus
6	D	* (K, R, H, S, T, Q, N, D)
7	V	* (A, I, L, V, M, C)
8	I	–
9	V	* (A, I, L, V, M, C)
10	V	–
11	G	* (G)
12	A	–
13	G	* (G)
14	M	–
15	A	–
16	G	* (G)
17	N	–
18	A	–
19	A	–
20	A	–
21	Y	* (A, I, L, V, M, C)
22	T	–
23	L	–
24	A	* (A, I, L, V, M, C)
25	K	–
26	G	–
27	G	–
28	L	–
29	K	–
30	V	–
31	L	–
32	Q	–
33	I	* (A, I, L, V, M, C)
34	E	–
35	R	* (A, I, L, V, M, C)
36	G	–
37	E	* (E, D)

Sec. str.: β-sheet 1 — α-helix — loop — β-sheet 2

Fig. 4. Homology of the amino acids 6-37 of the FixC protein with the consensus sequence for ADP binding. Numbers indicate residues in the FixC sequence. "Consensus" is consensus sequence for ADP binding [according to Wierenga et al., 1986]. "-" indicate that any amino acid is allowed at this position; "*" indicate homologous amino acids. "Sec. str." is secondary structure; "β-sheet 1", "α-helix" and "β-sheet 2" indicate that these are elements of the βαβ structure, involved in ADP-binding.

The fixX gene

Downstream the *fixC* gene an open reading frame is found, which may encode a protein of 10.7 kDa. This protein is homologous with the FixX protein of various symbiotic bacteria: *R. leguminosarum* [Grönger *et al.*, 1987], *A. caulinodans* [Arigoni *et al.*, 1991], *B. japonicum* [Gubler *et al., 1989*], *R. trifolii* [Iismaa and Watson 1987] and *R. meliloti* [Earl *et al.*, 1987] and also with ferredoxin I from *A. vinelandii* [Howard *et al.*, 1983; Morgan *et al.*, 1988] (Figure 5).

```
                                                                CLUSTER PATTERN
              10        20        30        40        50
FixX:  MSKIEEKLFQDRYRVDSGRPHIRIKDPDH.CTELSEKQCTVCCPAGC.YTRE      C-X7-C-X3-C
                                     ||         ||  |
FrdI:                  MAFVVTDN.CIKCKYTDCVEVCPVDCFYE.G           C-X7-C-X3-C
                                ||    |  |   || |||      |
FrdN:                  MALKIVES.CVNC..WACVDVCPSEAISLAG           C-X2-C-X2-C-X3-C
                            | ||  | |    |    |  |    ||
FixP:                  MPYKINGSECTAC..AACESECPNDAIHEKN           C-X2-C-X2-C-X3-C
                            |  | | |||| |    || ||   |
FrdV:                  MAMAIDGYECTVC..GDCEPVCPTGSIVFRD           C-X2-C-X2-C-X3-C
                                   CLUSTER 1

              60        70        80        90
FixX:  TNGKVTLVTDGCLECG......TCRIICQ..DSGNLEWEWPRGGFG.ILFK       C-X2-C-X2-C-X3-C
          |  |    |  |       |     |     | | |        |
FrdI:  PNFLVIH.PDECIDCA......LCEPECP.AQAIFSEDEVPEDMQEFIQLN       C-X2-C-X2-C-X3-C
        |  |   || |    |     | ||  |    || |  |
FrdN:  PHFE.IS.ASKCTECDGDYAEKQCASICPVEGAILLADGTPANPPGSLTGI       C-X2-C-X8-C-X3-C
          |     ||| ||  | || | ||
FixP:  GVYA.IK.SELCTECVGDHDEPQCVSNCPVD.CVRIDKTTPRYQAL*           C-X2-C-X8-C-X3-C
        |  |     ||  | ||| || |||| |
FrdV:  DHYA.IE.ADSCNECT.DVGEPRCLGVCPVDLCIQPLDD*                  C-X2-C-X7-C-X3-C
                                   CLUSTER 2

              100       110       120
FixX:  FG*

FrdI:  AE..LAEVWPNITEKKDPLPDAEDWDGVKGKLQHLER*
          |||    |
FrdN:  PPERLAEAMREIQAR*
```

Fig. 5. Homology between the FixX and FixP proteins of *A. vinelandii* and various 7Fe-ferredoxins of *A. vinelandii*. Abbreviations are as follows: FixX: *A.vinelandii* FixX; ; Frd I: *A.vinelandii* ferredoxin I [Howard *et al.*, 1983, Morgan *et al.*, 1988]; FrdN: *A. vinelandii* ferredoxin in *nif* cluster [Joerger and Bishop, 1988]; FixP: *A.vinelandii* FixP; FrdV: *A. vinelandii* ferredoxin in *vnf* cluster [Joerger *et al.*, 1990]. Numbering of the amino acid residues is according to the FixX protein.

In all FixX proteins, five cysteine residues are conserved (Cys42, Cys46, Cys62, Cys65 and Cys68), three of which are found in a motif Cys-X_2-Cys-X_2-Cys, the consensus motif for ligation of a [4Fe-4S] cluster [Bruschi and Guerlesquin, 1988]. Eight cysteine residues are conserved between *A. vinelandii* FixX and *A. vinelandii* FdI (Figure 5). Two of these conserved cysteine residues (Cys 30 and Cys 38) are not present in the other FixX proteins. Ferredoxin I contains both a [4Fe-4S] and a [3Fe-4S] cluster. From the 3D-structure of FdI it is known, that Cys20, Cys39, Cys42 and Cys45 are the ligands for the [4Fe-4S] cluster and Cys8, Cys16 and Cys49 are involved in ligation of the [3Fe-4S] cluster [Stout, 1989]. All these cysteines are conserved in the *A. vinelandii* FixX protein: Cys42, Cys62, Cys65, Cys68 ([4Fe-4S] cluster) and Cys30, Cys38, Cys72 ([3Fe-4S] cluster). We therefore suggest that *A. vinelandii* FixX contains, in contrast to the rhizobial FixX proteins, not only a [4Fe-4S] cluster, but also a [3Fe-4S] cluster.

The fixP gene.

Upstream of the start codon of the *fixA* gene, an open reading frame was found, encoding a protein of approximately 8.5 kDa, which we called *fixP*. The nucleic acid derived protein sequence of *fixP* contains two motives typical for 7Fe-ferredoxins having a [4Fe-4S] and a [3Fe-4S] cluster. The homology with the other known 7Fe-ferredoxins of *A. vinelandii* is shown in Figure 5. This implies that *A. vinelandii* possesses probably at least five 7Fe-ferredoxins: ferredoxin I [Howard *et al.*, 1983, Morgan *et al.*, 1987], a ferredoxin (FrdN) in the *nifB-nifQ* cluster [Joerger and Bishop, 1988], a ferredoxin (FrdV) in the *vnf* gene cluster [Joerger *et al.*, 1990] and FixX and FixP.

The *fixP* gene product does not have any homology with the "ORF35-protein", found upstream of the *fixBCX* genes in *B. japonicum* [Gubler *et al.*, 1989]. In *B. japonicum*, the polypeptide encoded by ORF35 (35 amino acid residues in size), affects the level of transcription of the *fixBCX* operon, and a deletion of this open reading frame decreases the nitrogen fixation activity.

A. vinelandii is the only organism, in which a *fixPABCX* gene cluster has been found to contain more than one gene encoding a ferredoxin-like protein. All other bacteria possessing the *fixABCX* gene cluster lack this second ferredoxin-like coding gene.

ORF6

The putative gene product of the sixth open reading frame (ORF6) revealed no homology with the gene product downstream of the *fixX* gene of *A. caulinodans* [Arigoni *et al.*, 1991]. No genes homologous with ORF6 have been found in *fix* gene clusters of other species.

Putative regulatory sequences

Sequences homologous with the consensus sequence for ribosome binding [Shine and Dalgarno, 1974] are found upstream of all five genes, as indicated in Fig. 2.

63 bp upstream of the start codon of the first open reading frame (*fixP*) a sequence is found, 5'-TGGTACGGCTGTTGCA-3', bearing homology with the consensus for σ^{54} (RpoN)- dependent promoters [GGYRYR-N_4-TTGC/A ; Buck, 1990]. *Nif* genes are known to have a σ^{54}-dependent promoter [Buck, 1990].

At position -164 to -150 upstream of the start codon of *fixP*, a sequence homologous with the consensus for NifA binding [Buck *et al.*, 1986] is present (TGT-N_9-ACA). However, the spacing between the TGT- and ACA- elements of this sequence is one base shorter than in the consensus (TGT-N_{10}-ACA).

No terminator sequence could be found downstream of any of the open reading frames. This could indicate that the complete cluster is transcribed as a single polycistronic mRNA.

We suggest that amongst the *fixPABCX* genes of *A. vinelandii* at least three genes encode proteins involved in electron transport: the FixB protein is highly homologous with the α-subunit of ETF and both FixP and FixX proteins have the characteristics of 7Fe ferredoxins of *A. vinelandii*. The fact that genes, homologous with the *fixABCX* genes were found in *E. coli* [Yura *et al.*, 1992], indicates that these genes might be involved in a more general electron transfer process. In the *E. coli* genome, the *fixABCX* genes are followed by a gene encoding a NAD(P)H dehydrogenase and they are preceded by genes, involved in fatty acid metabolism. This could be an indication of the function of the *fixPABCX* genes in fatty acids metabolism.

Another possible function of the *fixPABCX* genes could be regulation of gene expression. It has been suggested that the 7Fe-ferredoxin of *A. vinelandii* is involved in regulation of gene expression in response to cellular iron(II) and the redox state of the cell [Thomson, 1991]. Ferredoxin is expected to belong to a novel class of DNA binding proteins. The FixP and FixX gene products could also belong to this new class of regulatory proteins, based on the fact that they belong to the group of 7Fe-ferredoxins. In *B. japonicum* the *fixB* gene is preceded by an open reading frame encoding a polypeptide of 35 amino acids, ORF35, that controls transcription of the *fixBCX* operon. Although no homology of *fixP* with ORF35 was found, a functional relationship could be present. When the 7Fe-ferredoxins are involved in regulation of gene expression, FixP could be involved in the regulation of the expression of the *fixABCX* genes, identical to ORF35.

Interpreting Figure 5, we suggest that there are two different groups of 7Fe-ferredoxins. FixX and ferredoxin I form the first group, whereas the ferredoxin from the *nif* gene cluster (FrdN), the ferredoxin from the *vnf* gene cluster (FrdV) and the FixP protein

form the second group of 7Fe-ferredoxins. This distribution is based on the organisation of the cysteine residues in the two [Fe-S] cluster binding domains of the ferredoxins. Cluster 1 of both ferredoxin I and FixX contain the cysteine residues in a $Cys-X_7-Cys-X_3-Cys$ motif, which is mainly involved in the ligation of the [3Fe-4S] cluster in ferredoxin I [Stout, 1989]. Cluster 2 of both proteins contain the cysteine residues in the motif $Cys-X_2-Cys-X_2-Cys-X_3-Cys$, in ferredoxin I mainly involved in the ligation of the [4Fe-4S] cluster [Stout, 1989]. In the three other ferredoxins, the second motif appears in the first cluster and the $Cys-X_7-Cys-X_3-Cys$ motif is present in cluster 2. This could imply that the ligation of the [3Fe-4S] and the [4Fe-4S] clusters is different in both types of ferredoxins. In both types of ferredoxin, the cysteine residues at position 46 and 74 might not directly involved in the [Fe-S] cluster ligation, but they might be able to take over the function of residues 42 and 72 respectively. In ferredoxin I, cysteine residue 25 can take over the function of cysteine residue 21 (46 and 42 respectively in Figure 5) [Stout, 2989].

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CHAPTER 3.

THE *FIXA, FIXB, FIXC* AND *FIXX* GENES OF *AZOTOBACTER VINELANDII*: PHYSIOLOGICAL ANALYSIS.

THE *FIXA, FIXB, FIXC* AND *FIXX* GENES OF *AZOTOBACTER VINELANDII*: PHYSIOLOGICAL ANALYSIS.

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Index entries:

Azotobacter vinelandii fixABCX genes, effect of mutations of the *fixABCX* genes on nitrogen fixation.

Abbreviations:

Restriction enzymes are abbreviated as follows: *Asp*718, A; *Bam*HI, B; *Bst*EII, Bs; *Eco*RI, E; *Eco*RV, EV; *Rsa*I, R; *Sal*I, Sa; *Sma*I, S; *Sst*I, Ss; *Stu*I, St; *Xho*I, X.

TES-NaOH (pH 7.5) is Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid adjusted to pH 7.5 with NaOH. UAS is Upstream Activator Sequence. IPTG is isopropyl-ß-D-thiogalactopyranoside; AV2 is *A. vinelandii* nitrogenase (E.C. 1.1.1.37) component 2 (Fe-protein).

SUMMARY

Azotobacter vinelandii strains mutated in the *fixA*, *fixB*, *fixC* and *fixX* genes were constructed by marker exchange. Therefore, the copies of these genes in the *A. vinelandii* genome were exchanged with plasmid derived copies that had been interrupted by insertion of the gene encoding kanamycin-resistance.

All *fix⁻* mutants showed normal growth characteristics in nitrogen-free medium under all conditions tested. *In vivo* and *in vitro* activities of acetylene reduction of mutants were comparable to wild-type activities. Growth on several sugars, dicarboxylic acids and fatty acids or amino acids indicated that the *fixABCX* cluster is not necessary for the catabolism of these components.

From experiments using a chromosomally integrated *fixA::lacZ* gene fusion, it was observed that the low expression level of the *fixABCX* genes of *A. vinelandii* cells, grown in the presence of ammonium acetate, was not significantly increased when these cells were transferred to nitrogen-free medium. It is concluded that the expression of the *fixABCX* genes is very low. Antibodies against the purified FixA protein did not show the presence of the FixA protein in extracts of *A. vinelandii* cells, either grown on nitrogen-free medium or medium containing ammonia.

It is concluded from the experiments that the *fixABCX* genes are not essential for nitrogen fixation in *A. vinelandii* under the variety of conditions tested.

INTRODUCTION

Nitrogen fixation, which occurs in a variety of micro-organisms, requires the involvement of a number of genes. Besides the genes encoding the structural proteins of nitrogenase, a wide variety of other genes is involved in nitrogen fixation. Some of these genes are not common to all nitrogen fixing organisms. A number of genes, essential for biological nitrogen fixation in *Rhizobium*, is not present in *Klebsiella pneumoniae*. These genes are called *fix* genes [Gubler and Hennecke, 1986].

The *fixA*, *fixB*, *fixC* and *fixX* genes have been found in a number of organisms, most of which live in symbiosis with higher plants. Recently, they have also been found in the non-diazotroph *Escherichia coli* [Yura *et al.*, 1992]. The effect of mutations in the *fixA*, *fixB*, *fixC* and *fixX* genes has so far been studied in the symbiotic diazotrophic bacteria *Rhizobium meliloti*, *Rhizobium leguminosarum*, *Bradyrhizobium japonicum* and *Azorhizobium caulinodans* ORS571 [Ruvkun *et al.*, 1982; Schetgens *et al.*, 1985, Gubler and Hennecke, 1986, Kaminski *et al.*, 1988; Masepohl *et al.*, 1990; Arigoni *et al.*, 1991]. In all cases, these mutations, mostly Tn5-insertions, resulted in a Fix⁻ phenotype, but normal nodules were formed when tested on plants. The *in vivo* nitrogenase activity of these Fix⁻ mutants was reduced to 0-10% of the wild-type activity. In *A. caulinodans* and *B. japonicum*, the *fixABCX* genes were also found to be necessary for nitrogenase activity in the free-living state [Kaminski *et al.*, 1988; Gubler and Hennecke, 1986; Arigoni *et al.*, 1991].

The function of the *fixABCX* genes was investigated by biochemical experiments. The nitrogenase polypeptides were reported to be present in *A. caulinodans* and *R. leguminosarum* FixA⁻, FixB⁻, FixC⁻ and FixX⁻ mutants [Kaminski *et al.*, 1988; Schetgens *et al.*, 1985], but no *in vitro* nitrogenase activity could be observed in cell-free extracts of the mutants of *A. caulinodans*, *R. leguminosarum* and *R. meliloti* [Schetgens *et al.*, 1985; Kaminski *et al.*, 1988; Masepohl *et al.*, 1990] with dithionite as an artificial electron donor. Addition of either one of the purified nitrogenase components to cell-free extracts of *R. leguminosarum* FixA⁻, FixB⁻ or FixC⁻ mutants did not restore nitrogenase activity, which was also observed for FixA⁻ and FixB⁻ mutants of *A. caulinodans*. Restoration of activity to wild-type levels was observed in *R. leguminosarum* mutants when both purified nitrogenase components were added, indicating that the system did not contain inhibitors for nitrogenase [Schetgens *et al.*, 1985]. Kaminski and coworkers found that the low nitrogenase activity in a FixC⁻ mutant of *A. caulinodans*, could be partially restored by addition of purified nitrogenase Fe-protein. It was therefore suggested that the FixC protein is involved in the maturation of the Fe-protein of *A. caulinodans* [Kaminski *et al.*, 1988].

Gubler and Hennecke [1986] proposed that the *fixABCX* genes are involved in electron transport to nitrogenase in microaerobic bacteria. Later, it was found that the *fixB* gene product is homologous to the α-subunit of the Electron Transfer Flavoprotein (ETF)

of human and rat origin [Arigoni *et al.*, 1991; Wientjens *et al.*, 1993], whereas the FixX protein and the FixP protein, recently discovered by Wientjens *et al.* [1993] are homologous to bacterial ferredoxins [Earl *et al.*, 1987; Kaminski *et al.*, 1988; Arigoni *et al.*, 1991; Grönger *et al.*, 1987; Iismaa and Watson, 1987]. These observations suggest that the FixABCX proteins are part of an electron transfer chain.

The fact that the *fixABCX* genes are also present in the *E. coli* genome, in which they are followed by a gene encoding a NAD(P)H dehydrogenase, could be an indication that these genes are all involved in an electron transfer process using NAD(P)H as reductant.

The *fixPABCX* genes could on the other hand be involved in a regulatory process, possibly involved nitrogen fixation, using the FixP and FixX proteins (7Fe-ferredoxins) as regulatory proteins as proposed by Thomson [1991]. In this paper, the results of an investigation of the function of the *fixABCX* genes in nitrogen fixation in *A. vinelandii* is reported.

METHODS

Bacterial strains, vectors and growth conditions.

A. *vinelandii*, strain ATCC 478 and derivatives, and A. *vinelandii* strain OP (ATCC 13705) derivatives were cultured on Burks nitrogen-free basic salt medium, as described earlier [Newton *et al.*, 1953]. Bacterial strains and plasmids are listed in Table 1. For non-diazotrophic growth, ammonium acetate was supplied to a final concentration of 15 mM. Instead of sucrose, various other carbon sources (glucose, fructose, acetate, butyrate, ß-hydroxybutyrate, L-lactate, L-malate, or succinate) or amino acids (L-asparate, L-glutamate, L-glycine or L-threonine) were used as sole carbon and energy sources in final concentrations of 1% (w/v), after adjustment of the pH of the stock solutions to 7.0.

For induction of either of the two alternative nitrogenases, strains were grown on N-free medium prepared by extracting metals and adding back $FeSO_4$ (30 µM) with or without V_2O_5 (100 nM) [Kennedy and Dean, 1992]. Growth was assessed visually after colonies from the several strains were patched and replica plated onto agar medium.

E. *coli* TG2 (Table 1) was used as a host for pUC9-, pUC18-, and pACYC184-derived recombinant plasmids (Table 1). E. *coli* was grown at 37°C in TY medium or on TY plates containing 1.5% agar [Miller, 1972].

Antibiotics were added, when appropriate, in the following concentrations (in $\mu g.ml^{-1}$): for A. *vinelandii*, ampicillin (Amp) 100; kanamycin (Km), 2; streptomycin (Sm) 2; chloramphenicol (Cm) 50; for E. *coli*: Amp 100; Km 15; Sm 15; Cm 10.

Construction of plasmids with interrupted *fix*-genes.

The construction of an A. *vinelandii* gene library in E. *coli* and the isolation of clones containing the *fixPABCX* genes of A. *vinelandii* has been described previously [Wientjens *et al.*, 1993].

Plasmid pRW4 [Wientjens *et al.*, 1993] and derivatives were used to make mutations in the *fixA*, *fixB* and *fixC* genes and in the *fixABCX* gene cluster (Fig. 1[a]). The 2.2 kb *SstI-StuI* fragment of pRW4, made blunt ended with T4 polymerase, was ligated into the *SmaI* site of pUC18, resulting in plasmid pRW44. The *fixA* gene on this plasmid was then interrupted by insertion of the gene encoding kanamycin resistance from Tn5 [Beck *et al.*, 1982] into the unique *XhoI* site, creating plasmid pRW44KmA (Fig. 1[b]).

The *fixB* gene was interrupted by insertion of the 1.6 kb *SstI* fragment of plasmid pKIXX [Pharmacia], containing the genes encoding kanamycin and bleomycin resistance, into the unique *SstI* site of plasmid pRW4, resulting in plasmid pRW4KmB (Fig. 1[c]).

Plasmid pRW43KmC was constructed by insertion of the 1.2 kb *SmaI* fragment of pKIXX into the unique *Asp718* site of pRW43 [Wientjens *et al.*, 1993], which was made blunt ended by Klenow polymerase. This resulted in a construct in which the *fixC* gene was interrupted by the gene encoding kanamycin resistance (Fig. 1[d]).

Plasmids, in which the *fixABCX* genes were deleted, were made by substitution of the 3.2 kb *Bst*EII fragment of plasmid pRW41 [Wientjens *et al.*, 1993] with either the 1.2 kb *Sma*I fragment of pKIXX, or with the 7 kb promoterless *lacZ*-Km^r-Sm^r-cartridge of pGS100 (Table 1), resulting in plasmids pRWΔABCX (Fig. 1^e) and pRWΔABCX-*lacZ*-KS (Fig. 1^f), respectively.
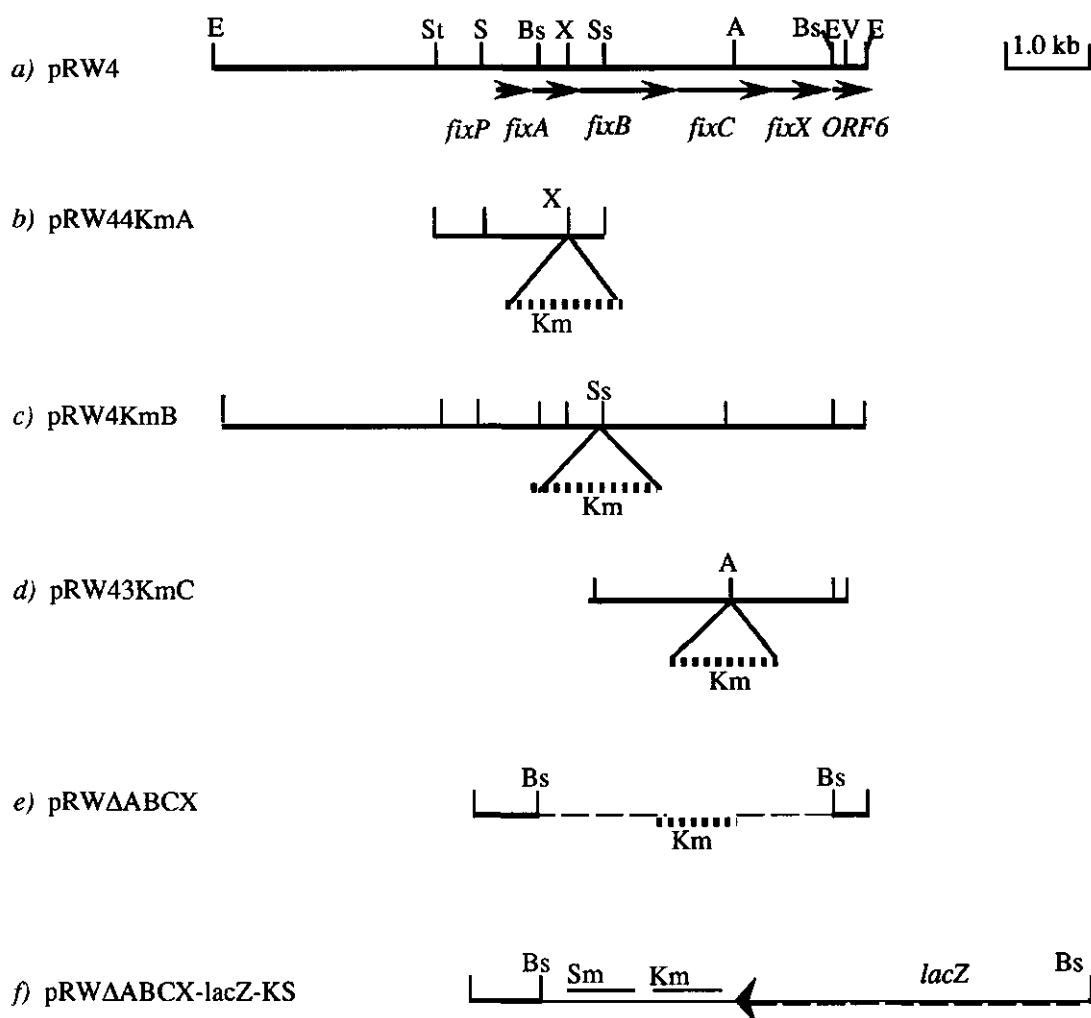

Fig. 1. Physical map of the inserts of plasmids containing insertions of the kanamycin resistance gene in the *fixABCX* region of *A.vinelandii*. Construction of the plasmids is described in Materials and Methods. Restriction enzymes are abbreviated as follows: *Asp*718, A; *Bst*EII, Bs; *Eco*RI, E; *Eco*RV, EV; *Sma*I, S; *Sst*I, Ss; *Stu*I, St; *Xho*I, X. "Km" is the gene for kanamycin resistance, "Sm" is

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the gene for streptomycin resistance; *lacZ* is the complete *lacZ* gene, encoding ß-galactosidase.

Construction of a plasmid with a *fixA::lacZ* gene fusion.

The construction of the *fixA::lacZ* gene fusions is shown in Figure 2. Plasmid pRW44 was digested with *Sal*I, which resulted in a 1.7 kb fragment, containing the *fixPABCX* promoter region, *fixP* and the first 457 bp of the *fixA* gene. This fragment was ligated into the *Sal*I site of pACYC184, resulting in plasmid pRW45. pRW45 was linearised by digestion with *Bst*EII, which cuts 60 base pairs downstream of the ATG start codon of the *fixA* gene, followed by treatment with Klenow polymerase in the presence of 0.2 mM dNTPs. The 7 kb *lacZ*-Km^r-Sm^r cartridge, containing the promoterless *lacZ* gene and genes encoding kanamycin and streptomycin resistance, was isolated from pGS100 [Walmsley and Kennedy, 1991] by digestion with *Bam*HI, and ligated into the linearised pRW45, resulting in plasmid pRW46 (Fig. 2). pACYC184 was chosen as cloning vector, since this vector contains no *lacZ'*-fragment, thereby preventing unwanted recombination between this fragment and the *fixA::lacZ* and *nifH::lacZ* gene fusions.

The construction of the *nifH::lacZ* gene fusion, located on plasmid pJAW2, has been described previously by Walmsley and Kennedy [1991].
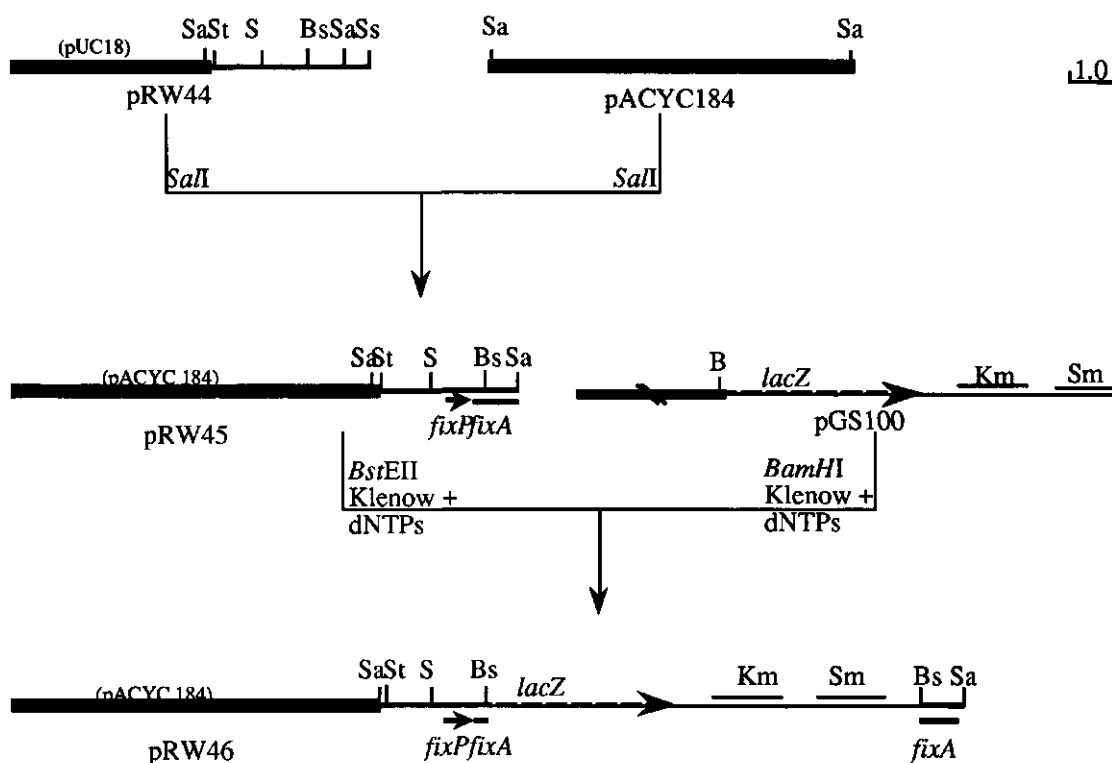
Fig. 2. Construction of pRW46 with a *fixA::lacZ* fusion. Restriction sites are abbreviated as described. *lacZ* is the complete *lacZ* gene, "Km" is the gene for kanamycin resistance, "Sm" is the gene for streptomycin resistance.

Construction of *A. vinelandii* mutants with disrupted *fix genes* by marker exchange.

Plasmids, pRW44KmA, pRW4KmB, pRW43KmC and pRWΔABCX containing the mutated genes, and plasmids pRW46 and pJAW2, containing the *fixA::lacZ* and the *nifH::lacZ* gene fusions in pACYC184, respectively, were introduced into *A. vinelandii* by transformation according to a modification of the method of Page [1985]. Therefore, *A. vinelandii* cells were streaked twice on agar plates containing modified Burks medium in which iron and molybdenum were omitted and to which 15 mM ammonium acetate was added (competence medium plates). Cells were grown for 2 days at 30°C until the colonies were bright green. All cells from the plate were resuspended in 40 ml of Fe- and Mo-free Burks medium with ammonium acetate (competence medium) and grown for 48 hours at 30°C in an incubator shaker at 180 rpm. Cells were harvested from the bright green medium by centrifugation at 6000*g for 10 min. and resuspended in 2 ml of competence medium (30°C), supplemented with MgSO₄ in a final concentration of 16 mM. Approximately 1 μg of DNA in a volume of 10 μl was added to 100 μl of these competent cells and the mixture

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was spotted on a competence medium plate, that was incubated for 24 hours at 30°C. About 5.10^7 cells from this spot were plated on Burks agar medium with 15 mM ammonium acetate and the appropriate antibiotic for the selection of crossing-over.

After subcloning colonies, that became visible after 2-3 days of incubation, three times by streaking on selective agar to segregate the wild-type gene, recombinants were further analysed. As pUC- and pACYC184-derived plasmids do not replicate in *A. vinelandii*, colonies expressing the insert-encoded antibiotic resistance arise either by double crossing-over or by integration of the entire plasmid in the bacterial genome. Less than 5% of all isolates had the vector-encoded resistance, suggesting that a double cross-over, resulting in marker exchange, was the predominant event.

For further analysis of the mutant strains chromosomal DNA was isolated and digested with suitable restriction enzymes. After electrophoresis the DNA was transferred to Immobilon-N sheets (Millipore) and hybridised with the insert of pRW4, made radioactive by nick-translation with [α^{32}P]dATP as described previously [Wientjens *et al.*, 1993]. Mutants in which double crossing-over had occurred, were identified by their restriction pattern.

As Azotobacters contain multiple copies of their genome per cell (20-25 for *Azotobacter chroococcum* [Robson *et al.*, 1984] and even up to 80 for *A. vinelandii* [Punita *et al.*, 1989]), it was necessary to verify that the cross-over had occurred in all copies of the genome. Therefore, digested DNA from wild-type *A. vinelandii*, digested DNA from the respective mutant strains, and mixtures of digested DNA from mutant and wild-type strains (in ratios up to 100:1) were electrophoresed, blotted, and hybridised with the radioactive pRW4 insert.

A. vinelandii mutants with the kanamycin gene inserted in the *fixA*, *fixB* or *fixC* genes, replacing the *fixABCX* genes or containing *fixA::lacZ* and *nifH::lacZ* fusions are listed in Table 1.

FixA⁻ mutants of *A. vinelandii* strains MK8 and MK12 were constructed essentially in the same way, except that streptomycin was used as selective agent (as both strains are kanamycin resistant) and that MK12, which is defective in cytochrome *d*, was made competent under microaerobic conditions. The plasmid used for transformation was pRWΔABCX-*lacZ*-KS.

Construction of *fixA⁻* mutants of strain OP derivatives.

In order to determine the effect of the deletion of the *fixABCX* genes on the alternative nitrogenases, the interrupted *fixA* gene was also introduced into mutants of *A. vinelandii* strain OP, that were unable to synthesise the MoFe-nitrogenase. Therefore, *A. vinelandii* UW136, UW10 , and UW1 (Table 1) were made competent for DNA transformation according to Bali *et al.* [1992]. A crude lysate with total genomic DNA was prepared from strain AvfixA⁻ by suspending approximately 10^9 cells in lysing buffer (1

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mM MgSO$_4$) and heating at 65°C for 60 min. Ten µl of the crude preparation was mixed with 50 µl of competent cells on competence medium plates and the mixture was incubated for 2 days at 30°C. About 5.10^7 cells from this mixture were plated on Burks medium with ammonium acetate and kanamycin. Several hundred kanamycin resistant colonies grew from the transformation of all three recipients, UW136, UW10 and UW1. A few of each were picked, subcultured three times by streaking on medium with kanamycin and ammonium acetate, before testing growth properties.

Physiological analysis of the mutants.

Growth of wild-type and mutant *A. vinelandii* strains in liquid Burks medium was carried out in 600 ml Burks medium in 2 litre Erlenmeyer flasks, incubated at 30°C in a New Brunswick gyratory shaker (180 rpm). When appropriate, kanamycin was added. Growth curves were obtained by monitoring the protein concentration determined by the method of Sedmak and Grossberg [1977], using bovine serum albumin as a standard. Growth on a modified Burks medium, containing carbon sources other than sucrose, was carried out on plates.

Microaerobic growth was assessed on Burks plates in a 3 litre jar with an oxygen concentration of 1.5-2.0% (v/v) oxygen, as determined by gaschromatography. Mutants MK12ΔfixABCX and MK8ΔfixABCX (Table 1) were tested for growth under both aerobic and microaerobic conditions. Iron-free growth was carried out on Burks nitrogen-free agar, from which FeSO$_4$ was omitted.

In vivo and *in vitro* nitrogenase activities of wild-type and mutant *A. vinelandii* strains were determined by measuring the acetylene reduction activities from cells grown diazotrophically or grown in the presence of ammonium acetate. *In vivo* acetylene reduction activities were determined with intact cells in a setup described by Haaker *et al.* [1974], in which the buffer was replaced by Burks medium.

For determination of *in vitro* nitrogenase activities, intact cells were washed twice in 50 mM TES-NaOH, 5 mM MgCl$_2$, pH 7.5 (wash buffer), and concentrated in a minimal volume of the wash buffer. Cells were disrupted by ultrasonic treatment under an argon atmosphere, using a Soniprep 150 ultrasonic disintegrator (MSE). Cell-free extract was obtained by centrifugation at 10000*g for 15 minutes. The supernatant (cell-free extract) was used in the *in vitro* acetylene reduction assay as described by Haaker and Veeger [1977]. Protein concentrations of the cell-free extracts were determined with the microbiuret method [Goa, 1953]. Bovine serum albumin was used as a standard.

Antibodies against the FixA protein.

Antibodies were raised against the *fixA* gene product, which was overproduced in *E. coli* TG2 (Table 1). Therefore, the *fixA* gene was inserted downstream of the *lacZ* promoter of pUC18. The resulting plasmid pRW47 was introduced in *E. coli* TG2 and

expression of the FixA protein was induced with IPTG (20 $\mu g \cdot ml^{-1}$). Cells were disrupted by sonication in the presence of 4% Triton-X100 and inclusion bodies containing the FixA protein were separated from cytoplasmic and solubilised membrane proteins by centrifugation for 30 min at 100000*g. The FixA protein was further purified from the inclusion bodies by electroelution from a 12.5% polyacrylamide gel containing 0.1% SDS. The purified FixA protein was used to illicit antibodies in mice. Antibodies were used for detection of FixA in *A. vinelandii* cell lysates by immunoblotting.

SDS-polyacrylamide gel electrophoresis, electroelution, and immunoblotting were carried out as described by Ausubel *et al.* [1987]. The alkaline phosphatase catalysed reaction, using 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium chloride (NBT) as chromogenic substrates, was used as detection method in the immunoblotting assays.

β-galactosidase activity measurements.

The *A. vinelandii* strains containing either the *fixA::lacZ* gene fusion or *nifH::lacZ* gene fusions were grown in 10 ml cultures in 50 ml Erlenmeyer flasks in Burks medium with 15 mM ammonium acetate and 2 $\mu g.ml^{-1}$ kanamycin to mid-exponential phase. Cells were harvested and resuspended in Burks medium with 2 $\mu g.ml^{-1}$ kanamycin and, when required, 15 mM ammonium acetate, and grown for another 6 hours. 100 μl samples were taken from the cultures and the cells were lysed with 0.1% SDS and chloroform as described [Miller, 1972]. β-Galactosidase activities were measured, using *o*-nitrophenyl-ß-D-galactoside as substrate, according to the method developed by Miller [1972]. The ß-galactosidase activities are expressed as Miller-units.

Genetic analysis

Several *A. vinelandii* mutants with disrupted *fix* genes have been constructed by marker exchange (Table 1). In order to check that all *fix* genes were replaced in all (up to 80) copies of the genome, which are present per cell, chromosomal DNA of the *A. vinelandii* mutants was isolated, digested with restriction enzymes and hybridised with the ^{32}P-labelled insert of pRW4. Figure 3 shows the hybridisation pattern of *Eco*RI-digested DNA from wild-type *A. vinelandii* and the AvfixA⁻ mutant. No wild-type copies are visible in the DNA of the AvfixA⁻ mutant (lanes 12 and 13). A control experiment, in which DNA from mutant and wild-type strain were mixed in ratios of 30:1 and 100:1 indicated, that a single wild-type copy of the gene among 100 mutated copies would have been detectable (see lanes 9 and 10). This indicates, that all copies of the *fixA* gene per cell had been mutated. All other mutations described in this paper were checked in a similar way (data not shown).
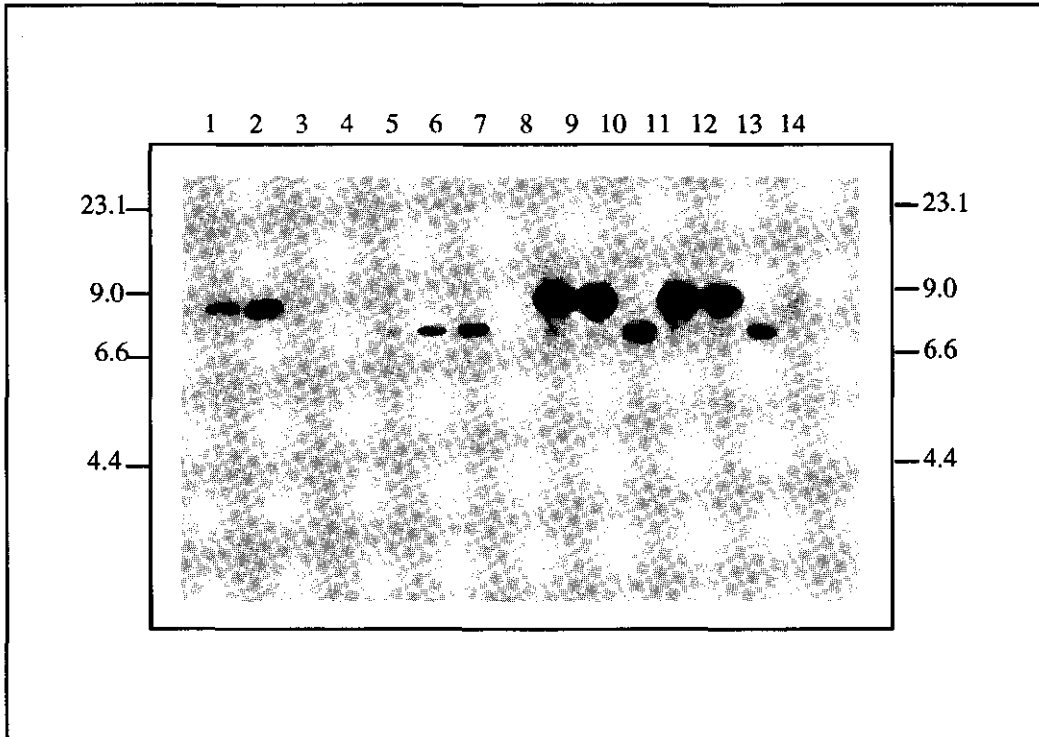
Fig. 3. Hybridization of *Eco*RI-digested DNA from wild type *A.vinelandii* and AvFixA⁻ with ^{32}P-labeled pRW4. Lane 1 and 2: AvFixA⁻ DNA, 5 μg; lane 3: lambda DNA, *Hind*III digested (not visible); lane 4: wild type DNA, 0.3 μg; lane 5: wild type DNA, 0.6 μg; lane 6: wild type DNA 1.3 μg; lane 7: wild type DNA, 2.5 μg; lane 8: lambda DNA, *Hind*III digested (not visible) ; lane 9: wild type DNA (0.33

μg) + AvFixA⁻ DNA (10 μg); lane 10: wild type DNA (0.1 μg) + AvFixA⁻ DNA
(10 μg); lane 11: wild type DNA: 7.5 μg; lane 12 and 13: AvFixA⁻ DNA, 10 μg;
lane 14: wild type DNA: 7.5 μg.

Phenotype of the mutants.

The *in vitro* nitrogenase activities of mutants with inactivated *fix* genes were
determined (Table 2).

Strain	Nitrogenase activity[¶]	
	No addition of AV2	Addition of AV2[†]
ATCC478	60	120
AvfixA⁻	75	166
AvfixB⁻	61	137
AvfixC⁻	41	75
AvΔfixABCX	58	136

Table 2. *In vitro* nitrogenase activities of wild-type and mutant strains cell-free extracts
of *A.vinelandii*.

¶: nmoles C_2H_2 reduced·min^{-1}·mg extract protein^{-1}.

†: AV2: *A.vinelandii* Fe protein of nitrogenase, added in saturating
concentrations.

The results indicate that the acetylene reducing activities of the mutants are
comparable to the wild-type activities. The specific activities of the AvfixC⁻ mutant may be
caused by slightly different growth conditions, which cause the specific activity of the
nitrogenase in the cell to decrease. The doubling of the acetylene reduction activity after
addition of saturating concentrations of nitrogenase Fe protein indicates that both
nitrogenase proteins have wild-type characteristics. These data clearly show that the mutants
produce active nitrogenase. No *in vitro* nitrogenase activity was detected in extracts of the
AvΔfixABCX⁻ mutant, grown in the presence of ammonium acetate, indicating that the
fixABCX gene products do not act as repressors of the nitrogen fixation system in response
to ammonia, as does the *nifL*-like gene product of *A. vinelandii* [Bali *et al.*, 1992].

In order to test the nitrogenase activity *in vivo*, we determined growth of the mutant
strains under N-fixing conditions and we determined the *in vivo* nitrogenase activity,
measured as acetylene reduction at different oxygen input rates. Growth curves of wild-type
and mutant strains in Burks nitrogen-free medium with sucrose as carbon source are shown

in Figure 4. No difference in growth rates was observed between AvfixA⁻, AvfixB⁻, AvfixC⁻ and wild-type strains.
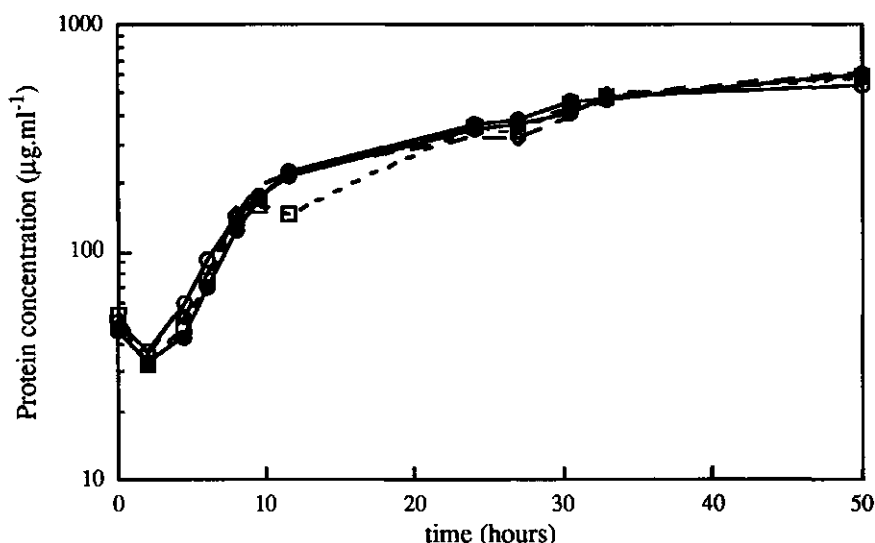
Fig. 4. Growth curves of wild-type *Azotobacter vinelandii and* Fix⁻ mutant strains. Cells were grown at 30°C in Burks medium using sucrose as carbon source and without fixed nitrogen. Growth, assessed as increase in cellular protein, is shown for ─●─ : ATCC478 (wild-type); ─○─ : AvfixA⁻; ─□─ : AvfixB⁻; ─◇─ : AvfixC⁻.

The *in vivo* nitrogenase activities of the wild-type *A. vinelandii* and the AvΔfixABCX mutant are shown in Figure 5. The *in vivo* nitrogenase activity is dependent on respiration and is therefore controlled by the oxygen input [Klugkist *et al.*, 1986]. At low oxygen input rates the *in vivo* activity is limited by the activity of the respiratory chain. At high oxygen input rates the nitrogenase is inactivated by excess oxygen. This gives a bell-shaped curve when the *in vivo* nitrogenase activity is plotted as a function of oxygen input rates.
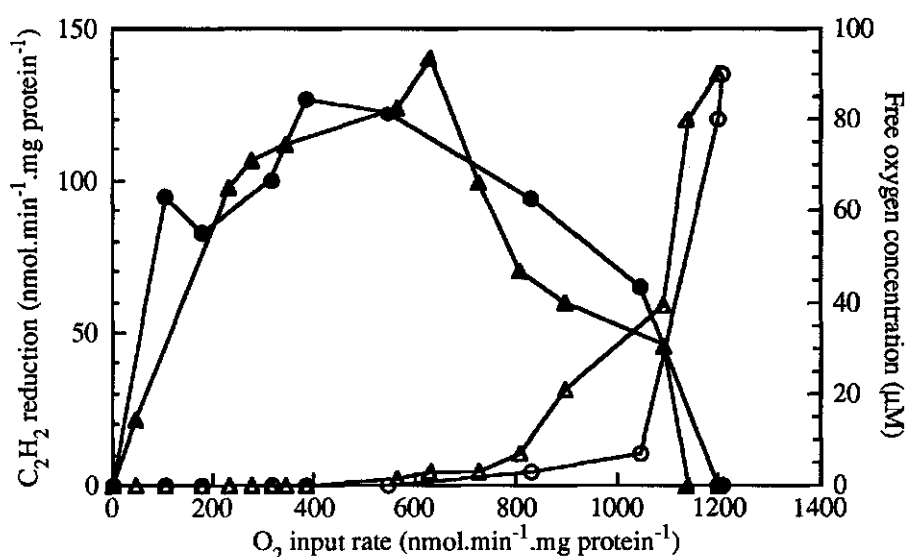
Fig. 5. *In vivo* nitrogenase activities of wild-type *A.vinelandii* and the AvΔfixABCX mutant as function of the O₂-input in the assay. Nitrogenase activity (closed symbols) was measured as C₂H₂ reduction. Open symbols represent the concentration of free oxygen. —●— or —○— : wild type; —▲— or —△— : AvΔfixABCX.

As shown in Fig. 5, no difference in *in vivo* activity is detected between the wild-type strain and the AvΔfixABCX mutant; both strains have maximal nitrogenase activity (approx. 130 nmol acetylene reduced per min per mg protein) at the same O₂-input rate (300-600 nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$).

Strains UW10 and UW1, unable to express the MoFe-nitrogenase, because of deletions in the *nifD* and *nifA* genes, are dependent on one of the two alternative nitrogenases for diazotrophic growth. Inactivation of the *fixA* gene in these strains (resulting in strains MV732 and MV734, Table 1) did not influence diazotrophic growth, neither under conditions under which the VFe-nitrogenase is induced (Mo-free, V-containing medium), nor under conditions inducing the all-Fe-nitrogenase (Mo-free, V-free medium).

The *fix⁻* mutants were also tested for growth on different carbon sources on nitrogen-free medium. C₄-dicarboxylic acids (succinate and malate) were tested, as these sources are the main energy supply for nitrogen fixation in symbiotic nitrogen fixing bacteria [Ronson, *et al.*, 1981; Ronson and Primrose, 1979]. Butyrate, ß-hydroxybutyrate

and threonine were used, as acylCoA dehydrogenases, involved in the degradation of these substrates, require the presence of an electron transfer flavoprotein [Davidson *et al.*, 1986] (the *fixB* gene product is homologous to the α-subunit of electron transfer flavoprotein [Arigoni *et al.*, 1991; Wientjens *et al.*, 1993]). No differences in growth were detected however, when wild-type and mutant strains were grown on these substrates. There was neither a difference in growth of wild-type and mutants under microaerobic conditions (1-2% oxygen, v/v). Growth of the cytochrome *d* mutants MK8ΔfixABCX and MK12ΔfixABCX (Table 1) was also not altered by deletion of the *fixABCX* genes. Both under microaerobic and aerobic conditions, growth of single and double mutants was similar.

Iron was omitted from the medium to test whether the *fixABCX* genes were hampered in their iron-uptake systems. None of the mutants growth characteristics differ from the wild-type characteristics, so iron uptake is not distorted. None of these qualitative tests led to a difference between wild-type and mutants.

Expression of the *fixABCX* genes.

The expression of the *fixABCX* operon in *A. vinelandii* was tested by two independent methods. The presence of the FixA protein was investigated by immunoblotting and the activity of the *fixA* promoter was tested by measuring the ß-galactosidase activity in the *fixA::lacZ* gene fusion strain, AvproA.

When the lysates of diazotrophically grown *A. vinelandii* cells were assayed for the presence of the FixA protein by immunoblotting, a very weak signal could only be detected when 300 µg of total protein was loaded in one slot of the polyacrylamide gel (data not shown). Approximately half of the signal found for wild-type extract was found in cell extracts grown in the presence of ammonia. A signal, approximately one third of the signal found for the wild-type extract, was found when an AvfixA⁻ extract was assayed. This implies that if the FixA protein is present in these cells, the concentration is very low.

The activity of the *fixABCX* promoter was assessed by measuring the ß-galactosidase activity in the *fixA::lacZ* fusion strain, AvproA. The results are summarised in Table 3. The ß-galactosidase activity of AvproA cells grown in medium containing ammonia was low and was not significantly increased when cells were transferred to a medium without ammonia. As a control, the *nifH* promoter showed a more than fifty fold increase when cells of AvproH containing a chromosomally integrated *nifH::lacZ* gene fusion were transferred to medium requiring diazotrophic growth.

DISCUSSION

There appears to be a major difference between the function of the *fixABCX* genes of *Rhizobia* and those of *A. vinelandii*. The rhizobial genes are unequivocally involved in the process of nitrogen fixation, both symbiotic and free-living, whereas for the *A. vinelandii fixABCX* genes no function in nitrogen fixation could be established at present. Both *in vivo* and *in vitro* nitrogenase activities of the *A. vinelandii fix⁻* mutants are similar to those of the wild type strain. Since no *in vitro* nitrogenase activity of mutants was observed when cells were grown in the presence of ammonia, the *fixABCX* gene products are not involved in repression of nitrogenase synthesis in response to ammonia, as does the *nifL* gene product of *A. vinelandii* [Bali *et al.*, 1992].

We investigated various conditions to detect a phenotype of the mutations in the *fixABCX* genes. The influence of the carbon source, by growing *fixA⁻*, *fixB⁻*, *fixC⁻*, and *ΔfixABCX⁻* mutants under N-fixing conditions on different carbon sources was investigated. All *fix-* mutants grew normally on different carbon sources, among these C4-dicarboxylic acids (used as C-source by bacteroids and known have a separate regulatory system, involving the *dctA*, *dctB* and *dctD* genes in *Rhizobia* [Ronson *et al.*, 1987]) and C-sources that require ETF for degradation (ETF is involved in electron transport from acylCoA dehydrogenase to ubiquinone [Davidson *et al.*, 1986]). Also under other conditions (low oxygen concentrations, since in *Rhizobia*, nitrogen fixation requires microaerobic conditions, or low iron content of the medium, which induces *e.g.* the iron uptake systems), growth of *A. vinelandii* under nitrogen fixing conditions was not dependent on intact *fix* genes. All experiments indicate that deletion of either one or all of the *fixABCX* genes does not influence the *in vivo* nitrogenase activity. Obviously, this also means that the *fixABCX* genes are not essential for the electron transport to nitrogenase in *A. vinelandii*.

In the experiments, in which the functions of *fixABCX* genes were investigated, the genes have been interrupted by insertion of a marker gene or marker genes. As a consequence of this insertion there may be no translation of genes downstream of the mutated gene, caused by a termination signal in the insert, assuming that the genes are all controlled by the *fixPABCX* promoter. When any of these genes has a function in repression of the nitrogenase system, the blockade of the translation of these genes might influence the measurements, but this is not observed.

In symbiotic bacteria, expression of the *fixABCX* genes (or in *B. japonicum*, the *fixA* gene and the *fixBCX* operon, since the *fixA* gene is separated from the *fixBCX* genes [Fuhrmann *et al.*, 1985]) has been studied [Szeto *et al.*, 1984; Gubler and Hennecke, 1988; Gubler, 1989; Gubler *et al.*, 1989]. Expression of the genes in these organisms appears to be co-regulated with the expression of the *nif*-operon: both operons are regulated by the NifA-protein. The NifA protein functions as an activator by binding to an Upstream

Activator Sequence (UAS), with the consensus sequence TGT-N_{10}-ACA. Although the *B. japonicum fixA* lacks such an UAS and the *fixBCX* operon has an imperfect UAS (9-instead of 10-nucleotide spacing between the conserved TGT- and ACA- element of the UAS [Gubler, 1989]), their transcription is NifA- and sigma factor 54 (σ^{54})- dependent. In *A. vinelandii*, although a sequence with perfect homology to the consensus sequence for σ^{54}-dependent promoters and an imperfect UAS (TGT-N_9-ACA) are found upstream of the *fixP* gene [Wientjens *et al., 1993*], the expression of the *fixPABCX* genes does not seem to be co-regulated with expression of the *nif* genes: the promoter activity of the *fixA* gene does not increase under N_2-fixing conditions, as opposed to that of the *nifH* gene (Table 3). Both the low promoter activity of the *fixA* gene (2% of the *nifH* promoter) and the fact, that the FixA protein can hardly be detected with antibodies in wild type-cells under both nitrogen fixing and nitrogen replete conditions indicate that expression of the *fixABCX* genes is low under nitrogen fixing conditions in *A. vinelandii*.

The finding of sequences with perfect homology to both a possible σ^{54}-dependent promoter and an -although imperfect- UAS upstream of the *A. vinelandii fixA* gene, but low expression under diazotrophic conditions, suggests a second regulatory system acting as a repressor of expression of the *fixABCX* genes and possibly overruling NifA-dependent expression. No regulatory systems responding to carbon sources, levels of oxygen or iron content in the environment have been found.

It remains unclear what the function of the *fixABCX* genes in *A. vinelandii* is. Based on the homology of the *A. vinelandii fixP, fixB, fixX* genes with electron transfer proteins [Wientjens *et al.,* 1993], a function in some kind of electron transfer reaction is most likely. This reaction is not directly linked to nitrogen fixation in *A. vinelandii*, since mutations in the genes do not influence the nitrogen fixing ability, opposite to the rhizobial *fixABCX* gene products. Further evidence for this hypothesis is given by the fact that in the genome of *E. coli* the *fixABCX* genes are surrounded by genes encoding proteins involved in fatty acid metabolism and oxidation of NAD(P)H [Yura *et al.,* 1992]. No evidence for a regulatory function of the *fixPABCX* genes in *A.vinelandii* was found, so it seems therefore reasonable to assume that the *fixPABCX* genes of *A. vinelandii* are involved in an electron transfer process which is possibly linked to fatty acid metabolism.

ACKNOWLEDGEMENTS

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CHAPTER 4

THE NADH:VIOLOGEN OXIDOREDUCTASE OF *AZOTOBACTER VINELANDII.*

THE NADH:VIOLOGEN OXIDOREDUCTASE OF *AZOTOBACTER VINELANDII.*

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Abbreviations

MV is methyl viologen, BV is benzyl viologen, DAPV is Di-(*n*-aminopropyl) viologen, MAPV is Mono-(*n*-aminopropyl) viologen, MPSV is Monopropyl sulphonic acid viologen, DPSV is Di-propyl sulphonic acid viologen. Tes/NaOH pH 7.4 is N-TRIS-(hydroxymethyl)-methyl-2-aminoethanesulphonic acid, adjusted to pH 7.4 with NaOH.

Summary

Substrates that reduce ubiquinone in *Azotobacter vinelandii* cytoplasmic membranes were tested for the reduction of viologen. Only with NADH, viologen reduction could be detected. The other substrates tested, NADPH, malate, succinate and lactate, were unable to reduce viologen.

The kinetic properties of the NADH:viologen oxidoreductase were determined and compared with those of the NADH:ferricyanide oxidoreductase activity, an activity of the NADH:ubiquinone oxidoreductase complex of the respiratory chain. As found for the beaf heart enzyme system, the kinetics of the NADH:ferricyanide oxidoreductase are consistent with a ping pong bi bi reaction mechanism with double substrate inhibition, while the kinetics of the NADH:viologen oxidoreductase could not be described with this mechanism. The kinetics of the NADH:viologen oxidoreductase activity at low NADH concentrations (≤ 0.5 mM) follow Michaelis-Menten kinetics, whereas at higher concentrations, NADH acts as an an activator of the NADH:viologen oxidoreductase activity.

The reactivity of the NADH:viologen oxidoreductase activity with different viologens was studied. The larger the charge of the viologen, the higher the NADH:viologen oxidoreductase activity. When the charge of the viologens was the same, a more positive midpointpotential resulted in a higher oxidoreductase activity.

A remarkable phenomenon was the observation that viologen is reduced during NADH oxidation by the respiratory chain. This reaction was studied. It was found that the distribution of electrons over viologen reduction or the reduction of dioxygen by the terminal oxidases was not influenced by the input rate of electrons at the level of the NADH dehydrogenase, when this activity was modified by the redox potential of the NADH/NAD$^+$ couple applied. When the activity of the NADH:viologen oxidoreductase activity was increased (higher viologen concentration or a better reducable viologen), more electrons were used for viologen reduction. However, even under the most favourable conditions for viologen reduction, maximally 50% of the electrons from NADH were transferred to the viologens.

A model is proposed, by which the distribution of electrons from NADH over viologens and respiration can be explained. A link to the reduction of low potential electron carriers, required for nitrogen fixation, in the presence of oxygen, is made.

Introduction

Nitrogen fixation, the reduction of N_2 to ammonia, requires the enzyme nitrogenase, an anaerobic environment, MgATP and a strong reductant. This reductant should have a redox potential of -400mV or lower, in order to be able to reduce oxidised Fe protein with MgADP bound. This reduction is part of the catalytic cycle of nitrogenase [Ashby and Thorneley, 1987]. While *in vitro* dithionite is almost exclusively used as reducing agent, *in vivo* only two classes of electron carriers with sufficiently low redox potentials, flavodoxins and ferredoxins, are known to be able to serve as electron donor in nitrogen fixation [Yates, 1971; Haaker, 1977].

In the facultative anaerobic bacterium *Klebsiella pneumoniae*, the reduction of flavodoxin is catalysed by a pyruvate:flavodoxin oxidoreductase (*nifJ* gene product), that transfers the electrons from pyruvate to flavodoxin (*nifF* gene product) [Hill and Kavanagh 1980; Nieva-Gomez *et al.*, 1980; Bogusz *et al.*, 1981]. This thioclastic reaction is probably the source of reducing power for nitrogen fixation in anaerobic bacteria in general [Haaker, 1986].

In obligate aerobic nitrogen fixing organisms, such as *Azotobacter vinelandii* and *Rhizobium leguminosarum*, it is not known how electrons are transported to nitrogenase. Aerobic nitrogen fixing bacteria lack a pyruvate:flavodoxin oxidoreductase activity. It was suggested that the so-called *fixABCX* genes are involved in electron transport to nitrogenase in *Bradyrhizobium japonicum* [Gubler and Hennecke, 1986], both in symbiotic and free-living state. However it has been shown for *Azorhizobium caulinodans* ORS571 [Kaminski *et al.*, 1988; Arigoni *et al.*, 1991] and *A.vinelandii* [Wientjens *et al.*, chapter 3, this thesis] that these genes are not essential for electron transport to nitrogenase in these organisms.

A number of enzyme activities in *A.vinelandii* have been described, that could be involved in electron transport to nitrogenase:

(1) A NAD(P)H:flavodoxin oxidoreductase activity was measured in membranes of *A.vinelandii* [Haaker *et al.*, 1977]. This finding led to the proposal that the pH gradient across the cytoplasmic membrane, generated during respiration, would supply the energy to enable the NAD(P)H:flavodoxin oxidoreductase to reduce flavodoxin. Experimental evidence for this hypothesis is lacking.

(2) Naik and Nicholas [1966] measured in membranes, isolated from *A.vinelandii*, a NADH:benzyl viologen oxidoreductase activity. This activity was suggested to be involved in electron transport to nitrogenase. A similar suggestion was made by Yates [1971], who investigated a NADH dehydrogenase, isolated from *Azotobacter chroococcum* membranes. In the presence of benzyl viologen, MgATP, nitrogenase and NADH dehydrogenase, acetylene reduction was observed. Although

flavodoxin or ferredoxin stimulated the NADH:benzyl viologen oxidoreductase at pH 7.4, no acetylene reduction was accomplished using flavodoxin or ferredoxin instead of benzyl viologen.

(3) Klugkist *et al.* [1986] found that a NADPH dehydrogenase complex, that is predominantly expressed during the induction of nitrogen fixation, contains a 29kDa polypeptide. This NADPH dehydrogenase, solubilised from the cytoplasmic membranes, might also be a part of the NAD(P)H oxidase system, described by Ackrell and coworkers [1972]. They showed that *A.vinelandii* membranes contain two pyridine nucleotide oxidising complexes, one specific for NADH and one for NADPH. Both dehydrogenases are linked to oxidative phosphorylation [Ackrell and Jones, 1971; Ackrell *et al.*, 1972].

Not only *A.vinelandii* contains more than one pyridine nucleotide oxidising complex, also in other bacteria more than one type of NADH dehydrogenase have been found. From *Thermus thermophilus* HB-8 [Yagi *et al.*, 1988] and from *Escherichia coli* cytoplasmic membranes [Hayashi *et al.*, 1989], two enzymatically distinct NADH dehydrogenases were isolated. *R.leguminosarum* also contains two NADH dehydrogenases, one of which is exclusively found in nitrogen fixing bacteroids [Klein Lankhorst *et al.*, 1988].

Up to now, only studies with intact cells provide information about the nature of electron transport to nitrogenase in obligate aerobic bacteria. From experiments with intact cells of *A.vinelandii*, a relation between respiration and nitrogen fixation was found [Klugkist *et al.*, 1986]. Based on this result, Haaker and Klugkist proposed a model, in which the NADPH dehydrogenase distributed electrons over respiration and flavodoxin reduction [1987]. It will be demonstrated in this paper that the NADH:viologen oxidoreductase catalyses this type of proposed reaction. It will be shown that electrons from NADH are distributed over viologen and ubiquinone during respiration.

Materials and Methods

Bacterial strain and isolation of membranes.

Azotobacter vinelandii (strain ATCC478) was grown in a 200 litre fermentor in a modified, nitrogen free Burk's medium with sucrose as sole carbon source.

Cells were harvested in the early logarithmic phase and resuspended in 100 mM Tris-HCl, pH 8.0. DNase I and RNase were added and the cells were disrupted by passage through a French pressure cell at 8000 psi. This extract was stored at -80°C.

Unbroken cells and cell debris were removed by anaerobic centrifugation for 30 min at 20000 g. Na_2SO_4 was added to a final concentration of 2 mM and the cell free extract was loaded on a DEAE column equilibrated with 50 mM Tris-HCl, 2 mM $Na_2S_2O_4$, pH 7.8. The column was washed with the same buffer and membranes were eluted directly from the column, whereas other proteins, amongst which nitrogenase, bound to the column. All further procedures were carried out aerobically. The membranes were sedimented by centrifugation between 30 min at 20,000 g and 60 min at 100,000 g. The pellet was resuspended in 50 mM Tes/NaOH (N-TRIS-(hydroxymethyl)-methyl-2-aminoethanesulphonic acid, adjusted to pH 7.4 with NaOH), 5 mM $MgCl_2$, pH 7.4 to a concentration of 10 mg.ml^{-1}, sonicated four 30 seconds intervals on ice and centrifuged as before. Washed membranes were resuspended in the wash buffer at a protein concentration of 10 mg.ml^{-1} and stored at -80°C.

Analytical methods.

Protein concentrations were measured by the microbiuret method as described by Goa [1953]. Bovine serum albumin was used as a standard.

NADH:ferricyanide reductase activity was assayed spectrophotometrically at 420nm in 50 mM Tes/NaOH, 5 mM $MgCl_2$ buffer (pH 7.4) containing 10 µg.ml^{-1} of membrane protein, using an extinction coefficient of ferricyanide of 1.03 mM^{-1}.cm^{-1}. Ferricyanide and NADH were added at various concentrations as indicated.

Rates of respiration were measured at 30°C using a Clark-type oxygen electrode in a reaction vessel with a total volume 1.6 ml, connected to a MSE-Spectroplus. An analogue to digital converter [Poptronics, type 417], interfaced with an Atari 1400ST computer, was used to collect the data, using the program Popspec. This program was also used for data analysis. All experiments were carried out at 30°C in 50 mM Tes/NaOH, 5 mM $MgCl_2$, pH 7.4 at a membrane protein concentration varying between 0.05 and 0.15 mg·ml^{-1}. Superoxide dismutase, the Fe-enzyme, was isolated from *Escherichia coli*, purified according to the method of Slykhouse and Fee [1976] (specific activity: 3000U. mg^{-1}) and added in a final concentration of 1.6 µg.ml^{-1}.

Both respiration and viologen reductase activity were assayed by measuring the oxygen consumption using NADH, NADPH, L-malate, succinate, L+D-lactate, or mixtures of these donors at saturating concentrations. When the maximal viologen reductase activity was measured, 5 mM KCN was present to inhibit oxygen reduction by the terminal oxidases. KCN also inhibits katalase activity. The stoichiometry of NADH oxidation by NADH:viologen oxidoreductase in the presence of CN^- is given in equation 1.
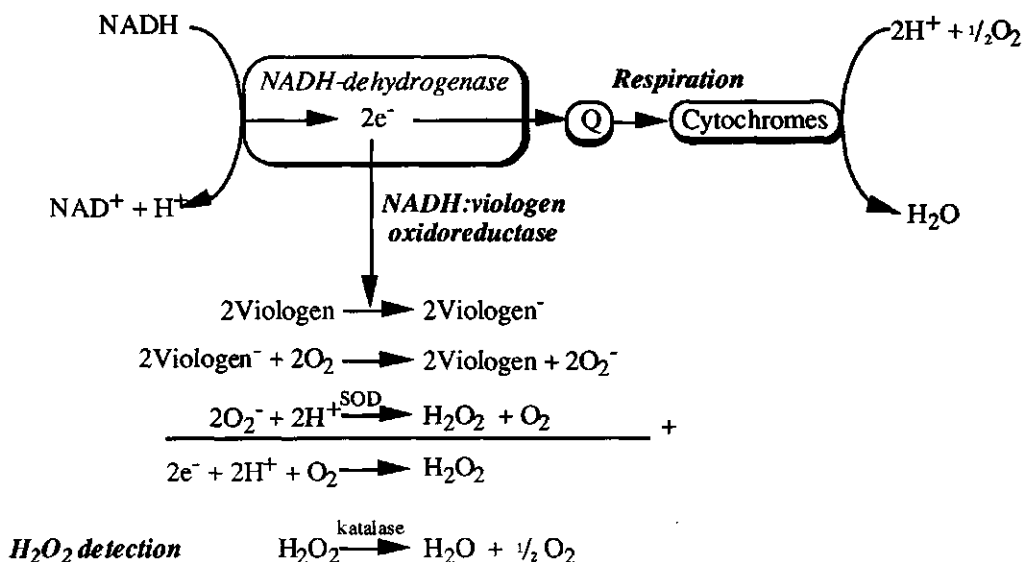
$$NADH + H^+ + O_2 \Rightarrow NAD^+ + H_2O_2 \quad \text{(See scheme 1).}$$

Equation 1: Oxidation of NADH by *A.vinelandii* cytoplasmic membranes in the presence of
viologen and 5 mM KCN.

The midpoint potential of the various viologens was measured by cyclic voltametry according to Hagen [1989], using a BAS CV27 voltammograph instead of the original voltammograph described. The concentration of the viologens was 2 mM in all experiments, unless indicated otherwise. In experiments, in which the initial redox potential was varied, the sum of the concentrations of the pyridine nucleotides ([NADH]+[NAD$^+$]) was 100 μM.

Detection of hydrogen peroxide.
The formation of hydrogen peroxide due to the activity of the NADH:viologen oxidoreductase under aerobic conditions was measured directly in the oxygraph reaction vessel. All solutions used were prepared freshly and were, as were the membranes, free of katalase activity. A limited, known amount of electron donor was added to the reaction mixture consisting of 50 mM TES/NaOH, 5 mM $MgCl_2$ pH 7.4, membranes, viologen and superoxide dismutase (to ensure all superoxide radicals to react to form hydrogen peroxide). At the end of the reaction, indicated by a stabilisation of the oxygen concentration in the reaction vessel, 20 μl katalase (from Boehringer, 0.5 mg.ml^{-1}) was added, causing the accumulated hydrogen peroxide to react to form water and oxygen. The increase in oxygen concentration was monitored and the amount of hydrogen peroxide accumulated due to the NADH:viologen oxidoreductase activity was calculated from the amount of oxygen formed. The reaction equations of the different modes of NADH oxidation are shown in Scheme 1.

$$\text{NADH} \quad \text{NADH-dehydrogenase} \quad \textbf{\textit{Respiration}} \quad 2H^+ + \tfrac{1}{2}O_2$$

$$2\text{Viologen} \longrightarrow 2\text{Viologen}^-$$

$$2\text{Viologen}^- + 2O_2 \longrightarrow 2\text{Viologen} + 2O_2^-$$

$$\underline{2O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2} \qquad +$$

$$2e^- + 2H^+ + O_2 \longrightarrow H_2O_2$$

$$\textbf{\textit{H}}_2\textbf{\textit{O}}_2 \textbf{\textit{ detection}} \qquad H_2O_2 \xrightarrow{\text{katalase}} H_2O + \tfrac{1}{2}O_2$$

Scheme 1: Reactions of the NADH dehydrogenase of *A.vinelandii*. SOD is Superoxide dismutase, Q is ubiquinone.

EPR spectroscopy.

Normal-mode-X-band EPR data were taken on a Bruker EPR 200 D spectrometer. The microwave frequency was measured with a Systron Donner frequency counter, model 1292A. The direct-current magnetic field was measured with an AEG Kernresonanz Magnetfeldmesser, type GA-EPR 11/21-02. The field was modulated with a frequency of 100 kHz. The amplitude of modulation was calibrated on the modulation broadening of the signal of the Bruker-strong-pitch sample. The temperature was measured with two 5-kΩ Allen-Bradley carbon resistors immersed in the EPR sample just below and above the measuring area of the TE_{102} cavity. The spectrometer was interfaced with a DASH-16 card to an Olivetti M24 PC with software written in ASYST for 1024-point data acquisition, correction for background signals (with frequency alignment), double integration procedures, and *g*-value determination.

Membranes, used for the EPR studies, were concentrated to a final concentration of 100 mg.ml^{-1} by centrifugation at 100,000 g for 1 hour. Membranes were either incubated with succinate and NADH or with succinate, NADH and dithionite, all at concentrations of 5 mM in 50 mM Tes/NaOH, 5 mM $MgCl_2$, pH 7.4. All samples were incubated at 20°C for 2-3 minutes and then frozen in liquid nitrogen. NaN_3 was added to inhibit the respiratory chain.

Results

Relation between oxidase and viologen reductase activities present in A.vinelandii cytoplasmic membranes.

Substrates, known to donate electrons to ubiquinone, were investigated for their ability to reduce viologens. Hydrogen was not tested since the cytoplasmic membrane hydrogenase was inactivated during the aerobic isolation of the membranes. The viologen reductase activity was measured as oxygen consumption in a system, in which the respiratory chain was inhibited by cyanide. The stoichiometry of this reaction is given in equation 1. It must be emphasised, that for each O_2 consumed by viologen oxidation, two electrons are consumed, whereas the reduction of one molecule of dioxygen by the respiratory chain requires four electrons.

Donor	O_2 consumption (μmol.min^{-1}.mg^{-1})	
	-	DAPV+ CN$^-$
NADH	2.30	4.80
NADPH	2.80	0.07
L-Malate	2.50	0.02
Succinate	0.17	0.01
L+D- Lactate	0.16	0.03
NADH + NADPH	2.40	4.60
NADH + L-Malate	4.50	4.80
NADPH + NAD$^+$	0.09	0.04
NADPH + L-Malate	4.20	0.06

Table 1: Respiratory and viologen oxidoreductase activities of *A.vinelandii* cytoplasmic membranes. The concentrations of electron donors used were: NADH and NADPH: 1 mM; L-Malate: 5 mM; Succinate: 10 mM and L+D-Lactate: 50 mM. DAPV is added at 2 mM. NAD$^+$ and NADP$^+$: 1 mM.

As shown in table 1, only NADH was able to reduce viologen. All other substrates, although having respiratory activities, lacked the possibility to reduce viologen. The fact that NADH is capable of reducing viologen, whereas NADPH is not, implies that NADH and NADPH must have different dehydrogenases in the cytoplasmic membrane of *A.vinelandii* and that the reactions are not reversible between ubiquinone and the dehydrogenases. The transhydrogenase activity of *A.vinelandii* cytoplasmic

membranes does not play a significant role in the viologen reduction, since the NADPH dependent viologen reduction is not stimulated by NAD^+. The inhibition of the pyridine nucleotide oxidation with the oxidised pyridine nucleotides also indicates two different dehydrogenases. $NADP^+$ hardly inhibits NADH or NADPH oxidation, whereas NAD^+ inhibits the NADPH oxidation for 96% and the NADH oxidation for only 35%.

Addition of both malate and NADH resulted in an increase of the respiratory activity, compared to the respiratory activity observed in the presence of either one of the electron donors. Similar results were obtained when malate and NADPH were added simultaneously, but no stimulation of the respiratory activity was found when NADH and NADPH were added simultaneously to the reaction mixture. These results indicate, that the NADH and NADPH dehydrogenases must have the same electron output site to ubiquinone. Malate dehydrogenase on the other hand, must have a distinct reduction site for ubiquinone, since addition of malate increased both NADH- and NADPH-respiration.

Kinetic properties of the NADH:viologen oxidoreductase of A.vinelandii cytoplasmic membranes.

To determine if the NADH:viologen oxidoreductase activity is the same as the NADH:ferricyanide oxidoreductase activity, which is a diaphorase activity of the NADH dehydrogenase of *A.vinelandii* cytoplasmic membranes, the kinetic properties of both activities were analysed. Typical results are shown in figure 1. It should be noted that the reduction of ferricyanide requires one electron, whereas the oxygen consumption is caused by the oxidation of two viologen molecules which are reduced each by one electron. This implies that under conditions where there is no substrate inhibition, the NADH dehydrogenase activities of both reactions shown in Fig. 1 are almost the same.
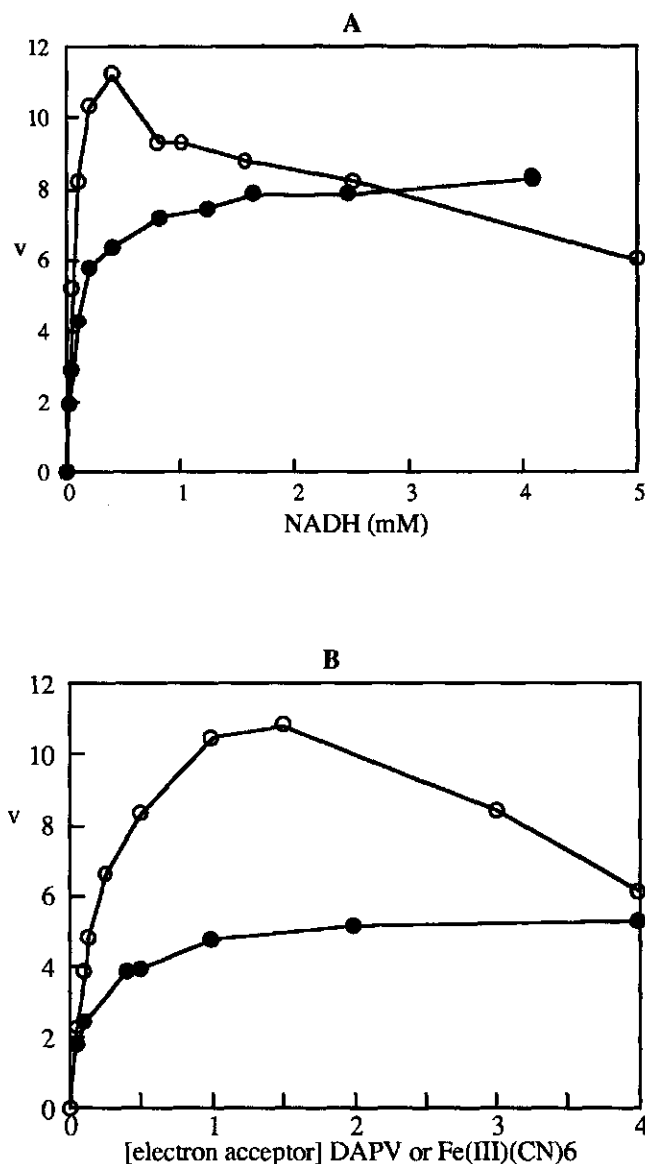
Figure 1. Kinetics of the NADH:ferricyanide oxidoreductase activity and the NADH:viologen oxidoreductase activity of *A.vinelandii* cytoplasmic membranes. Dependence of the NADH:ferricyanide and NADH:viologen oxidoreductase activities on **(A)** the NADH concentration (concentration of electron acceptors: Fe(III)(CN)$_6$, 1 mM, or DAPV, 0.25 mM) and on **(B)** the concentration of the electron acceptor used (concentration NADH 100 µM). Membranes were used at a final concentration of 10 µg.ml^{-1}. When DAPV was present, 5 mM KCN was added. "v" is either NADH:viologen oxidoreductase (—●—) (µmolO$_2$ consumed.min^{-1}.mg^{-1}) or

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NADH:ferricyanide oxidoreductase (—⊖—) (μmolFe(III)(CN)$_6$ reduced.min^{-1}.mg^{-1}).

The data demonstrate that the NADH:ferricyanide oxidoreductase activity of *A.vinelandii* cytoplasmic membranes show double substrate inhibition, as was found for the bovine heart mitochondria enzyme complex [Dooijewaard and Slater, 1976)] According to Dooijewaard and Slater, this implies that NADH and ferricyanide react at the same site. As shown in figure 1, this is clearly not the case for the NADH:viologen oxidoreductase activity.

The dependence of the NADH:viologen oxidoreductase activity on the substrate concentrations was investigated. As can be seen in Fig. 2A the dependence of the NADH:viologen oxidoreductase activity on the NADH concentration does not follow Michaelis-Menten kinetics. Also the dependence on the DAPV concentrations at fixed NADH concentrations (< 1 mM) show deviation from Michaelis-Menten kinetics at higher DAPV concentrations (insert in Fig. 2B). These data show that the kinetic properties of the NADH:viologen oxidoreductase are different from that of the NADH:ferricyanide oxidoreductase activity. The NADH:ferricyanide activity is inhibited at higher substrate concentrations while the NADH:viologen oxidoreductase activity is stimulated. A more detailed kinetic analysis is beyond the scope of this paper and will be the subject of a following paper.
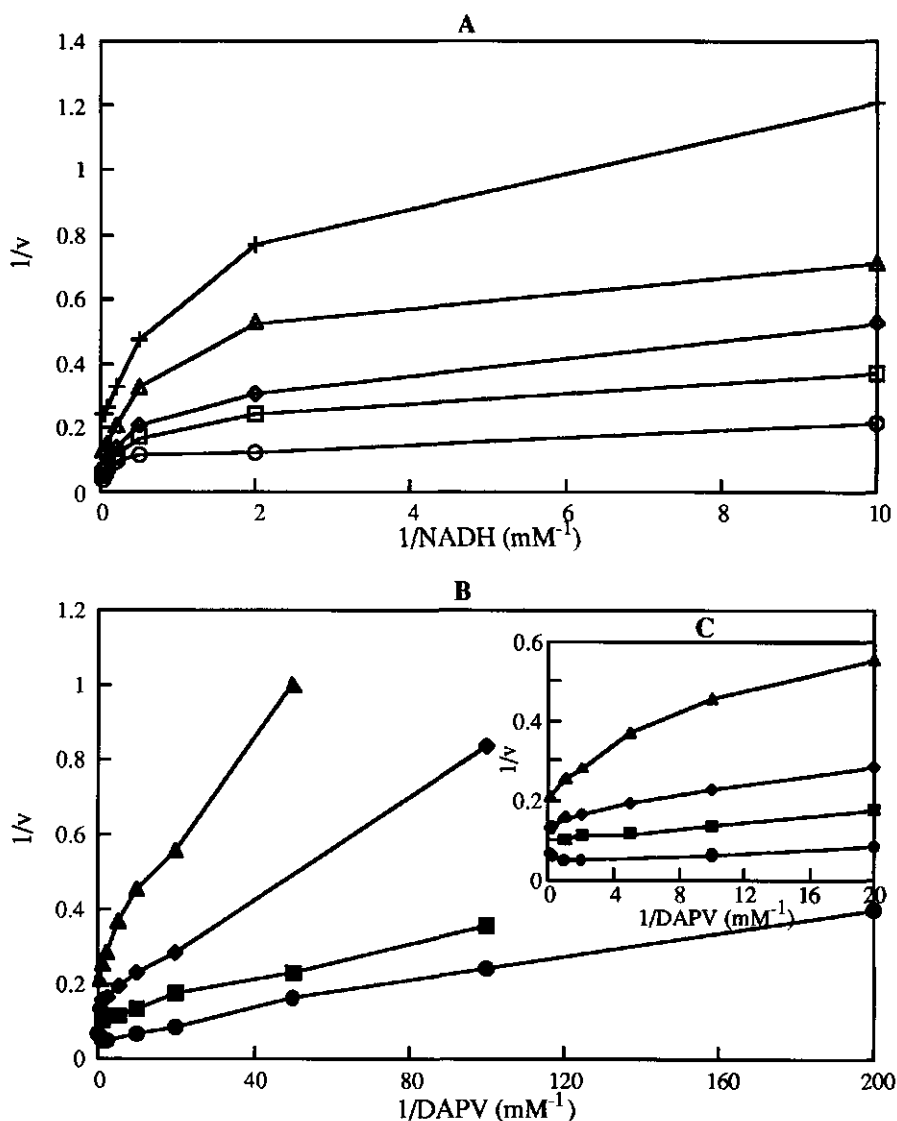
Figure 2: Lineweaver-Burk plots of the NADH:viologen oxidoreductase activity. **(A)** Reciprocal NADH:viologen oxidoreductase activity *versus* reciprocal NADH concentration at various fixed concentrations DAPV. ——⊖—— : 4 mM DAPV; ——□—— :0.2 mM DAPV; ——◇—— : 0.1 mM DAPV; ——△—— : 0.04 mM DAPV and ——+—— : 0.02 mM DAPV. **(B)** Reciprocal NADH:viologen oxidoreductase activity *versus* reciprocal DAPV concentration at various fixed concentrations NADH. ——●—— : 20 mM NADH; ——■—— :6 mM NADH; ——◆—— : 1 mM NADH and ——▲—— : 0.1 mM NADH. **(C)** The insert is a magnification of the 0-20 region of Fig. 2B.

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The NADH:viologen oxidoreductase activity was determined for different viologens. The results are summarised in table 2. As can be seen, the NADH:viologen oxidoreductase activity increased with both an increase in charge of the viologen and with an increase in redoxpotential. It was found that 2 mM MV, MPVS and DPVS were not saturating. The Km^{app} for MV was under the conditions 1.1 mM. The activity increased linearly with the concentration (tested to 20 mM, not shown). It is therefore concluded that the charge of the viologens influences the affinity of the viologens for the NADH:viologen oxidoreductase.

Species	Viologen Midpoint potential (mV)	Charge (oxidised form)	O_2 consumption (μmol.min^{-1}.mg protein^{-1})
DAPV	-320	4+	7.1
MAPV	-360	3+	5.9
BV	-360	2+	4.9
MV	-446	2+	2.8
MPSV	-395	1+	0.5
DPSV	-370	0	0.4

Table 2.NADH:viologen oxidoreductase activity of *A.vinelandii* cytoplasmic membranes. The midpoint potentials (vs SHE) were determined as described in Materials and Methods. NADH and viologens were added to a final concentration of 2 mM. DAPV: Di-(*n*- aminopropyl) viologen; MAPV is Mono-(*n*- aminopropyl) viologen; BV is Benzyl viologen; MV is Methyl viologen; MPSV is Mono-propyl sulphonic acid viologen; DPSV Di-propyl sulphonic acid viologen.

Reactivity of the NADH:viologen oxidoreductase during respiration.

When, in the presence of viologen, a limited amount of NADH was oxidised by the cytoplasmic membranes, the formation of H_2O_2 was demonstrated by the addition of katalase (Figure 3). No H_2O_2 was formed when the viologen was omitted from the reaction mixture. This implies that the viologen is responsible for the accumulation of H_2O_2. The small increase in the oxygen concentration after addition of katalase is caused by the oxygen present in the katalase solution itself. These results indicate that the NADH:viologen oxidoreductase, present in cytoplasmic membranes of *A.vinelandii*, is active during respiration.
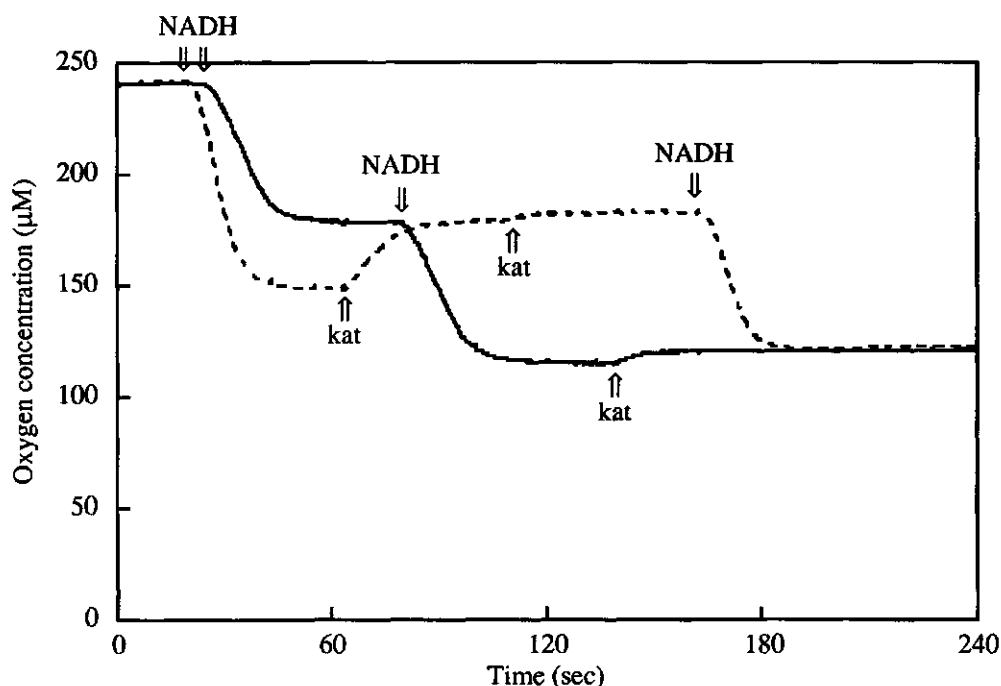
Figure 3: Measurement of hydrogen peroxide formation by the NADH:viologen oxidoreductase activity of *A.vinelandii* membranes (30 μg.ml^{-1}) in the absence of 2 mM methyl viologen (———), or in the presence of viologen (- - - - -). "NADH" indicates the addition of 207 nmol NADH; "kat" indicates addition of katalase (20 μl of 0.5 mg.ml^{-1} solution).

As can be seen in figure 3, the stoichiometry of the O_2 consumption per NADH oxidised also changes by the addition of viologen. This is caused by the formation of H_2O_2 via NADH:viologen oxidoreductase which leads a higher O_2 consumption for the same amount of NADH oxidised. From the amount of oxygen formed after the addition of katalase, or from the amount of O_2 consumed in the reaction with viologen compared to the O_2 consumption in the absence of viologen, the percentage of electrons from NADH reacting with the viologen was calculated according to the reactions depicted in Scheme 1. In the case of figure 3, the percentage was calculated as follows: The amount of oxygen formed after the addition of katalase is 31 μM (concentration increase) * 1.6ml (volume oxygraph vessel) = 50 nmol O_2. According to Scheme 1, twice the amount of electrons are required to form this amount of oxygen, namely 100 nmol. 207 nmol NADH was added, so 414 nmol electrons were available. 100 nmol of these 416 nmol electrons are used for viologen reduction, which is 25% of the electrons available.

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Relation between the H_2O_2 formation and the activity of the NADH:viologen oxidoreductase.

The activity of the NADH:viologen oxidoreductase was modulated in two ways (by altering the $NADH/NAD^+$ ratio and by using different viologens) and the effect on the viologen-dependent H_2O_2 formation was determined. A decrease in $NADH/NAD^+$ ratio inhibited respiration and viologen dependent O_2 uptake in the presence of cyanide, but it hardly affected the electron distribution between viologen and ubiquinone (Figure 4).
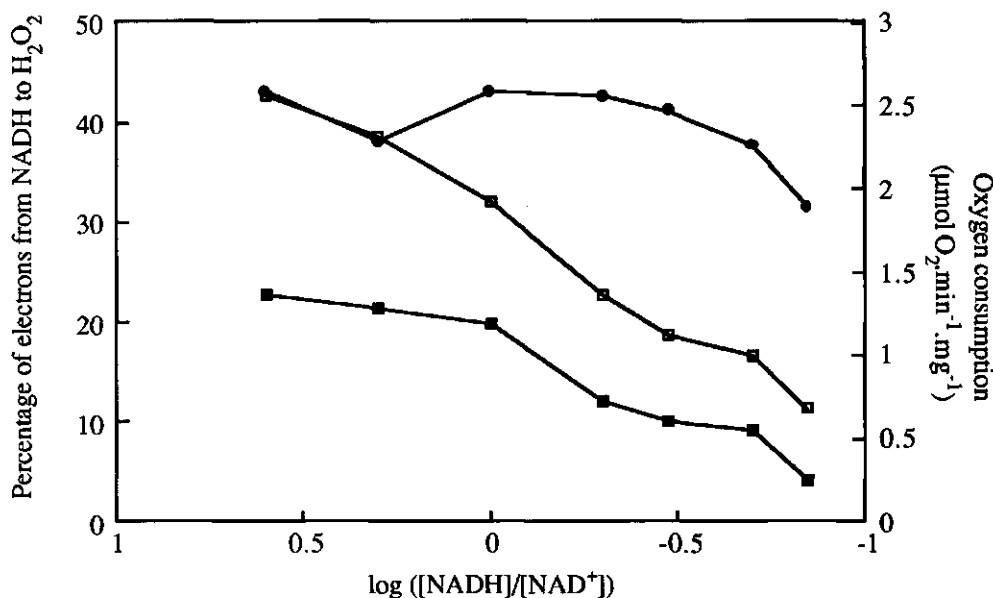
Figure 4: Influence of the initial $[NADH]/[NAD^+]$-ratio applied on the electron allocation to viologens to form H_2O_2 (—●—), the NADH:viologen oxidoreductase (NVOR) activity (—□—), and the respiration rate (—■—). The initial redox potential was varied by changing the $NADH/NAD^+$ ratio. Concentrations: $[NADH]+[NAD^+]$, 100 µM; Methyl viologen, 2 mM; membrane protein, 100 µg.ml^{-1}; Superoxide dismutase, 1.6 µg.ml^{-1}. When all NADH had reacted, katalase was added to a final concentration of 6.25 µg.ml^{-1}. The NADH:viologen oxidoreductase activity was measured under the conditions described in the presence of 5 mM CN$^-$. Under these conditions, O_2 is reduced by NADH to H_2O_2.

This implies that the distribution of electrons over respiration and viologen reduction is fixed at a certain viologen concentration, and is not altered by the electron input rate.

The activity of the NADH:viologen oxidoreductase activity can also be altered by the concentration of viologen (not shown) or by the the type of viologen. An increase in NADH:viologen oxidoreductase activity, measured in the presence of cyanide, due to a more active viologen, also increases the viologen-dependent hydrogen peroxide formation. The relation between the activity of the NADH:viologen oxidoreductase, modulated by different viologens and the hydrogen peroxide formation during respiration is shown in figure 5. It is clear that there is a linear relationship between the percentage of electrons of NADH used for hydrogen peroxide formation during respiration and the NADH:viologen oxidoreductase activity with different viologens. This indicates that the NADH:viologen oxidoreductase activity and the H_2O_2 formation during respiration are catalysed by the same enzyme.
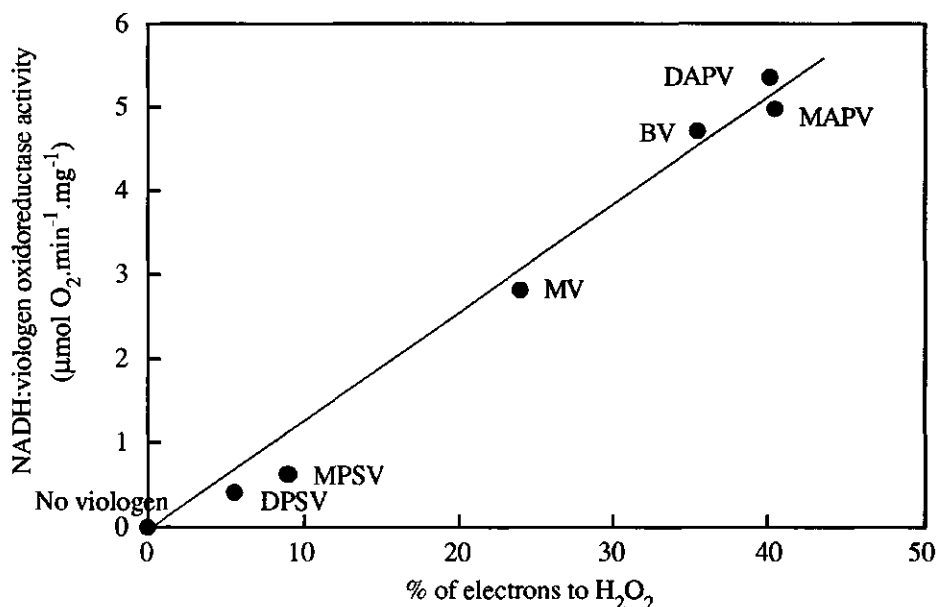
Figure 5: Relation between the NADH:viologen oxidoreductase activity and the percentage of electrons of NADH used for hydrogen peroxide formation during respiration with different viologens. The reactions were measured as described in materials and methods. The concentrations of the viologens was a 2 mM, NADH was supplied at 100 μM. Membrane protein concentration was 100 μg.ml^{-1}. The NADH:viologen oxidoreductase activity was measured under the conditions described in the presence of 5 mM CN$^-$.

EPR spectroscopy.

The site at which viologen is reduced is not known. In the bovine heart mitochondrial NADH dehydrogenase, the [Fe-S] clusters are called N-1, N-2, N-3 and N-4. N-2 is the [Fe-S] cluster, which transfers electrons to ubiquinone. The other [Fe-S] clusters are involved in intramolecular electron transfer [Weiss *et al.*, 1991]. It is reported that the N-1 [Fe-S] cluster is possibly composed of two clusters, N-1a and N-1b [Ohnishi *et al.*, 1972; Ohnishi, 1976], of which N-1a has a very low redox potential (-400mV [Ohnishi *et al.*, 1981; Ohnishi and Salerno, 1982; Ohnishi *et al.*, 1985]). Other groups have contradicted the existence of the N-1a cluster [Beinert and Albracht, 1982; Kowal *et al.*, 1986]. But if this "low-potential" cluster is present in *A.vinelandii*, then it is possible that this cluster is the site where the viologens are reduced by the NADH dehydrogenase complex. The EPR difference spectrum of NADH/succinate/dithionite reduced *A.vinelandii* cytoplasmic membranes minus NADH/succinate reduced membranes demonstrated the appearance of a signal of a [Fe-S] cluster with *g*-values of 2.038, 1.926 and 1.823 Gauss (Figure 6).
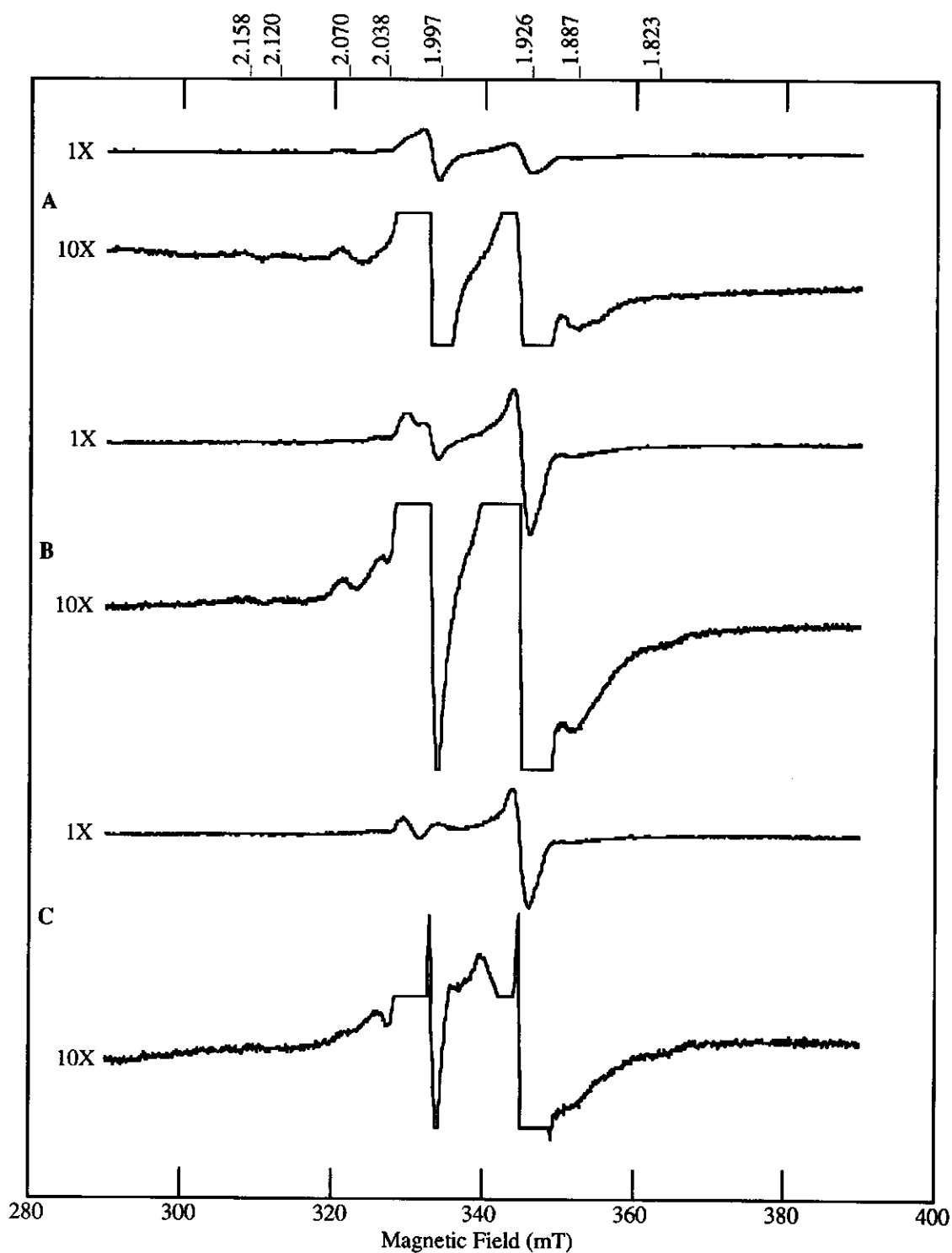
Figure 6: EPR spectra of *A.vinelandii* membranes (100 mg.ml^{-1}) in 50 mM TES/NaOH, 5 mM MgCl$_2$, pH 7.4, treated as described in Materials and Methods. **(A)** Spectrum of membranes reduced with succinate and NADH, **(B)** Spectrum of membranes reduced with succinate, NADH and dithionite. **(C)** Difference spectrum of NADH/succinate/dithionite minus NADH/succinate. The difference spectrum was constructed by subtracting the NADH/succinate spectrum from the NADH/ succinate/dithionite spectrum. EPR conditions: microwave frequency, 9.30 GHz; modulation amplitude, 1.0 mT; microwave power, 3.2mW; temperature, 16-17K.

It is clear that this cluster could not be reduced by NADH. This can be due to either a thermodynamical unfavourable condition (the redox potential of the cluster is too low) or it can have a kinetical reason, namely that there is no electron pathway from NADH leading to this cluster. Dithionite reduces this cluster directly. More work is required to characterise the cluster.

Discussion

In several papers, the properties of the pyridine nucleotide dehydrogenases, present in *A.vinelandii* cytoplasmic membranes have been described [Naik and Nicholas, 1966; Yates, 1971; Ackrell and Jones, 1971; Ackrell *et al.*, 1972]. Our experiments with viologens as electron acceptors indicate, that the NADH- and NADPH dehydrogenases have different electron pathways which come together at ubiquinone. This is concluded from the experiments where NADH can act as electron donor for viologen reduction, whereas NADPH can not. The inhibition of the NADPH dehydrogenase by NAD^+ and the absence of inhibition of the NADH dehydrogenase by $NADP^+$ confirms this hypothesis. The absence of a stimulation of the rate of respiration by addition of both NADH and NADPH indicates that both dehydrogenases have the same output site towards ubiquinone. Malate dehydrogenase on the other hand, must have a different output site toward ubiquinone, since addition of malate increased both NADH- and NADPH-dependent respiration.

It can also be concluded from the data presented in table 1 that there is no reversible electron flow from ubiquinone to the NADH:viologen oxidoreductase, since no viologen reduction occurred when NADPH was present with an inhibited respiratory chain.

The NADH:viologen oxidoreductase activity reacts differently compared to the NADH:ferricyanide oxidoreductase activity of mitochondria [Dooijewaard and Slater, 1976] and that of *A.vinelandii* membranes [This work]. In contrast to the double substrate inhibition found in the case of both NADH:ferricyanide oxidoreductase activities, no such inhibition was found for the NADH:viologen oxidoreductase activity. Instead at higher substrate concentrations an activation of the NADH:viologen oxidoreductase was found. The kinetic analysis indicates that the NADH:ferricyanide oxidoreductase and the NADH:viologen oxidoreductase are two different activities of the NADH dehydrogenase complex.

The effectiveness of the reduction of the viologens was found to depend on the charge and on the midpoint potential of the viologen (Table 2): 2 mM MAPV with a charge of 3+ was reduced at a higher rate than was BV (charge: 2+), although the midpointpotentials are not very different. It seems therefore reasonably to assume that the affinity for the viologen at the binding site increases with the charge of the viologen. In the case of DAPV, it was observed that at membrane concentrations higher than 10 $\mu g.ml^{-1}$, a significant amount of semiquinon was present during respiration which might be stabilised by binding to the membrane (data not shown). The increased reactivity of viologen with a higher midpoint potential can be explained when taken into account that

the reactivity also depends on the difference in midpoint potential between the donor and acceptor.

A method was developed to measure the NADH:viologen oxidoreductase activity with an active respiratory chain. This method allowed an investigation of the relation between respiration and viologen reduction during respiration. It was observed that maximally 50% of the electrons from NADH were transferred to viologen. The reaction rate of the NADH-dehydrogenase, as modulated by the initial redoxpotential applied (by altering the NADH/NAD$^+$ ratio), did not influence the distribution of electrons over viologen and ubiquinone, indicating that competition for the available electrons commences only after the dehydrogenation of NADH.

The concomitant flow of electrons through the respiratory chain and electron transport to low potential redox mediators is shown in this work, which supports the electron pair splitting model of Haaker and Klugkist [1987]. The model supposes two electrons from NADH to be accepted by the FMN group of the NADH dehydrogenase, which are subsequently distributed over two different electron pathways, respiration and a pathway leading to a low potential electron carrier.

Investigations to the site where viologen is reduced resulted in an EPR signal of a [Fe-S] cluster in *A.vinelandii* cytoplasmic membranes, which could be identical to the "low-potential" electron transfer site (N1a) of the mitochondrial NADH dehydrogenase complex I [Ohnishi *et al.*, 1972; Ohnishi, 1976; Ohnishi *et al.*, 1981; Ohnishi and Salerno, 1982; Ohnishi *et al.*, 1985]. This site might transfer electrons from NADH to viologen.

It was suggested that the NADH:benzyl viologen oxidoreductase could be the primary electron-transfer-site on the pathway to nitrogenase in *A.chroococcum* [Naik and Nicholas, 1966; Yates, 1971]. However, although nitrogenase was reduced in the presence of benzyl viologen and the NADH dehydrogenase, flavodoxin could not substitute for benzyl viologen in the electron transfer to nitrogenase from NADH. Reduction of flavodoxins by the NADH:viologen oxidoreductase of *A.vinelandii* cytoplasmic membranes was also not observed in this work. It was tested whether purified flavodoxin could replace viologen to produce hydrogen peroxide during NADH oxidation. The results were negative (data not shown). However, it can not be excluded that the reduction of flavodoxin requires a soluble factor, not present in the experimental setup described here, or that the presence of a membrane bound oxygen sensitive protein is required, which was inactivated during the isolation procedure of the membranes. More research is required to investigate the possibility to link flavodoxin reduction to respiration.

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CHAPTER 5.

General discussion

Introduction.

The work in this thesis is mainly focused on the electron transport route to nitrogenase in the free-living, obligate aerobic, nitrogen fixing organism *Azotobacter vinelandii*. For many years now, this topic has been the subject of research. Several hypotheses, which would explain the mechanism of electron transport to nitrogenase in obligate aerobic bacteria, have been postulated. None of these hypotheses have been proven yet.

The electron transport to nitrogenase in *A.vinelandii* has been investigated both biochemically and genetically. It is known in *Klebsiella pneumoniae*, which fixes nitrogen anaerobically or microaerobically, that the gene products of two genes are responsible for the electron transport to nitrogenase, the *nifF* gene and the *nifJ* gene. They encode a flavodoxin and a pyruvate:flavodoxin oxidoreductase, respectively. Electrons are transferred from pyruvate to flavodoxin through this oxidoreductase, and are then passed on to the nitrogenase proteins. This reaction, nor the two genes involved, have been found in *A.vinelandii*. There must therefore be different pathway for electrons to reduce nitrogenase in this organism.

Gubler and Hennecke [1986] discovered a number of genes, the *fixA, B* and *C* genes, which were required for nitrogen fixation in the obligate aerobic *Bradyrhizobium japonicum*, both in symbiotic and free-living state. Later, they found that another gene, linked to the *fixBC* cluster, the *fixX* gene, was also involved in nitrogen fixation. Since the nodules of plants, infected with mutants in these genes were normal, but no nitrogenase activity was observed, a function in the electron transport to nitrogenase was suggested. This was contradicted later by Kaminski and coworkers [1989], who found that both *in vivo* and *in vitro* nitrogenase activity was absent in mutants in the *fixABCX* genes in *Azorhizobium caulinodans* ORS571.

The fixPABCX genes of A.vinelandii: genetic analysis.

In order to find out whether these genes are also present in *A.vinelandii*, a 4.4 kb part of the genome of this organism, which hybridised to a heterologous *fixA* probe from *Rhizobium leguminosarum* was isolated. The nucleotide sequence of the 4.4 kb *Smal-EcoRI* fragment of *Azotobacter vinelandii* was determined. Five open reading frames (ORF's) and the beginning of a sixth one were found. The proteins encoded by the open reading frames were investigated for their homologies with other known gene products in the Genbank® databank.

The nucleic acid derived protein sequence of the first open reading frame contains two cysteine patterns, [Cys-X_7-Cys-X_3-Cys] and [Cys-X_2-Cys-X_2-Cys-X_3-Cys], indicative for the ligation of both a [3Fe-4S] and a [4Fe-4S] cluster. These sequences are characteristic for 7Fe-ferredoxins, such as ferredoxin I of *A.vinelandii* (FdI). Besides the

conserved cysteine residues, no other regions of homology between the *fixP* gene product and ferredoxin I were found, and no apparent homology with any other protein in the database was found

The second open reading frame showed a high degree of homology with the *fixA* gene product of various other aerobic nitrogen fixing bacteria. The *A.vinelandii fixA* gene product did not have any homology with other proteins in the database, so its function could not be determined from the nucleic acid derived protein sequence.

Homology searches revealed that the product of the third open reading frame not only had a high degree of homology with the sequence of FixB proteins, but also with the protein sequence of the α–subunit of the Electron Transfer Flavoprotein (ETF) of both human and rat origin. No other significant homologies with the *A.vinelandii fixB* gene product were found in the database.

The fourth open reading frame was homologous with *Rhizobial* FixC proteins. The N-terminal domain of the *A.vinelandii fixC* gene product was found also to contain a sequence homologous with the consensus sequence for an ADP binding site, as found in NAD^+ or FAD dependent enzymes. In many FAD-containing enzymes (*e.g.* lipoamide dehydrogenase and mercuric reductase), the FAD-binding site is located close to the N-terminus. This suggests, that the FixC protein might be a FAD-containing protein. It is known, that the β-subunit of ETF contains FAD. However, as no primary structure of this subunit has been published yet, it is not known if FixC has homology to the ß-subunit of ETF.

The protein encoded by the fifth open reading frame is also homologous with ferredoxin I of *A.vinelandii* and also with the FixX proteins of various *Rhizobia*, all ferredoxin-like proteins. The *A.vinelandii* FixX protein is the only FixX protein, that contains the two cysteine motifs that are found in ferredoxin I and the FixP protein, whereas the FixX proteins of other nitrogen fixing bacteria lack the cysteine motif, involved in ligation of the [3Fe-4S] cluster.

The fact that *A.vinelandii* contains at least five different 7Fe-ferredoxins (FdI, FdN (7Fe-ferredoxin in the *nif* gene cluster), FdV (7Fe-ferredoxin in the alternative nitrogenase gene region), FixP and FixX) is indicative for a special function of these proteins in this organism. Recently, a hypothesis has been proposed by Thomson [1991], stating that the function of the 7Fe- ferredoxins in *A.vinelandii* is to regulate gene expression by binding to the DNA. This binding is controlled by the iron(II) levels and the redox state of the cell. When the 7Fe-ferredoxin-DNA complex binds iron(II), it becomes an 8Fe-ferredoxin. The affinity of the 8Fe-ferredoxin for the DNA is less than that of the 7Fe ferredoxin, so the ferredoxin no longer binds to the DNA, and mRNA synthesis is possible. The fact that *A.vinelandii* contains five genetically distinct 7Fe-ferredoxins could be clue for this model, whereas the existence of two different 7Fe-ferredoxins in what is most likely one operon,

might give reason to assume that the *fixPABCX* cluster is involved in regulation of some kind of process, possibly involved in nitrogen fixation.

Recently, the *fixABCX* genes have also been found in the 0-2.4 min region of the *Escherichia coli* genome. *E.coli* is unable to grow diazotrophically. The *E.coli fixABCX* genes were followed by an open reading frame encoding a NAD(P)H dehydrogenase and preceded by a number of genes encoding proteins, involved in fatty acid metabolism. This might be a clue for a function of these genes in fatty acid metabolism, which could be of vital importance for nitrogen fixing organisms.

Downstream of the *fixX* gene, the start of a sixth open reading frame was found, but the N-terminal sequence did not show any homology to other proteins in the database.

A sequence motif with high homology to the promoter consensus for RNA polymerase complexed with sigma factor 54 (σ^{54}) was found 63 bp upstream of the ATG start codon of the *fixP* gene. A putative binding site for the regulatory NifA protein (TGT-N_9-ACA) is found 164 bp upstream of the start codon. However, the spacing between the TGT- and ACA- elements of this sequence is one base shorter than the consensus (TGT-N_{10}-ACA). The reason for this mismatch is not known, but it is known that a mismatch like this still functions in other organisms. No terminator sequence was found downstream of the stop codon of any of the genes.

It is concluded that amongst the *fixPABCX* genes of *Azotobacter vinelandii* at least three genes encode proteins, which are possibly involved in electron transport: FixB is highly homologous to the α-subunit of ETF and both FixP and FixX are homologous to *A. vinelandi* ferredoxin I. Whether these genes are actually involved in an electron transport system, fatty acid metabolism or whether they fulfil a function in the proposed regulation of gene expression in *A.vinelandii*, is the objective of the research described in chapter 3.

The fixPABCX genes of A.vinelandii: physiological analysis

Chapter 3 describes the construction and characterisation of mutants of *A.vinelandii* with alterations in the *fixA*, *fixB*, *fixC* and/or *fixX* genes. The gene of interest was exchanged with a plasmid derived copy that had been interrupted by insertion of the gene encoding kanamycin-resistance. A mutant lacking the *fixABCX* genes was constructed by replacing these genes by a DNA fragment containing the gene encoding kanamycin resistance.

The mutants were tested under a large number of conditions. All *fix⁻* mutants showed normal growth characteristics in nitrogen-free medium under all conditions tested. *In vivo* and *in vitro* activities of acetylene reduction of mutants were comparable to wild-type activities. Growth on several sugars, dicarboxylic acids and fatty acids or amino acids was not different from wild type bacteria, which indicated that the *fixABCX* cluster is not obligatory in the catabolism of these components.

Our results indicate that there is a major difference between the *fixABCX* genes of various *Rhizobia* and those of *A.vinelandii*. In contrast to *A.vinelandii*, deletion of the genes in symbiotic nitrogen fixing organisms results in loss of both *in vivo* and *in vitro* nitrogenase activity. The finding, that in *R.leguminosarum*, the polypeptides from which the nitrogenase enzyme complex is composed, are present in FixA⁻, B⁻, and C⁻ mutants but inactive, suggests that a step in the biosynthesis of active nitrogenase enzyme is hampered in these bacteria or that the proteins are inactivated by oxygen damage during growth. In *A.caulinodans*, a FixC⁻ mutant still had 10% of wild type nitrogenase activity, which could be elevated to 36% by adding saturating amounts purified nitrogenase Fe protein. This is an indication that the *fixC* gene product is necessary for the maturation of nitrogenase of *A.caulinodans*. In *A.vinelandii* no similar function for the *fixABCX* genes could be demonstrated.

The hypothesis of Thomson that the 7Fe-ferredoxin of *Azotobacter* might be a DNA binding protein, involved in regulation of protein synthesis in response to iron(II) levels in the cell, could be an indication for the function of the *fixPABCX* genes in *A.vinelandii*. The *fixPABCX* gene cluster contains two 7Fe-ferredoxins, but evidence for the iron(II) dependent regulation has not been found.

The fixPABCX genes of A.vinelandii: promoter analysis.

The expression of the *fixPABCX* genes was investigated using two methods. A chromosomally integrated *fixA::lacZ* gene fusion was made and it was observed that expression of the *fixABCX* genes of *A.vinelandii* was not significantly increased when cells, grown in the presence of ammonium, were transferred to a nitrogen free medium. It was concluded that the expression of the *fixABCX* genes, if occurring, was very low. From experiments, in which the promoter activity was investigated by immunological techniques, using antibodies against the purified FixA protein, similar observations were made. A very low signal of the FixA protein on the Western blots was found. Approximately half of this signal was found in cell extracts, grown in the presence of ammonia, and even in a FixA⁻ mutant, a weak signal was detected. This signal was probably caused by a-specific binding of the antibodies, since 300 μg of total cellular protein was loaded in one slot of the gel.

It cannot be ruled out, that downstream of the *fixX* gene, one or more genes are located that are co-transcribed with the *fixPABCX* genes. A mutation in the *fixPABCX* genes might cause polar effects on the downstream genes. The fact however, that no effect of any of the mutations was found, is either evidence that no polar effect is present, or that the gene(s) downstream of the *fixX* gene is/are a negative regulatory gene(s).

It is concluded, that, the *fixABCX* genes, which are of vital importance for nitrogen fixation in symbiotic organisms, are not essential for nitrogen fixation in *A.vinelandii*. Despite all investigations the function of the *fixABCX* genes is not known.

Electron transport to nitrogenase: biochemical investigations.

Biochemical investigations on the electron transport to nitrogenase are the subject of chapter 4. In order to elucidate the electron pathway to nitrogenase, a model system was used, in which the flavodoxins were replaced by artificial low potential electron carriers. The respiration and the reduction of viologens by different substrates, catalysed by *Azotobacter vinelandii* cytoplasmic membranes was investigated. Only with NADH, viologen oxidoreductase activity could be detected; NADPH, malate, succinate and lactate were unable to reduce viologens. From the oxygen consumption experiments with different substrate combinations it is concluded, that NADH and NADPH are oxidised by different dehydrogenases, although they have the same output site in the respiratory chain towards ubiquinone. Malate dehydrogenase on the other hand, has a different output site to ubiquinone than the dehydrogenases that oxidise NADH and NADPH.

The kinetic parameters of the NADH:ubiquinone oxidoreductase of the respiratory chain were investigated and compared with the NADH:ferricyanide oxidoreductase activity, which is an activity of complex I of the respiratory chain. In contrast to the NADH:ferricyanide oxidoreductase activity, the NADH:viologen oxidoreductase activity did not show double substrate inhibition, which indicates that the viologen reducing site of the NADH dehydrogenase complex is different from the NADH binding site. EPR studies on the presence of paramagnetic centers in cytoplasmic membranes demonstrated a "low-potential" electron accepting site, which could only be reduced using dithionite. This indicates that either the site is not accessible for NADH, or the redox potential is too low to enable NADH to reduce this site.

Viologen reduction did not only take place under conditions of an inhibited respiratory chain, but also under aerobic conditions with an active respiratory chain. This shows that electrons from NADH are transferred to the viologens, when at the same time electrons from NADH are transferred through the respiratory chain to oxygen.
The NADH:viologen oxidoreductase activity was modulated by using different viologens and by changing the viologen concentration. It was observed that the hydrogen peroxide formation increased linearly with the NADH:viologen oxidoreductase activity. It was also observed that maximally 50% of the electrons from NADH were transferred to the viologens and that the NADH-dehydrogenase activity (modulated by the $NADH/NAD^+$) had no influence on the distribution over viologens and ubiquinone.

The results of the experiments can be used as an example for a model, as proposed by Haaker and Klugkist [1987]. Some modifications should be made to update the model to the current knowledge. In the model, a NADPH dehydrogenase is the central part,

which, according to the results of this work, should be altered to a NADH dehydrogenase, since no viologen reduction was observed when NADPH was used as electron donor. No statements can be made to whether or not the 29kDa protein is involved in the reduction of low potential mediators. The concomitant flow of electrons through the respiratory chain and electron transport to low potential redox mediators, the central dogma of the model of Haaker and Klugkist, is shown in this work. This supports the observation of Klugkist *et al.* [1986], that electron transport to nitrogenase and respiration are coupled. According to the revised model, shown in figure 1, two electrons from NADH are accepted by the FMN group of the NADH dehydrogenase, operating at -320mV. During respiration, these electrons are subsequently distributed over respiration and a route, leading to the reduction of viologen. In the presence of cyanide and oxygen, the electrons can only be directed to the viologen reducing cluster. The viologens are oxidised efficiently by dioxygen to form H_2O_2, thereby maintaining a high concentration of oxidised viologen. The reduced FMN group of the NADH dehydrogenase can also be oxidised by ferricyanide.
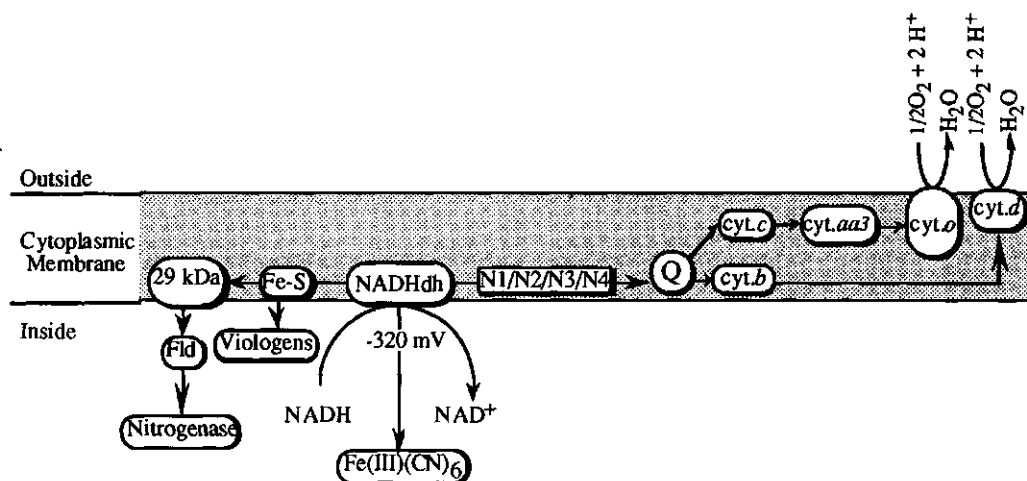

Figure 1: Electron pair splitting model, updated from Haaker and Klugkist [1987]. NADHdh is NADH dehydrogenase; Fld is flavodoxin; N1, N2, N3 and N4 are the paramagnetic [Fe-S] centers in the complex I; Q is ubiquinone and cyt is cytochrome.

The electron transport pathways as suggested in Figure 1 explain why viologen reduction and respiration are coupled and why not more than 50% of the electrons from NADH are used for viologen reduction. Unfortunately, the formation of H_2O_2 was not observed when the viologens were replaced by purified flavodoxin. The fact that flavodoxin reduction is not observed under the conditions applied, could be an indication

for the existence of a factor, which is absent under the experimental conditions. This factor could be an oxygen sensitive and/or a soluble protein. A possible candidate for an O_2 sensitive, membrane bound protein could be the 29kDa protein, found by Klugkist and coworkers. It is also possible that this factor is present in a complex of proteins, which is lost apart during the isolation of the membranes. Future research is required to investigate the possibility to link flavodoxin reduction to respiration.

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# CHAPTER 6.

Nederlandse samenvatting.

Inleiding.

"Maar leg nu eens uit, wat onderzoek je nu precies?" "En wat is het nut ervan, ik bedoel, wat kun je nu met jouw resultaten?" Een ieder die fundamenteel onderzoek verricht, dit is onderzoek niet gericht op toepassing, heeft deze vragen ongetwijfeld één of meerdere malen gesteld gekregen. In dit hoofdstuk hoop ik degenen, die deze vragen aan mij gesteld hebben, een bevredigend antwoord te kunnen geven.

Allereerst wil ik uitleggen wat het Onderzoeker In Opleiding (OIO)-schap inhoudt. Veel mensen denken dat dit een studie is, waardoor mij meerdere malen gevraagd is wanneer ik afstudeerde, of hoe het met mijn studie ging. Het OIO en AIO (Assistent In Opleiding) gebeuren is in het leven geroepen toen de twee fasen structuur in het universitaire onderwijs werd ingevoerd. Als OIO of AIO word je de mogelijkheid geboden om in een vierjarige periode onderzoek te verrichten en de resultaten te bundelen tot een proefschrift. Het is een betaalde baan, alhoewel vooral in het begin de honorering matig te noemen is, zodat bij veel mensen het idee leeft van een arme student(e) die moet zien rond te komen van zijn/haar studiefinanciering. Ondanks de salariëring is het toch een betaalde baan. Het resultaat van viereenhalf jaar experimenteel werken staat beschreven in dit proefschrift.

Het belang van biologische stikstofbinding voor het leven op aarde.

Er bestaan verschillende vormen waarin het element stikstof in de natuur voorkomt. Één vorm is het stikstofgas. Dit molecuul bestaat uit twee stikstofatomen. Daarnaast komt er in de natuur ook stikstof voor in moleculen samen met andere atomen, in eenvoudige (anorganische) en complexe (organische) moleculen. Voor organismen is het element stikstof een essentiële bouwsteen voor veel verbindingen, bijvoorbeeld eiwitten en nucleïnezuren (dit zijn lange moleculen waarin de genetische informatie van een cel is vastgelegd). De verschillende vormen van stikstofverbindingen doorlopen een cyclus: "de stikstofcyclus" (Figuur 1). Planten halen via hun wortels anorganische stikstofverbindingen uit de bodem (de tweede vorm waarin het element sitkstof kan voorkomen) en maken hieruit ingewikkelde organische verbindingen, zoals aminozuren en eiwitten (dit is de derde vorm waarin het stikstofatoom kan voorkomen). Dieren (en dus ook mensen) gebruiken planten als voedsel en krijgen zo deze organische stikstofverbindingen binnen. Door middel van uitscheidingsprocessen en ook door afsterven van planten en dieren komen de organische stikstofverbindingen weer terug in de grond, waar bacteriën deze verbindingen omzetten in anorganische verbindingen. De stikstofkringloop lijkt nu gesloten.
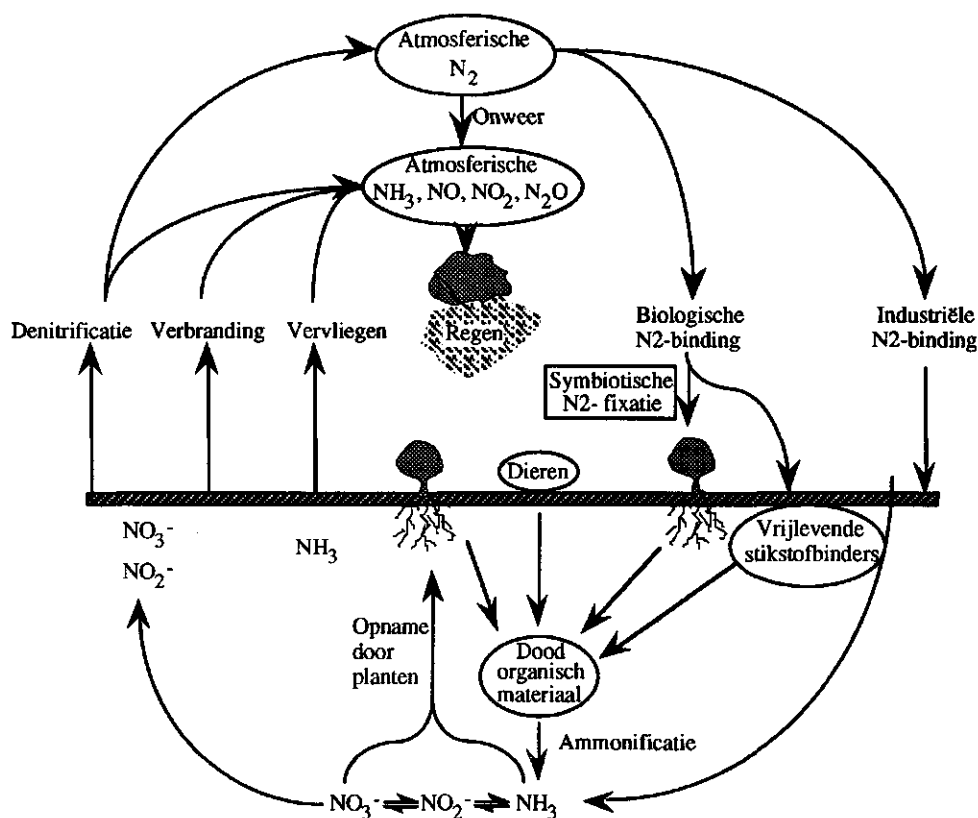
**Figuur 1:** De stikstofkringloop.

Dit lijkt echter alleen, want er zijn verschillende mogelijkheden waardoor gebonden stikstof uit de bodem stikstofkringloop weglekt. Omdat dit op den duur zou leiden tot een beperking van de levende wereld, is het van levensbelang dat deze lekkage wordt aangevuld. Nu bestaat de ons omringende lucht voor circa 80% uit stikstof, dus het lijkt het meest logisch dat deze bron als voorraad gebruikt wordt. Echter, de vorm waarin dit stikstof zich bevindt, is niet bruikbaar als stikstofbron voor organismen als mens en dier. Het zal dus eerst "bruikbaar" moeten worden gemaakt door het om te zetten in eenvoudige, anorganische verbindingen. Het begrip "stikstofbinding" doet hier zijn intrede. Zoals met veel processen is stikstofbinding op twee manieren te bewerkstelligen: op chemische wijze en op biologische wijze. Een van de verschillen tussen beide processen is de gebruikte reactieversneller, de katalysator. Als energie- en milieuaspecten in acht genomen worden, krijgt de biologische stikstofbinding een steeds belangrijkere rol. Het chemisch binden van stikstof kost namelijk veel energie: een grote druk en een hoge temperatuur zijn vereist om het proces, het zogenaamde "Haber-Bosch-proces", te laten verlopen (vergelijking 1). Het verkrijgen van de reactanten is een energetisch kostbaar proces. Het kost veel energie om waterstofgas te produceren, waarbij

ook koolmonoxide gevormd wordt, hetgeen een milieubelasting met zich meebrengt (aantasting van de ozonlaag).

$$N_2 + 3H_2 \Leftrightarrow 2NH_3 \qquad\qquad T \geq 400°C; p \geq 10^4 \text{ kPa}$$
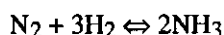
Vergelijking 1: Het Haber-Bosch proces: chemische stikstofbinding, gekatalyseerd door ijzer.

Biologische stikstofbinding wordt uitgevoerd door bacteriën; zij katalyseren een gelijksoortige reactie bij een normale omgevingstemperatuur en een normale druk. In de biologische stikstofbindingsreactie, te zien in vergelijking 2, wordt MgATP als energiebron gebruikt. ATP is een algemene energiebron voor alle cellen en wordt uit ADP en anorganisch fosfaat gerecycled door verbranding van suikers binnen de cel.

$$N_2 + 8H^+ + 8e^- + 16\,MgATP \Rightarrow 2NH_3 + H_2 + 16\,MgADP + 16\,P_i$$
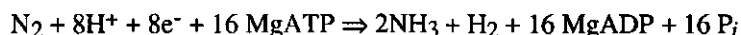
Vergelijking 2: Biologische stikstofbinding door micro-organismen (P_i is anorganisch fosfaat).

Stikstofbindende bacteriën.

Het feit dat bacteriën in staat zijn om stikstof te binden onder normale condities, danken zij aan de aanwezigheid in de cel van het enzym nitrogenase. Dit enzym zorgt ervoor dat stikstof wordt omgezet in ammoniak (vergelijking 2). In alle tot nu toe bekende en onderzochte stikstofbindende bacteriën is dit enzym gevonden.

Er zijn twee belangrijke soorten van stikstofbindende bacteriën te onderscheiden: symbiotische en vrijlevende stikstofbinders. Symbiotische stikstofbinders binden stikstof wanneer zij samenleven met planten (Symbiose komt van het grieks: Sym= samen, bios = leven). De bacteriën en de plant herkennen elkaar en samen zorgen zij ervoor, dat knolletjes op de wortels ontstaan, waarin de bacteriën de stikstofbinding uitvoeren. Het gevormde ammoniak wordt vervolgens naar de plant getransporteerd. In ruil zorgen de planten ervoor dat de bacteriën alle andere voor groei noodzakelijke stoffen krijgen. Het zijn voornamelijk vlinderbloemigen die optreden in dit proces als gastheer van de bacterie. Een groot deel van het stikstofbindingsonderzoek richt zich op deze bacteriën, waartoe onder andere *Rhizobium* bacteriën behoren. Dit is verklaarbaar als men weet dat deze groep voor de landbouw het meest belangrijk is: op de plaats waar het nodig is, in de plant, wordt relatief veel stikstof gebonden door een kleine hoeveelheid bacteriën. Het ligt in de bedoeling om de stikstofbindende bacteriën ook met andere planten dan vlinderbloemigen in symbiose te laten leven, teneinde het werkgebied van de bacteriën te vergroten. Om dit te kunnen verwezenlijken zal echter wel bekend moeten zijn, hoe het stikstofbindingsproces in al zijn aspecten in elkaar steekt.

Een van de manieren om de kennis van de biologische stikstofbinding te vergroten, is de mogelijkheid om bacteriën, die ook stikstof binden maar niet in symbiose leven, te onderzoeken. Dit levert een aantal praktische voordelen op, zoals bijvoorbeeld het feit dat men gemakkelijk een grote hoeveelheid bacteriën kan verkrijgen. Deze groep van vrijlevende bacteriën, waartoe ook de in dit proefschrift onderzochte bacterie *Azotobacter vinelandii* behoort, is van minder belang voor de landbouw, maar is van belang voor het fundamentele onderzoek naar verschillende reacties van het stikstofbindingsproces.

Doel van het promotieonderzoek.

Zoals vergelijking 2 laat zien, vereist het proces van stikstofbinding naast N_2 ook nog waterstofionen, energie in de vorm van ATP en een reduktiemiddel, dat de electronen aan nitrogenase doneert. Juist over het reduktiemiddel is nog weinig bekend in *A.vinelandii*. Men weet dat er twee eiwitten in de *Azotobacter* cel aanwezig zijn, die in staat zijn om de electronen naar nitrogenase te brengen, namelijk flavodoxine en ferredoxine. De manier waarop de electronen op deze twee electronentransporteurs terechtkomen, is nog onbekend. Het doel van het promotieonderzoek was dan ook, om opheldering te verschaffen hoe dit electronentransport naar nitrogenase zou kunnen verlopen.

Het promotieonderzoek: de genetische benadering.

Het probleem van het promotieonderzoek is op twee verschillende manieren benaderd: op genetische wijze en langs biochemische weg. In hoofdstuk 2 is de isolatie beschreven van een aantal genen, aanwezig in *A.vinelandii*, waarvan verondersteld werd, dat zij coderen voor eiwitten, die betrokken zijn bij het electronentransport naar nitrogenase. Een gen is een stukje erfelijk materiaal (ook wel DNA genoemd), dat een recept is voor een eiwit. Zo heeft elk eiwit een eigen recept, dus een eigen gen. De veronderstelling dat deze genen betrokken zouden zijn bij het electronentransport naar nitrogenase, was gedaan naar aanleiding van experimenten met een symbiotisch stikstofbindende bacterie, *Bradyrhizobium japonicum*. Hieruit bleek dat een verandering (mutatie) van deze genen leidde tot een bacterie, die niet meer in staat was tot stikstofbinding, ondanks dat het nitrogenase eiwit zelf wel aanwezig was.

Deze genen, de *fixABCX* genen, zijn geïsoleerd uit *A.vinelandii*, gekloneerd (dit is het vermenigvuldigen van een stukje genetisch materiaal) en de basevolgorde van de genen is bepaald. Naast deze genen bleek een nieuw gen voorafgaand aan het *fixA* gen aanwezig te zijn in *A.vinelandii*, dat niet in andere organismen voorkomt. Dit gen is *fixP* genoemd. Er is een databankonderzoek uitgevoerd om uit te vinden waarmee de *fixPABCX* genprodukten (= de FixP, FixA, FixB, FixC en FixX eiwitten) overeenkomsten vertonen (=homoloog zijn). Het bleek, dat de eiwitten waarvoor de *fixABCX* genen van *A.vinelandii* coderen, een grote homologie vertonen met de *fixABCX* genprodukten van andere stikstofbindende bacteriën, en dat daarnaast het *fixB* genprodukt een hoge mate van homologie vertoonde met de α-subunit van het humane en ratte Electron Transfer Flavoprotein (ETF). Dit ETF is, zoals zijn naam

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reeds doet vermoeden, ook betrokken bij transport van electronen in verschillende fysiologische processen. Daarnaast geeft de aminozuurvolgorde van het FixC eiwit aan, dat er mogelijk een FAD-bindingsplaats aanwezig is. FAD is een factor, die zorgt dat een eiwit een electronenoverdrachtsreactie kan katalyseren.

Zowel het FixP als het FixX eiwit uit *A.vinelandii* vertonen in hun aminozuurvolgorde, een structuur, welke ook voorkomt in ferredoxine I van *A.vinelandii*. Dit ferredoxine kenmerkt zich door het feit, dat het 7 ijzeratomen bindt. De FixX eiwitten van de andere bacteriën kunnen maximaal slechts 4 ijzeratomen binden, op grond van hun primaire sequentie. Nu is verondersteld, dat het (7 ijzer) ferredoxine uit *A.vinelandii* een DNA-bindend eiwit is, waardoor het het overschrijven/vertalen van informatie kan beïnvloeden. De FixP en FixX eiwitten kunnen samen met de FixA, FixB en FixC eiwitten ook een dergelijke functie vervullen in *A.vinelandii*.

Zoals hierboven is beargumenteerd zijn twee functies voor de *A.vinelandii fixPABCX* cluster mogelijk. De *fixPABCX* genen van *A.vinelandii* kunnen betrokken zijn bij een electronentransport proces, mogelijk naar nitrogenase, of de genencluster bezit een regulerende functie op DNA niveau. In welk proces zij betrokken zijn kan niet geconcludeerd worden uit de homologiestudies.

Om achter de functie van de genen te komen, zijn mutaties in de *fixABCX* genen van *A.vinelandii* gemaakt. Dit werk staat beschreven in hoofdstuk 3. De genen zijn uitgeschakeld door er een stukje vreemd DNA in te zetten (insertie mutatie), waardoor het gen geen functioneel eiwit meer kan voortbrengen (het "recept" is niet goed meer) Ook zijn de genen verwijderd (deletie mutatie). In tegenstelling tot wat gevonden was voor de symbiotische bacteriën waarin de *fixABCX* genen voorkomen, gaf een uitschakeling van de genen, zowel afzonderlijk als te samen, in *A.vinelandii* geen verschil te zien met de wild-type bacterie; de mutanten groeiden onder alle geteste condities precies hetzelfde als de bacterie met de functionele *fixABCX* genen.

Mede hierdoor is het vertalen van de *fixABCX* genen tot eiwit, het bereiden van het recept (figuur 2), aan een diepergaande studie onderworpen.
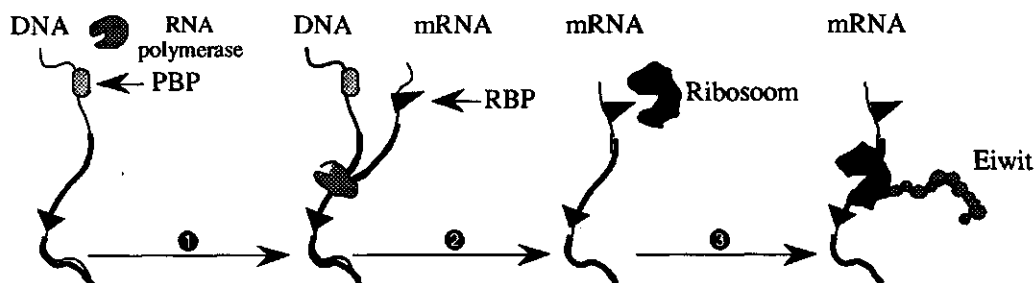
Figuur 2: Het vertalen van gen tot eiwit:

De vette pijl is een gen, een gebied op het DNA dat codeert voor een eiwit. ❶: Het RNA polymerase bindt aan het DNA op de promoter bindingsplaats (PBP) en synthetiseert het messenger RNA (mRNA). Dit proces heet transcriptie. Het mRNA is een duplicaat van het DNA. ❷: Het eiwitsynthetiserend apparaat (ribosoom genaamd, zelf ook een eiwit-RNAcomplex) bindt aan de ribosoombindingsplaats (RBP) op het mRNA en ❸ zorgt voor de synthese van eiwit, waarvan de karakteristieken door het gen bepaald zijn. Dit proces heet translatie.

Het gebied op het DNA waar het RNA polymerase bindt, het promotergebied, is nader onderzocht door achter dit gebied een reportergen te plaatsen. Dit reportergen wordt vertaald (translatie) wanneer de promoter geactiveerd wordt. Zoals de naam reeds zegt, kan het vertalen van het reportergen aangetoond worden omdat via stappen 2 en 3 het reportereiwit gemaakt wordt, waarvan de aanwezigheid door een specifieke kleurreactie kan worden aangetoond. De hoeveelheid kleur die per tijdseenheid gevormd wordt is dus een maat voor de activiteit van de promoter. Het bleek dat de promoter van de *fixPABCX* genen niet gebruikt werd onder de condities, waaronder stikstofbinding plaatsvindt. Blijkbaar heeft *A.vinelandii* de *fixPABCX* genen niet direkt nodig voor de stikstofbinding. Alle andere geteste condities leverden hetzelfde resultaat op.

Antilichamen, gemaakt tegen het FixA eiwit, bevestigden het resultaat van de experimenten van de promoteractiviteit. Hiervoor is het FixA eiwit gesynthetiseerd zoals in figuur 2 beschreven staat, gezuiverd en ingespoten in muizen. Deze muizen herkennen het FixA eiwit als niet-eigen en maken er antilichamen tegen. Deze antilichamen zitten in het bloed en door het bloed van de muis op te vangen, worden antilichamen tegen het FixA eiwit verkregen. Met behulp van speciale technieken, Western-blotting en immunokleuring, kan de aanwezigheid van het FixA eiwit in *A.vinelandii* cel extracten onderzocht worden. De aanwezigheid van het FixA-eiwit kon echter onder geen enkele conditie aangetoond worden in *A.vinelandii*. De conclusie van dit deelonderzoek was dan ook dat de *fixPABCX* genen niet aangeschakeld worden onder condities van stikstofbinding in *A.vinelandii*, maar het is

mogelijk dat onder andere omstandigheden de genen wel noodzakelijk zijn voor de stikstofbinding. Deze condities zijn (nog) niet gevonden.

Het promotieonderzoek: de biochemische benadering.

Het ophelderen van het electronentransport naar nitrogenase is ook op biochemische wijze benaderd. Dit is beschreven in hoofdstuk 4. Langs biochemische weg is onderzocht of een enzymsysteem in de cytoplasma membranen van *A.vinelandii* verantwoordelijk zou kunnen zijn voor de overdracht van electronen naar flavodoxines. Omdat bekend is dat het zuurstofverbruik van *A.vinelandii* gekoppeld is aan de stikstofbindingscapaciteit van de bacterie en dat de ademhaling van bacteriën plaatsvindt in de cytoplasma membranen, richtte het onderzoek zich met name op deze membranen van *A.vinelandii*. Ook is bekend dat NADH en NADPH, twee verwante moleculen, de sterkste reduktiemiddelen zijn in de *A.vinelandii* cel. Dit in acht genomen, is het onderzoek met name toegespitst op de enzymsystemen in cytoplasma membranen, die in staat zijn om de electronen van NADH en/of NADPH op te nemen en door te geven aan de ademhalingsketen of aan andere electronenacceptoren. Deze enzymsystemen zijn de NADH- en NADPH-dehydrogenases. Zowel NADH als NADPH dehydrogenase activiteiten zijn gevonden in de *A.vinelandii* membranen. Het bleek echter dat alleen de NADH dehydrogenase activiteit gekoppeld was aan reduktie van viologeen, de NADH:viologeen oxidoreductase activiteit. Viologenen zijn niet-fysiologische electronenacceptoren, die kunnen dienen als vervanger van electronenacceptoren die van nature in de bacterie voorkomen, omdat deze natuurlijk voorkomende electronenacceptoren moeilijk in grote hoeveelheden te verkrijgen zijn. In tegenstelling tot NADH leidde de oxidatie (=het afnemen van electronen) van NADPH niet tot reduktie van viologenen.

De NADH:viologeen oxidoreductase enzymactiviteit is verder onderzocht. Het effect van het type viologeen op de NADH:viologen oxidoreductase activiteit is bekeken. Hoe positiever de nettolading van het viologeen, hoe hoger de NADH:viologeen oxidoreductase activiteit. Als de lading van twee verschillende viologenen gelijk is, dan wordt waargenomen dat de activiteit van de NADH:viologeen oxidoreductase activiteit afhankelijk is van de redoxpotentiaal van de gebruikte viologenen. Een hogere redoxpotentiaal gaat samen met een hogere NADH:viologeen oxodoreductase activiteit. De redoxpotentiaal geeft informatie over de reactiviteit van een electronenoverdragend molecuul.

In zowel *A.vinelandii* cytoplasma membranen als in mitochondria van runderharten wordt een electronenoverdrachtsreactie van NADH naar ferricyanide waargenomen, de NADH:ferricyanide oxidoreduktase. Deze enzymactiviteit is een onderdeel van het NADH dehydrogenase complex I en volgt een reactiemechanisme, waarin zowel NADH als ferricyanide aan dezelfde plaats op het enzym binden. Dit resulteert in een remming van de reactie wanneer één van de substraten in hoge concentraties aanwezig is. Deze remming werd niet gevonden bij bestudering van de NADH:viologeen oxidoreductase reactie. Hieruit wordt

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geconcludeerd dat ferricyanide en de viologenen op verschillende plaatsen op het NADH dehydrogenase complex binden.

Een opmerkelijk fenomeen was het feit dat de NADH:viologeen oxidoreductase niet alleen onder condities met een geremde ademhalingsketen plaatsvond, maar ook als de ademhalingsketen actief was. Dit fenomeen is onderzocht met verschillende viologenen, waaruit bleek dat met een niet-geremde ademhalingsketen nooit meer dan 50% van de electronen van NADH naar de viologenen overgedragen werden. De andere electronen werden gebruikt voor de reduktie van zuurstof door de ademhalingsketen. De initiële redoxpotentiaal heeft geen invloed op de verdeling van electronen over de ademhalingsketen en over de viologenen, de concentratie viologenen heeft dit wel. Blijkbaar geldt, dat als de electronen eenmaal door het NADH-dehydrogenase complex zijn opgenomen, de verdeling van electronen niet meer verandert. Deze waarneming ondersteunt een model zoals voorgesteld door Haaker en Klugkist, waarin het NADPH dehydrogenase complex vervangen wordt door een NADH dehydrogenase complex. In dit model worden electronen van NAD(P)H verdeeld over de ademhalingsketen en over de reduktie van een electronenacceptor met een lage redox-potentiaal, zoals flavodoxine of ferredoxine. Aangezien reduktie van flavodoxine niet is waargenomen tijdens NADH oxidatie in onze experimenten, kan het niet uitgesloten worden dat voor de reductie van flavodoxine een factor is vereist, die tijdens de uitvoering van de experimenten afwezig is geweest. Deze factor kan aanwezig zijn in een andere celfractie dan de cytoplasma membranen of door zuurstof tijdens de isolatie van de cytoplasma membranen zijn geïnactiveerd. Een mogelijke kandidaat voor deze factor is het 29kDa eiwit, gevonden door Klugkist.

Curriculum vitae.

Marinus Jan Cornelis Wientjens werd geboren op 15 oktober 1963 te Breda. Hij behaalde in 1982 het diploma Atheneum B aan het Onze Lieve Vrouwe Lyceum te Breda. In datzelfde jaar begon hij met de opleiding tot medisch biochemisch analist aan het Dr. Struycken Instituut te Etten-Leur. Het diploma van deze opleiding werd behaald in 1986.

Na het vervullen van de militaire dienstplicht begon hij in oktober 1987 als promovendus aan de vakgroep Biochemie van de Landbouwuniversiteit te Wageningen. Vanaf 1 oktober 1992 was hij verbonden aan deze vakgroep als toegevoegd docent. Het in de periode oktober 1987 tot april 1992 uitgevoerde onderzoek heeft geleid tot dit proefschrift.