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**Review**

# Molecular aspects of the energetics of nitrogen fixation in *Rhizobium*-legume symbioses *

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## Contents



* The term '*Rhizobium*' without a species name refers collectively to the genera *Rhizobium, Bradyrhizobium* and, with some reluctance, *Azorhizobium*, all of which form symbioses with certain legumes.

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Abbreviations: ALA, δ-aminolevulinic acid; Hup, hydrogen uptake.

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## I. Introduction

Biological nitrogen fixation is an energy-intensive process; the amount of ATP used per $N_2$ fixed can be as high as 42 according to some growth yield measurements [203]. Under ideal conditions $N_2$ fixation via nitrogenase proceeds according to the following reaction:

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16(ADP + P_i) \quad (1)$$

The discrepancy in the ATP expenditure between the measured values and Eqn. 1 is that the latter does not take into account the cost of the high-energy electrons, nor does it consider that the ATP-dependent $H_2$ evolution can exceed $N_2$ reduction stoichiometrically under experimental and physiological conditions. Sixteen ATP molecules are hydrolyzed per eight electrons transported through the nitrogenase complex, and thus $H^+$ reduction diminishes the efficiency of $N_2$ fixation. Chemical $N_2$ reduction to ammonia requires energy in the form of heat and pressure, but it also requires the exclusion of $O_2$ and a nonaqueous environment [174], and hence it is miraculous that biological $N_2$ fixation occurs at all. This communication will not cover the enzymology of the nitrogenase complex, nor will it address the ATP requirements for nitrogenase at the level of that enzyme (these topics are reviewed in Refs. 166, 174 and 197). Rather, we wish to begin the discussion with the understanding that $N_2$-fixed bacteria need to synthesize a great deal of ATP and reducing equivalents, and proceed directly to problems in energetics which are unique to symbiotic $N_2$-fixing organisms.

The $N_2$-fixing root nodule in legumes is formed from the symbiosis of a *Rhizobium* or *Bradyrhizobium* species with a particular plant host. Nitrogen fixation is carried out by the bacteroids *, and they are also the direct source of ATP which drives the reaction. Although plant mitochondria in nodules almost certainly cannot synthesize the ATP necessary for $N_2$ fixation [175], the plant host is involved in every aspect of the energetics of this process. The carbon sources oxidized by the bacteroids for ATP synthesis are ultimately products of photosynthesis, and the leghemoglobin found in nodules, which prevents $O_2$ inactivation of nitrogenase, is a plant product. Also, the host plant apparently has some control over the efficiency of $N_2$ fixation at the enzyme level [22–24], and it can affect the expression of the rhizobial hydrogenase [22–24,40,111,151]. It is obvious then that strategies adopted to accommodate the restrictions and peculiarities of $N_2$ fixation are different in symbiotic associations than in free-living organisms.

* Bacteroids is the term given to the differentiated, $N_2$-fixing bacteria found in root nodules.

## II. Carbon metabolism

### *II-A. Physiological studies*

Unlike cell growth in culture, the carbon sources available to *Rhizobium* cells in root nodules are not subject to the whim of the investigator, and thus knowledge of the carbon sources which fuel nitrogen fixation symbiotically has not been easily ascertained. The energy for nitrogen fixation is ultimately derived from plant host photosynthate, and high concentrations of carbohydrate are found in nodules, but these compounds are not the immediate carbon-sources driving nitrogen fixation.

Carbon compounds have been tested for their ability to be taken up by isolated bacteroids [79,95,176,188, 189,193] or for their ability to support respiration and nitrogen fixation [27,171,216,217]. An early study by Tuzimura and Meguro [219] noted that *Bradyrhizobium japonicum* bacteroid respiration was enhanced by the addition of succinate to a much greater extent than by the addition of glucose, and thus they speculated that organic acids rather than carbohydrates may be the energy sources in symbiotically grown cells. Direct measurements of nitrogenase activity support this view [27,171,217], and although exceptions have been noted [216,217], it is generally believed that carbohydrate oxidation is not a major energy-source driving nitrogen fixation [27,188]. Carbohydrate uptake is inducible in free-living cells, but the symbiotic influx rate is slow compared with that of succinate or malate [95,176,188], and it is a passive process, at least in *B. japonicum* [176]. The uptake of $C_4$-dicarboxylic acids by bacteroids has been readily demonstrated [79,95,176,188,189,192], and the influx is an active process [176,189,192]. The transport of succinate, fumarate and malate shares a common system in bacteroids and in cultured cells of *Rhizobium leguminosarum* [72,79], *Rhizobium meliloti* [67], and *B. japonicum* [138,189], as discerned from kinetic data, and also from the study of mutants (subsection IIB).

In addition to dicarboxylic acids, there is evidence that glutamate is also involved in bacteroid respiration in *B. japonicum* [187]. Although succinate and malate are transported into *B. japonicum* more rapidly than is glutamate, the proportion of glutamate converted into $CO_2$ is substantial compared to the dicarboxylic acids. In fact, much of the succinate and malate (as well as aspartate) transported into cells is converted into glutamate.

### *IIB. Analyses of mutants*

The generation of mutants in carbon uptake and metabolism have confirmed many of the physiological studies, and they have provided considerable insight

into the carbon requirements of symbiotically grown cells. Ronson and Primrose [186] generated carbohydrate metabolism mutants of *Rhizobium trifolii* with lesions in glucokinase, fructose uptake, the Entner-Doudoroff pathway and in pyruvate carboxylase. These mutant strains formed effective symbioses with red clover, suggesting that neither glucose, nor fructose nor sucrose is required for nitrogen fixation by bacteroids, and thus the ATP and reductant required for this process must come from other carbon sources. Subsequent mutant analyses showed that $C_4$-dicarboxylic acids are required for effective clover nodule formation. *Rhizobium trifolii* mutant strains unable to grow on, or transport, succinate, fumarate or malate still nodulated clover, but those nodules were ineffective [183]. The isolation of bacterial mutants from *R. meliloti* [36,67,71,74] and *R. leguminosarum* [16,73,76] also demonstrate the importance of $C_4$-dicarboxylic acids in symbiotic nitrogen fixation. It should not be overlooked that these dicarboxylic transport (*dct*) mutants do form nodules that are invaded with bacteria, and therefore organic acids are not essential for bacterial growth symbiotically. It is not known what carbon sources are required for cell growth and maintenance as *R. leguminosarum* mutants defective in sugar metabolism [54,78] or strains with double mutations in organic acids and carbohydrate metabolism [15] still form root nodules on peas, and lesions in gluconeogenesis form effective nodules as well [140]. It seems that other carbon sources are available to the nodule, or that the lesions in the carbon mutants are circumvented by alternate pathways or by the ability of the plant host to rescue the mutant. Saroso et al. [191] argued that the peribacteroid membrane may be impermeable to sugars, since snake bean nodule bacteroids do not express enzymes which are inducible by sucrose in culture despite the presence of high concentrations of sucrose in nodules.

The transport of the dicarboxylic acids succinate, malate and fumarate occur via a common system as deduced from mutant analyses [36,67,183,226], which agrees with the kinetic data described above (subsection IIA). Mutants isolated as succinate transport mutants are invariably deficient in the uptake of other dicarboxylic acids, but they are able to incorporate carbohydrates as well as do the respective wild-type strains. These mutants, isolated in culture, are also *Dct* deficient as bacteroids, and thus it seems that $C_4$-dicarboxylic acids are transported by the same system under both growth conditions. Exceptions to this are noted below (subsection IIC) whereby Tn*5*-induced mutants in regulatory *dct* genes show different phenotypes symbiotically compared with cultured cells.

### *IIC. Genetics*

Ronson et al. [181] isolated cosmids containing $C_4$-dicarboxylic acid transport (*dct*) genes by complementing putative structural and regulatory *dct* mutants of *R. trifolii* with an *R. leguminosarum* gene bank. Three contiguous *dct* gene loci were identified within a 5.5 kb region of DNA, as discerned from mapping and transposon mutagenesis of subcloned fragments of the isolated cosmids. The *dctA* gene locus not only complemented a putative structural *dct* mutant of *R. trifolii* when expressed from its own promoter, but it also complemented a regulatory *dct* mutant of *R. trifolii* and of *E. coli* (*dctB*) when expressed from a constitutive vector promoter; these observations strongly suggest that the *dctA* locus codes for a structural gene. The *dctA* gene codes for a hydrophobic protein, and it is apparently the only structural gene necessary for $C_4$-dicarboxylic acid transport in *Rhizobium* grown symbiotically and in culture [180].

*dctB* and *dctD* code for positive regulatory elements which are necessary for the expression of the *dctA* gene product, and they are transcribed divergently from *dctA* [181]. Constitutive expression of *dctD* from a vector promoter complemented mutants with lesions in *dctB* which suggests that DctD expression may normally require the *dctB* gene product. The DNA sequences of the *dct* genes [180,182], and the inferred amino acid sequences, have revealed very interesting clues as to how $C_4$-dicarboxylic acid transport is regulated in *Rhizobium*. The *dctA* promoter is homologous to the consensus promoter sequence −26 CTGGYAYR–$N_4$–−TTGCA−10 found in genes which are under control of the *ntrA* (also called *rpoN*) gene product [180]. The *ntrA*, as well as *ntrB* and *ntrC*, gene product regulates the expression of genes involved in the assimilation of nitrogen under nitrogen-limiting conditions in enteric bacteria [118,130]. Ronson et al. [184] isolated an *ntrA*-like gene from *R. meliloti* using a scheme which assumed that the *ntrA* gene product (NtrA) does indeed regulate $C_4$-dicarboxylic acid transport. The deduced amino acid sequence of the *R. meliloti* NtrA shares 38% homology with NtrA from *Klebsiella pneumoniae*, and it is required for nitrate assimilation and nitrogen fixation, as well as for $C_4$-dicarboxylic acid transport [184].

The DNA sequences of *dctB* and *dctD* [182] show that DctB and DctD are homologous to NtrB and NtrC, respectively, and are in fact part of a conserved family of two-component regulatory systems which respond to stimuli from the environment [155,185,229]. It is thought that one component of these systems (constitutively expressed at a low level) acts as a sensor, and the second component receives a signal from the sensor which then activates the response (Fig. 1). According to this model, the sensor protein DctB is activated upon binding to a $C_4$-dicarboxylic acid which in turn activates DctD. Activated DctD would then act in concert with NtrA in the transcription of *dctA*. By analogy to the Ntr system [154], DctB may phosphorylate DctD in order to activate it; this idea is supported by the finding that

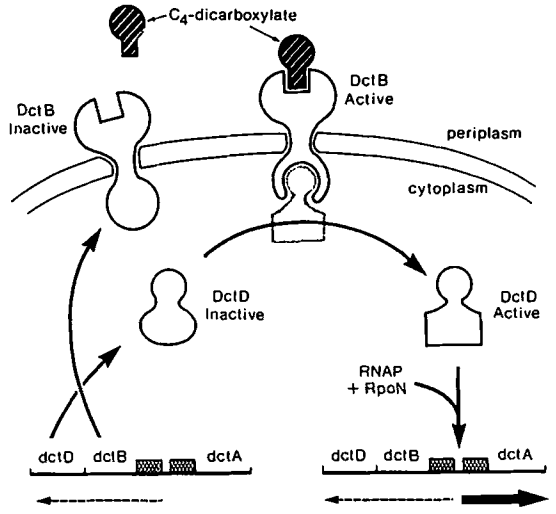


Fig. 1. Model for the activation of the *dctA* gene. Reprinted with permission from Ronson et al. (1987) Nucleic Acids Research 15, 7921.

DctD has a potential ATP-binding site in a region that is strongly conserved in NtrC [182]. However, the constitutive transcription of the cloned *dctD* gene by a vector promoter can overcome the need for the *dctB* gene product in $C_4$-dicarboxylic acid transport [181], thus DctD can be activated by a factor other than DctB, or else DctD can activate itself under those experimental conditions.

Most of the detailed genetic studies of $C_4$-dicarboxylic acid transport described above have been done with *R. leguminosarum*, but there are good reasons to believe that the Dct system is similar in other *Rhizobium* species. Regulatory, as well as structural, *R. leguminosarum dct* genes complement *dct* mutants of *R. trifolii* [181], and they can be regulated in *R. meliloti* [184]. *dct* genes isolated from *R. meliloti* [36] can be expressed in *B. japonicum* [33], and they confer increased ex planta nitrogen-fixing ability on the recipient strain. Watson et al. [226] identified two *dct* regions on a megaplasmid of *R. meliloti*. Interestingly, Tn*5*-induced mutants in one locus yielded ineffective nodules whereas mutations in the second locus resulted in nodules with wild type levels of nitrogen fixation activity even though mutations in either locus were Dct$^-$ in culture. These findings indicate that $C_4$-dicarboxylic acid transport may be regulated by different factors under free-living and symbiotic growth. Likewise, Ronson et al. found that Tn*5*-induced *dctB* and *dctD* mutants form nodules with good nitrogenase activity [182], although the chemically mutagenized *dctB* gene results in nodules which cannot fix nitrogen [183]. The latter group [182] speculated that the apolar mutation in the *dctB* gene caused by chemical mutagenesis would result in the synthesis of inactive DctD which could bind to the *dctA* promoter, and thus inhibit transcription by the symbiotic regulator. The polar mutation in the *dctB* gene, however, would result in no synthesis of DctD, since *dctD* is downstream from *dctB*, and thus the symbiotic regulator could activate *dctA* transcription.

### *IID. Carbon metabolism in free-living Rhizobium*

Carbon metabolism in *Rhizobium* cultured cells has been thoroughly reviewed [204], and only a brief mention of it is presented here. It is a topic in itself not only because carbon metabolism is quite different from that of symbiotically grown cells, but also because there are significant differences between the fast- and slow-growing species [204,235], and there are even differences among strains of the same species [119]. Since free-living species do not, for the most part, fix nitrogen, the rationale for understanding carbon metabolism in these cells is not so much from the perspective of its importance in supporting nitrogen fixation directly. Rather, it is useful in studying systems and pathways that are common to both growth modes, for understanding how cells change metabolically in the free-living and symbiotic states, and in understanding how *Rhizobium* behaves in the rhizosphere in general.

Fast-growing rhizobia can grow on hexoses, pentoses, disaccharides, trisaccharides and organic acids, whereas the slow-growing species do not grow on disaccharides or trisaccharides [204]. Growth on organic acids and sugar alcohols is variable among strains, and glycerol is the most universal carbon source. Fast- and slow-growing *Rhizobium* species are able to grow on a wide variety of aromatic compounds [167,204], which may be significant for survival in the soil.

## III. Leghemoglobin

### *IIIA. Properties and function*

Leghemoglobin is found in the root nodules of all legumes, and plant hemoglobins have also been identified in the non-legume symbioses of *Bradyrhizobium* sp. with *Parasponia* [12] and in the so-called 'actinorhizal' symbioses of *Frankia* with various plant hosts [212]. The leghemoglobin apoprotein is a plant product [20,198,225], whereas the heme prosthetic group may be synthesized by the bacterium (subsection IIIB). Leghemoglobin has been intensively studied genetically, physically and physiologically (reviewed in Refs. 7, 35 and 53), and it is a fascinating protein in many respects. Analysis of two soybean leghemoglobin genes show that each contains two introns in the same positions as those of animal hemoglobins [98], and the amino acid sequence of leghemoglobin also shows homology to animal globins in critical regions [96]. Since hemoglobin-like DNA sequences have also been found in non-nodulating plant species, Appleby et al. [10] speculated that

hemoglobins may occur in all plants, and that their origin, along with animal hemoglobin genes [8], are descended from a common ancestor.

A problem inherent to aerobic nitrogen-fixing organisms is that $O_2$ is essential for ATP synthesis, but the nitrogenase enzyme is $O_2$-labile, and thus a strategy must be adopted to deal with this paradox. The leghemoglobin found in root nodules facilitates the diffusion of $O_2$ to the bacteroids and buffers the free $O_2$ concentration in the nodule at a very low tension (reviewed in Refs. 8 and 230). The $O_2$ can be poised at a low concentration in nodules because leghemoglobin has a very high affinity for $O_2$. The dissociation binding constant of soybean leghemoglobin is 46 nM [99,231], and if an average partial oxygenation of 20% is assumed, then the free $O_2$ concentration in soybean nodules is 11 nM [8]. This value is reasonably consistent with the apparent dissociation binding constant of 6 nM of the efficient bacteroid oxidase for $O_2$ [30], and thus ATP-producing respiration can occur at the very low $O_2$ tension in the nodule. Also, if the leghemoglobin concentration in soybean nodule cytosol is 3 mM [26], and the other conditions described above are true, then the ratio of bound to free $O_2$ is 55 000 [8]. This means that leghemoglobin provides an enormous $O_2$ buffering capacity, and that the low free $O_2$ concentration can be maintained despite the rapid rate of bacteroid respiration. The high leghemoglobin concentration also allows the diffusion of $O_2$ to be facilitated by the much larger globin molecule. The high affinity of leghemoglobin for $O_2$ is due to a very fast association rate ($k_a = 1.2 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ for soybean leghemoglobin, Refs. 11, 99 and 231), rather than a very slow $O_2$ dissociation rate. The $O_2$ dissociation rate constant is 5.5 $s^{-1}$ [11], which is fast enough for soybean leghemoglobin to be kinetically competent to carry $O_2$ to the bacteroids [8].

Leghemoglobin was implicated in facilitated $O_2$ diffusion when Wittenberg et al. [232] found that the addition of leghemoglobin to isolated bacteroids resulted in a 10-fold increase in nitrogenase activity with only a 2-fold increase in the rate of $O_2$ uptake. Other $O_2$-binding proteins could substitute for leghemoglobin provided that the $O_2$ association and dissociation rates were sufficiently fast to support respiration. They postulated that leghemoglobin facilitates $O_2$ diffusion through the 'unstirred layer' surrounding the suspended bacteroids, and buffers $O_2$ at a concentration favoring electron transport through the efficient limb of a branched electron transport system (subsection IVB).

The nitrogenase enzyme complex is very well conserved and the $O_2$ lability of the purified proteins probably do not vary much between organisms. However, aerobic $N_2$-fixers show a range of sensitivities to $O_2$, and this is also true among the rhizobia. *Azorhizobium caulinodans* has very high nitrogenase activity in cultured cells grown in 2 $\mu$M $O_2$ [60,203], whereas *B. japonicum* bacteroids have no nitrogenase activity at this $O_2$ tension. Lupin bacteroids have nitrogenase activity when isolated from nodules aerobically [136], but activity in soybean bacteroids is irreversibly abolished in air. *R. leguminosarum* bacteroids have an $O_2$ optimum of 800 nM for nitrogenase activity [120], but *B. japonicum* bacteroids are not very active at this $O_2$ concentration, and have an $O_2$ optimum closer to 100 nM [28]. The low $O_2$ concentration optimum for *B. japonicum* nitrogenase activity is probably a direct result of ATP synthesis being more efficient at 100 nM $O_2$ than at 800 nM $O_2$ [29], which in turn is a function of the $O_2$ affinity of the terminal oxidases [30]. *R. leguminosarum* retains the 'free-living' oxidases cytochromes $aa_3$ and *o* symbiotically [9], which may reflect a higher free $O_2$ concentration in pea nodules compared with that of soybean nodules, where cytochromes $aa_3$ and *o* are repressed. *B. japonicum* has apparently evolved as an $O_2$-intolerant species compared with *R. leguminosarum* and *Bradyrhizobium* sp. (Lupinus) as judged by the $O_2$ lability of nitrogenase and by the low $O_2$ concentration at which maximal ATP synthesis and nitrogen fixation occurs in *B. japonicum*. Appleby [7] noted that the $O_2$ tolerance of *R. leguminosarum* compared with that of *B japonicum* is also reflected in the binding affinity of the respective leghemoglobins for $O_2$. The $O_2$ dissociation binding constant of soybean leghemoglobin is 46 nM [99,231] and 149 nM for pea leghemoglobin [221], and thus the pea hemoglobin would buffer $O_2$ at a higher concentration than is found in soybean nodules. Also, lupin bacteroid nitrogenase activity is greater at the myoglobin-buffered $O_2$ concentration than it is at the lower $O_2$ tension buffered by leghemoglobin [7]. It would be useful to rigorously establish the relationship between the leghemoglobin binding constants for $O_2$ and the $O_2$ binding properties of the respective bacteroid oxidases, and also to compare these data with the $O_2$ maxima at which $N_2$ fixation occurs in the different symbioses. It is also pertinent to know what factors confer $O_2$ tolerance on rhizobial species. The *Azotobacter vinelandii* nitrogenase is protected from $O_2$ inactivation when complexed with an iron-sulfur protein [88], but no such mechanism has been found in *Rhizobium*. Brief treatment of intact soybean roots with 1 atm $O_2$ diminishes nitrogenase activity, but this activity is partially restored in a very short time [169], and thus an $O_2$ protection mechanism may also occur in legume symbioses.

### *IIIB. The origin of leghemoglobin heme*

The prosthetic group of leghemoglobin is protoheme IX [41,64,125] and it is present in very high concentrations in legume nodules. The origin of the heme prosthetic group has been a subject of debate for some time, and three approaches have generally been taken in

addressing the question of which symbiont synthesizes heme for leghemoglobin: (i) measuring the incorporation of radioactively labelled heme precursors into heme by nodule fractions [48,70,84,85]; (ii) measuring enzyme activities of the heme biosynthesis pathway in plant and bacterial fractions [84,149,173]; and (iii) by quantitating heme in nodules formed from bacterial mutants deficient in heme biosynthesis [87,126,148,159]. δ-Aminolevulinic acid (ALA) synthase can be detected in bacteroid, but not in plant, fractions in soybean [149], serradella and lupin [84] nodules. Furthermore, the ALA synthase activity of soybean bacteroids parallels the heme concentration of developing soybean nodules [149]. ALA dehydratase activity is found in both the plant and bacteroid fractions in nodules from soybean [149], serradella and lupin [84] nodules, but the specific activity in the plant fraction decreases as the heme concentration increases in the soybean nodules [149]. These physiological data point to the bacterium as the source of leghemoglobin heme, which is easy to believe considering the large heme concentration in legume nodules and the large population of bacteria compared with the relatively few plant mitochondria in infected root cells. However, Guerinot and Chelm [87] mutated the ALA synthase structural gene (*hemA*) of *B. japonicum*, and found that soybean nodules formed from the mutant contains leghemoglobin heme. This latter finding is surprising, since most of the physiological data indicating that bacteroids synthesize leghemoglobin heme comes from work done with the soybean symbiosis [48,149,173]. It is equally interesting that a mutation in the *hemeA* gene of *R. meliloti*, from which the *B. japonicum* gene was derived, forms white nodules on alfalfa [126].

It is clear that neither the physiological data nor the mutant analyses of the first step in heme synthesis arrive at a unified conclusion as to which symbiont synthesizes leghemoglobin heme. More recently, a *B. japonicum* mutant defective in protoporphyrinogen oxidase activity, the seventh of eight enzymatic steps in heme synthesis, formed soybean nodules deficient in leghemoglobin heme [159]. Thus bacterial heme synthesis is essential for leghemoglobin expression in soybean nodules [159] even though a functional bacterial ALA synthase is not required [87]. It is important to note that nodules formed from the *B. japonicum* ALA synthase mutant have good nitrogenase activity as well as containing leghemoglobin heme [87]. These bacteroids must therefore contain cytochrome heme, otherwise the cells would not be able to support nitrogen fixation. It seems that the *B. japonicum* ALA synthase mutant can be rescued either by the plant host, or by an alternative bacterial pathway to ALA synthesis [87], whereas the protoporphyrinogen oxidase mutant cannot be rescued symbiotically [159]. It would also appear that the ALA synthase mutant of *R. meliloti* cannot be rescued as alfalfa nodules formed from the mutant do not contain leghemoglobin [126].

A secondary site suppressor mutation has been described in a *hemA* (the structural ALA synthase gene) mutant of *Azorhizobium caulinodans* strain ORS571 [170] suggesting that an alternative method of ALA synthesis is possible in $N_2$-fixing bacteria. The relevance of this finding, however, cannot be ascertained, since it is not yet known whether the suppressor mutant forms leghemoglobin-containing nodules on *Sesbania rostrata*. It is prudent not to extend these findings to other symbioses, since the *hemA* mutant of *R. meliloti* results in leghemoglobinless nodules [126], and thus an alternate pathway of ALA synthesis does not seem to function symbiotically in that bacterium.

It is possible that the *B. japonicum hemA* mutant was rescued symbiotically by the acquisition of ALA or by another heme precursor from the soybean host. Since the plant fraction of soybean nodules contains no measurable ALA synthase activity [149], then ALA would have to be synthesized via a different enzyme, or it would have to be made outside the nodule and then transported there. Although either of these situations could explain the apparent discrepancy in the soybean literature [87,149,159,173], neither hypothesis has been tested. It is known that plants synthesize ALA from glutamate via the five carbon pathway [21,43] rather than by ALA synthase, but the former pathway is confined to chloroplasts in eukaryotes [137]. Nevertheless, it is tempting to make an analogy between chloroplasts and infected plant roots with respect to the enormous amount of tetrapyrroles found in both structures.

Whereas arguments can be made against bacteroids as the provider of early heme precursors for leghemoglobin formation, the data thus far support a role for prokaryotic heme enzymes in catalyzing the late steps in heme biosynthesis [159,173]. Obligate aerobes use $O_2$ as a substrate in the two oxidative steps in heme synthesis, but the $O_2$ concentration in legume nodules is only about 10 nM, and thus these two enzymes, coproporphyrinogen oxidase and protoporphyrinogen oxidase, must either have a very high affinity for $O_2$, or else an oxidant other than $O_2$ is used in catalysis. Keithly and Nadler [110] found that *B. japonicum* bacteroids have $NADP^+$-dependent coproporphyrinogen oxidase activity under anaerobic conditions. This activity is known to occur in anaerobes [145], but *B. japonicum* is the only known obligate aerobe to have 'anaerobic' coproporphyrinogen oxidase activity [110]. It is not yet known whether rhizobial species have an anaerobic protoporphyrinogen oxidase; such activity has been found in *E. coli* [101,102] and in *Desulfovibrio gigas* [112], and the *D. gigas* protoporphyrinogen oxidase has been purified [113]. A mutation in $O_2$-dependent protoporphyrinogen oxidase activity in *B. japonicum* results

| | |
|---|---|
| *E. coli* FNR consensus | 5′-A___TTGA____TATCAAT_A-3′ |
| *B. japonicum hemA* | 5′-TTCTTTGATCGGGATCAAGTT-3′ |
| *R. meliloti hemA* | 5′-TTGCTTGACTTCGATCGATGT-3′ |

Fig. 2. Comparison of the *E. coli* FNR consensus sequence with the *hemA* promoter of *R. meliloti* ($P_2$) and of *B. japonicum*. Underlined regions of the *hemA* promoters are identical to the FNR consensus sequence. See text for details.

in a heme-deficient phenotype symbiotically [159], and thus if anaerobic activity exist in nodules, it is likely to occur via the same enzyme which catalyzes the $O_2$-dependent reaction.

### *IIIC. Regulation of heme biosynthesis*

Incubation of *B. japonicum* cultured cells under restricted aeration results in elevated levels of ALA synthase and ALA dehydratase activities, and in the excretion of porphyrins into the growth medium [17]. These observations suggest that heme synthesis is negatively regulated by $O_2$, which is consistent with the increased cytochrome synthesis [3,4] and possibly the leghemoglobin heme synthesis which occurs in the low $O_2$ environment of the nodule. In *E. coli*, the transcriptional activator FNR is required for the expression of numerous genes involved in anaerobic respiratory pathways, and an 'FNR consensus sequence' has been identified in the promoter regions of genes regulated by FNR [202]. The ALA synthase structural genes (*hemA*) of *B. japonicum* [139] and *R. meliloti* [127] have promoter sequences homologous to the FNR consensus sequence of *E. coli* * (Fig. 2). These findings indicate that rhizobia may contain FNR, and that it could be involved in the regulation of heme synthesis enzymes by $O_2$. The expression of FNR in *Rhizobium* and *Bradyrhizobium* would mean that anaerobic-type regulation occurs in an obligate aerobe. It seems that rhizobia have accommodated to the very low $O_2$ nodule environment in two ways: (i) by the expression of cytochrome oxidases which have a very high affinity for $O_2$, thereby allowing $O_2$-dependent oxidative phosphorylation in nodules; and (ii) by the development of anaerobic traits such as $NADP^+$-dependent coproporphyrinogen oxidase activity and possibly regulation by an FNR-type protein.

* To our knowledge the similarity between the *hemA* promoters and the FNR consensus sequence was first brought to the attention of the scientific community in a lecture by F. de Bruijn at the 7th International Congress on Nitrogen Fixation, 13–20 March 1988, Köln, F.R.G.

ALA synthase and ALA dehydratase activities are apparently regulated by iron as *B. japonicum* cells grown in iron-deficient media are depressed in these enzyme activities [178]. Addition of iron to extracts of iron-deficient cells does not restore ALA synthase or ALA dehydratase activities [178], and thus iron probably regulates enzyme synthesis at some level.

Heme does not seem to be a feedback inhibitor of ALA synthase in *B. japonicum*, as it is in *Rhodobacter sphaeroides* [42], since addition of heme does not repress ALA synthase activity in *B. japonicum* cells [178]. The incorporation of ALA into heme is inhibited by heme and protoporphyrin [49], but it takes 100 μM of porphyrin to inhibit incorporation by 50%, and thus heme may not be a physiological feedback inhibitor. Heme or protoporphyrin, however, may be a regulator of heme enzymes at the synthesis level as judged from the elevated levels of ALA synthase, ALA dehydratase and coproporphyrinogen oxidase activities in a heme synthesis mutant of *B. japonicum* deficient in protoporphyrinogen oxidase activity [159].

### *IIID. Heme as a regulator*

As stated above, heme may regulate the synthesis of enzymes of the heme biosynthesis pathway [159], but it is not an inhibitor of these enzymes [178]. It has also been speculated that bacterial heme induces leghemoglobin apoprotein synthesis by the plant (reviewed in Refs. 7 and 35), and there is evidence that heme controls synthesis at the post-transcriptional level [236]. According to the proposed scenario, rhizobia in the developing nodule consume most of the available $O_2$, which in turn triggers bacterial heme synthesis. The heme then induces apoleghemoglobin formation by the plant, and thus heme and apoprotein synthesis are coordinated in the respective symbionts. However, nodules formed from a protoporphyrinogen oxidase mutant of *B. japonicum* contain leghemoglobin despite the heme deficiency in those nodules [159]. This indicates that the heme moiety is not an essential prerequisite for apoleghemoglobin synthesis by the plant. Also, a porphyrin-excreting mutant of *R. leguminosarum* forms nodules on peas which contain some apoleghemoglobin [34].

Thummler and Verma [211] found that heme binds to nodule sucrose synthase in soybean, and that heme regulates that enzyme. The sucrose synthase tetramer dissociates in the presence of heme with a concomitant inactivation of activity. It was postulated that, in the nodule, free heme accumulates during senescence when leghemoglobin breaks down, and results in the inactivation of sucrose synthase. The supply of carbon sources to bacteroids would therefore decrease during nodule senescence.

## IV. Electron transport

### *IVA. Cultured cells*

Electron transport in *Rhizobium* has been studied most intensely in *B. japonicum*, and to a lesser extent in *Bradyrhizobium* sp. (*Lupinus*) and in *R. leguminosarum*. The complement of cytochromes expressed is significantly different in bacteroids compared with cells grown in culture. *B. japonicum*-cultured cells contain *b*- and *c*-type cytochromes, as well as the terminal oxidases cytochromes $aa_3$ and *o* [4]. Cytochromes *o* and $aa_3$ form complexes with carbon monoxide in a $CO-O_2$ atmosphere (95 : 5, v/v), but respiration is not completely inhibited under these conditions, and thus there is likely to be at least one other oxidase expressed in cultured cells [4]. Cytochromes *c* and $aa_3$ seem to be part of one branch of the electron-transport system, and cytochrome *o* is part of another branch which does not oxidize cytochrome *c* [4,158,160]. A mutant strain of *B. japonicum* which lacks cytochrome *c* and $aa_3$ has similar respiratory properties to a mutant which lacks only cytochrome $aa_3$ [158], suggesting that cytochromes *c* and $aa_3$ are a functional unit. Also, the isolation of *B. japonicum* cytochrome mutants based on a respiratory deficiency always yield lesions in the cytochromes *c* and $aa_3$ part of the electron transport system [65,158]. The electron transport system of free-living *R. trifolii* is similar to that of *B. japonicum*, with the branch point at cytochrome *b*, and with *a*- and *c*-type cytochromes forming a branch separate from that of cytochrome *o* [52]. *R. trifolii* [51] and *R. leguminosarum* [179] both express cytochrome *d* under restricted aeration, and although oxidase function has not been demonstrated in the cytochrome *d* of *Rhizobium*, this cytochrome is an oxidase in *E. coli* [75] and in other bacteria, and is expressed under $O_2$-limited growth in those organisms.

The expression of multiple oxidases is common in bacteria, and they allow the organism to survive in a wide range of environmental conditions. A cytochrome $aa_3$-deficient mutant of *B. japonicum* has greatly diminished NADH- and TMPD-oxidase activities, but it can oxidize succinate at wild-type levels [158]. Inhibitor data also show that NADH and TMPD are oxidized by a different oxidase than is succinate [158]. The substrate specificity may be due to the considerably higher rate of oxidation of NADH and TMPD by cell-free extracts of the parent strain compared with the oxidation rate of succinate. If this is true, it would suggest that cytochrome $aa_3$ has a higher turnover rate, and can support a higher electron flux than can cytochrome *o*. Cytochrome *o*, however, seems to be more suited to function under low $O_2$ conditions [162], and it probably has a higher affinity for $O_2$ than does cytochrome $aa_3$. Cells grown under restricted aeration have diminished , or no, cytochrome $aa_3$ [17,50,203], which is consistent with the idea that it is not functional under conditions of $O_2$ limitation.

Generally, cytochrome $aa_3$ and cytochrome *o* expression in *Rhizobium* is repressed in root nodules, and other oxidases unique to symbiotic growth are expressed [3,115,136,161,179]. The decrease in cytochrome $aa_3$ has been correlated with an increase in nitrogenase activity in *B. japonicum* bacteroids [45], and $aa_3$-type cytochrome is retained in ineffective (non-$N_2$-fixing) strains of *Bradyrhizobium* sp. (*Lupinus*) [136] and *R. leguminosarum* [116]. Despite these observations it is difficult to correlate cytochrome $aa_3$ and *o* with symbiosis, nitrogen fixation or even oxygen concentration. Cytochrome *o* has been observed in bacteroids from effective nodules of *B. japonicum* [109,161], *R. leguminosarum* and *B. parasponia* [9]. Similarly, cytochrome $aa_3$ is retained in effective nodule bacteroids of *B. parasponia* [9] and in an entire DNA homology class of *B. japonicum* [108], and also in cultured cells of *Bradyrhizobium* sp. strain 32H1 which fix nitrogen [9]. The symbiotic expression of cytochrome $aa_3$ in some effective *B. japonicum* strains [108,109] is most interesting in that these bacteria presumably function under the same leghemoglobin-buffered $O_2$ tension as those strains which do not express cytochrome $aa_3$ symbiotically, and thus oxygen cannot be the sole regulator of the expression of this oxidase. Cytochrome $aa_3$ seems to have a low affinity for $O_2$ not only in *Rhizobium* [50,115,162], but in other bacterial strains as well [190,193,201], and so it is somewhat surprising that the oxidase is retained in bacteroids of some rhizobial strains. The presence of bacterial cytochrome $aa_3$ in nodules, however, does not prove that it can function at very low oxygen tensions. Oxidase activity of cytochromes $aa_3$ or *o* has not been demonstrated in nodules, and a cytochrome $aa_3$-deficient mutant of a *B. japonicum* strain which normally expresses the oxidase symbiotically still produces effective nodules despite the lesion [152].

### *IVB. Bacteroids*

*Bradyrhizobium japonicum* bacteroids express cytochromes *c*-552, *c*-554, *P*-420, and *P*-450, all of which react with carbon monoxide and are thus putative oxidases [3,220]. *Bradyrhizobium* sp. (*Lupinus*) bacteroids also express cytochrome *P*-450 [136] as do, to a lesser extent, *R. leguminosarum* strain PRE and cultured cells of *Bradyrhizobium* sp. strain 32H1 induced for nitrogenase activity [9]. *B. japonicum* bacteroids also contain a non-heme component which has high cytochrome *c* oxidase activity; it is CO-insensitive and may be a flavin-containing metalloprotein [6,161].

Appleby [3] was unable to demonstrate that the CO-reactive proteins found in *B. japonicum* bacteroids

were cytochrome oxidases by photochemical action spectra; it was later discovered [29] that bacteroid respiration is insensitive to CO at the $O_2$ concentration (70 $\mu$M) at which the action spectra were performed. Carbon monoxide inhibits respiration at leghemoglobin-buffered $O_2$ concentrations (less than 0.1 $\mu$M; see Ref. 29), but it is not possible to maintain such a low $O_2$ concentration experimentally for performing action spectra without introducing an $O_2$ carrier protein such as leghemoglobin or myoglobin, which have their own CO-binding properties. Thus, elucidation of bacteroid electron transport has proceeded by other means, and proof of oxidase function of the numerous CO-reactive proteins has not yet occurred. Bergersen and Turner [30] identified four $O_2$ affinity states of *B. japonicum* respiration, which presumably correspond to four oxidases or to four $O_2$ binding sites with different affinities for $O_2$. The highest $O_2$ affinity state showed an apparent $K_s$ value of 6 nM [30], which is close to the estimated $O_2$ concentration of 10 nM within a soybean nodule [5,213]. Also, hydrogen oxidation by *B. japonicum* bacteroid membranes involved three cyanide-binding sites [161], supporting the CO-binding data and the respiration kinetics experiments for multiple oxidases. The multiple oxidase system of *B. japonicum* bacteroids apparently allows efficient and inefficient electron transport within the nodule with respect to ATP synthesis. The addition of leghemoglobin to bacteroid suspensions resulted in a large increase in nitrogenase activity with only a modest increase in $O_2$ consumption [31,232], indicating that the ATP/O ratio is greater at the low $O_2$ concentration that leghemoglobin maintains. It was directly shown that ATP synthesis is highest in *B. japonicum* bacteroids at leghemoglobin-buffered $O_2$ concentrations of 0.02–0.1 $\mu$M [29], showing that efficient respiration occurs at a low $O_2$ tension. This efficient phase of respiration is inhibited by carbon monoxide and *N*-phenylimidazole, whereas the less efficient respiration that occurs above 1 $\mu$M $O_2$ is insensitive to these inhibitors [13,29,30]. *N*-phenylimidazole has been shown to be an inhibitor of cytochrome *P*-450 [13], and thus this cytochrome, although perhaps not an oxidase, is essential for the efficient phase of respiration which supports nitrogen fixation.

At high $O_2$ concentrations (more than 1 $\mu$M) *B. japonicum* bacteroid respiration is uncoupled from ATP synthesis [29] and does not involve cytochrome *P*-450 as judged from inhibitor data [13,29,30]. It has been proposed that the uncoupled electron transport acts as 'protective respiration' to maintain a low intracellular $O_2$ concentration at the expense of high-energy electrons to prevent inactivation of the $O_2$-labile nitrogenase enzyme [29]. A protective respiration mechanism has been proposed for *Azotobacter* (see Ref. 174) which is an obligate aerobe with a very high respiratory rate and with no known hemoglobin-type protein to buffer the $O_2$ concentration. In these systems, electron flow through the cytochrome $b \rightarrow d$ branch is very rapid, but energy is not conserved by that pathway [57]. Appleby [6] discovered a very vigorous cytochrome *c* oxidase activity in *B. japonicum* which is sensitive to cyanide and other metal-chelating agents but not to carbon monoxide. The high turnover rate and the CO-insensitivity of this activity led to the conclusion that the oxidase terminated the inefficient branch of bacteroid electron transport [6]. Bacteroid cytochrome *c* oxidase activity is inhibited by Atebrin [6,161] which can be partially reversed by FMN or FAD [6], suggesting that the oxidase may be a flavoprotein. Also, $H_2$-reduced bacteroid membranes show a flavin absorption spectrum [161] that disappears in the presence of cyanide; the membrane-bound hydrogenase of *B. japonicum* is not a flavin-containing dehydrogenase, and thus the flavin must be due to another component. The insensitivity to CO suggested that the cytochrome *c* oxidase may be a non-heme metalloprotein [6]; this speculation was strengthened by the recent observation that a cytochrome-deficient mutant of *B. japonicum* retained almost wild-type levels of bacteroid cytochrome *c* oxidase activity [158]. The mutant analyses also show that bacteroid cytochrome *c* oxidase can be expressed independently of cytochrome *c* [158], even though cytochrome *c* is likely to be the physiological substrate of the oxidase [6].

A cytochrome $aa_3$-deficient mutant yielded bacteroids with elevated cytochrome *c* oxidase activity [158] which suggests that, even though the two oxidases are not expressed simultaneously, their regulation is somewhat related. Alternatively, protective respiration, and hence bacteroid cytochrome *c* oxidase, may only be necessary in young nodules where the $O_2$ concentration would be relatively high [7], and the cytochrome $aa_3$ mutation may result in less developed nodules with higher bacteroid oxidase activity. It should be noted, however, that those nodules formed from the cytochrome $aa_3$ mutant appear to be normal in several respects. The wild-type gene which was mutated to yield the cytochrome $aa_3$-deficient phenotype has been cloned [164]; it codes for a diffusible product that complements the cytochrome $aa_3$ mutant. It is not yet known if this gene product represses bacteroid cytochrome *c* oxidase in the mutant to wild-type levels.

## V. ATP synthesis and ion transport

The most accepted notion of how ATP is synthesized in bacteria, as well as in mitochondria and chloroplasts, is by the coupling of $H^+$ diffusion in the direction of an electrochemical gradient with the phosphorylation of ADP. The electrochemical gradient, or protonmotive force, is the sum of the electrical potential across the bacterial membrane ($\Delta\Psi$) and the proton chemical

gradient between the internal and external pH ($\Delta$pH). $\Delta\Psi$ is most commonly measured indirectly by quantitating the distribution of lipophilic ions (or ions made membrane-permeable by the addition of an ionophore) on both sides of the membrane, and calculating the membrane potential using the Nernst equation with the assumption that the ions are in equilibrium (see Ref. 104). Another model for ATP synthesis abandons the need for a potential across the membrane in favor of a localized proton current where $H^+$ diffusion is controlled at the level of the ATPase rather than at the membrane [228]. The latter model does not mandate a fixed correlation between $H^+$ flux and ATP synthesis, and thus the relationship between ATP/O ratio and $H^+$/O measurements cannot be assumed. Also, the interpretations of permeant ion distribution measurements inside and outside a cell as an indicator of its energy status can only be valid in terms of a protonmotive force as defined above for chemiosmosis. Although we do not intend to refute the chemiosmotic hypothesis here, it is our opinion that the alternate model described above [228], and also the criticisms raised against indirect measurements of $\Delta\Psi$ [209,228] cannot be ignored out-of-hand.

*VA. ATP synthesis in $N_2$-fixing cells*

ATP synthesis in *B. japonicum* bacteroids is most efficient at a low, leghemoglobin-buffered $O_2$ concentration, and the ATP/ADP ratio decreases as the $O_2$ tension is raised [29]. The control of ATP synthesis by $O_2$ in $N_2$-fixing organisms has been explained in terms of a branched electron-transport system with the ATP-efficient branch terminated with a high $O_2$-affinity oxidase, and with the inefficient branch terminated by an oxidase with a lesser affinity for $O_2$ [29,30]. However, high ATP/ADP ratios occur in *R. leguminosarum* bacteroids [120] at an $O_2$ concentration (approx. 1 $\mu$M) where respiration is uncoupled in *B. japonicum* [29]. Similarly, the ATP/ADP ratio increases markedly in *R. phaseoli* bacteroids when the $O_2$ concentration is raised from 3 to 7 $\mu$M [215]. Experiments with *R. leguminosarum* [120,121] and *B. japonicum* [29] show that ATP synthesis increases as a function of the $O_2$ concentration at very low $O_2$ tensions, but the optimal $O_2$ concentration for ATP synthesis and nitrogen fixation are quite different in the two systems. The different $O_2$ optima for ATP synthesis could be due to oxidases with different affinities for $O_2$, but the cytochrome profile of lupin bacteroids [136] looks very similar to that of soybean bacteroids [3], yet ATP synthesis is optimal at a higher $O_2$ concentration in lupin bacteroids compared with that of soybean bacteroids [7].

Appleby et al. [13] found a linear relationship between ATP/ADP ratio and nitrogenase activity in *B. japonicum* bacteroids and concluded that the ATP/ADP ratio controls that activity. Having established that the ATP/ADP ratio is dependent on the $O_2$ concentration [29,30], then nitrogenase activity is controlled by $O_2$ via ATP synthesis. However, Laane et al. reported that nitrogenase activity decreased with increasing $O_2$ without a concomitant decrease in the ATP/ADP ratio in *R. leguminosarum* [120]. They correlated a decrease in nitrogenase activity with a valinomycin- or nigericin-dependent decrease in the membrane potential, as measured by the distribution of tetraphenylphosphonium ion ($TPP^+$), and concluded that $\Delta\Psi$, independently of the protonmotive force, regulates nitrogenase activity [122]. Since the ATP/ADP ratio remained constant in these experiments, they assumed that $\Delta\Psi$ regulates nitrogenases by controlling the supply of electrons to the enzyme complex.

There are several other examples where the relationship between ATP synthesis, nitrogen fixation activity and protonmotive force measurements is not obvious. Trinchant et al. [215] determined that the ATP/ADP ratio increases under conditions where nitrogenase is suppressed in *R. phaseoli* bacteroids. The protonmotive force, as inferred by the distribution of benzoic acid and $TPP^+$ to deduce $\Delta$pH and $\Delta\Psi$, respectively, of *B. japonicum* was somewhat less in $N_2$-fixing bacteroids than in cultured cells [32]. Although this observation can be accounted for by the assumption that the assay was performed at high $O_2$ tensions where $N_2$ fixation is not likely to occur in either cell type, it should be noted that the electrochemical potential only decreased from $-173$ mV to $-136$ mV when 2.5 mM azide was added [32], and thus the protonmotive force is not severely affected by cell metabolism. Gober and Kashket [81] compared *Bradyrhizobium* sp. strain 32H1 grown in air and under 0.2% $O_2$ to derepress nitrogenase activity. The protonmotive force of the air-grown cells was $-237$ mV at pH 6.1, but it was only $-115$ mV in the $N_2$-fixing cells when assayed at 21% or 0.2% $O_2$. Despite the higher protonmotive force in the air-grown cells, the intracellular ATP concentration was higher in the $N_2$-fixing cells at pH 6.1, and the ATP/ADP ratio was about the same under both growth conditions. These findings are different from those of Laane et al. [120–122] in that the decrease in the membrane potential does not seem to shut off nitrogenase activity in *Bradyrhizobium* sp. strain 32H1 [81] as it does in *R. leguminosarum* [120,122]. Gober and Kashket [81] offered several hypotheses for the low membrane potential and protonmotive force in the $N_2$-fixing cells. One idea is that the cells grown under 0.2% $O_2$ ($N_2$-fixing) have a 'leakier' membrane with respect to protons than do the air-grown cells resulting in a collapse of the membrane potential. Subsequently, they found that a $K^+/H^+$ antiporter is induced at 0.2% $O_2$, which was credited with collapsing the membrane potential [82]. They also proposed that ATP synthesis may be driven

by localized proton currents as proposed by Williams [227,228], and thus the protonmotive force "does not reflect the true driving force for ATP synthesis" [81]. Indeed, the measured protonmotive force does not seem to correlate with the ATP concentration or the ATP/ADP ratio inside the cell. In those experiments, growth under 0.2% $O_2$ would result in an alteration in the proton circuit which drives ATP synthesis [81]. The induction of the $K^+/H^+$ antiporter would certainly alter the proton current, but under a chemiosmotic scheme the antiporter should have an uncoupling effect.

*VB. Ion transport*

*VB-1. Potassium*

A decrease in the membrane potential of *Bradyrhizobium* sp. strain 32H1 grown under 0.2% $O_2$ compared with air-grown cells led Gober and Kashket [81] to speculate that a $H^+$-linked flux of another ion occurs in the cells grown under low [$O_2$]. They found a $K^+/H^+$ antiporter that required high [$K^+$] and low [$O_2$] during growth for induction [82]. The antiporter was detected from the collapse of $\Delta\Psi$ when induced cells were loaded with $K^+$ and put in a low [$K^+$] medium; this treatment had no effect on the $\Delta\Psi$ in air-grown cells or in cells grown in low [$K^+$]. The $K^+/H^+$ antiporter was also discerned by a $K^+$-dependent increase in the proton conductance of the membrane of cells grown under low [$O_2$], high [$K^+$]. $K^+$ did not alter $H^+$ conductance in cells grown under high [$O_2$] and/or low $K^+$, and $Na^+$ did not increase $H^+$ conductance in cells where the antiporter is supposed to be expressed. This antiporter system is different from the $K^+$-ATPase of *Rhizobium* sp. strain UMKL20 [129] in that the latter is induced by 50 $\mu$M $K^+$ in air. The effects of $K^+$ in *Bradyrhizobium* also differ from other bacteria in that it alters the protonmotive force as well as the membrane potential in *Bradyrhizobium* sp. strain 32H1 [82]. In several other bacteria [19,91,107,116,117,150,200,214] a $K^+$-induced decrease in $\Delta\Psi$ is accompanied by an increase in the $\Delta$pH, and thus the magnitude of the protonmotive force is not significantly changed. The $K^+/H^+$ antiporter-dependent decrease in the protonmotive force in *Bradyrhizobium* sp. strain 32H1 is not accompanied by a decrease in ATP synthesis. Cells grown under conditions that induce the $K^+/H^+$ antiporter have nitrogenase activity, and have a higher or comparable intracellular ATP concentration and ATP/ADP ratio compared with cells repressed for the antiporter [81].

The effect of $K^+$ on cultured cells of *Bradyrhizobium* sp. strain 32H1, and also of *B. japonicum*, apparently have significance in the symbiosis as growth in $K^+$ and 0.2% $O_2$ resulted in traits normally associated with bacteroids. Cells grown in 8 mM $K^+$ and 0.2% $O_2$ expressed nitrogenase activity and an ammonium transport carrier, increased expression of $\delta$-aminolevulinic acid (ALA) synthase and ALA dehydratase activities, and repression of glutamine synthetase activity and of capsular polysaccharide synthesis [83].

*VB-2. Molybdenum*

Molybdenum is a component of the nitrogenase enzyme and, since it is a trace metal, it is reasonable to assume that $N_2$-fixing organisms have a mechanism for acquiring molybdenum from the environment. *Klebsiella pneumoniae* has a high affinity molybdenum uptake system that is constitutively expressed and is not energy-dependent [100,197], and *Clostridium pasteurianum* and *Azotobacter vinelandii* can store molybdenum in a protein-associated form [146,172]. Molybdenum uptake in *B. japonicum* cells induced for nitrogenase activity in culture showed biphasic kinetics indicative of a high- and a low-affinity system for molybdenum influx [133]. Mutants in molybdenum metabolism were isolated based on their ability to grow on nitrate only in the presence of added molybdenum. Two mutants were isolated which lack the high-affinity uptake system. Molybdenum uptake and nitrogenase activity were restored in these mutants by addition of spent media in which wild-type cells had grown. These data indicate that *B. japonicum* excretes an extracellular molybdenum-binding factor involved in the high-affinity uptake system. Nitrogenase activity was also restored in the molybdenum uptake mutants by the addition of 10–100 mM molybdenum to the depression medium [133]. The ability to accumulate molybdenum varies among naturally occurring strains of *B. japonicum* [86]; the variability can be accounted for by the differing affinities of the molybdenum uptake systems for molybdenum between the strains examined [86].

*VB-3. Nickel*

Hydrogenase is a nickel enzyme in *B. japonicum* (subsection VIB), and it must acquire this metal from the environment. Mutant strains of *B. japonicum* which express hydrogenase constitutively accumulate $Ni^{2+}$ at a much higher rate than does the parent strain [205]. The $K_m$ of $Ni^{2+}$ uptake for $Ni^{2+}$ is similar in both cell types, but the $V_{max}$ is much higher in the hydrogenase constitutive strains. $Ni^{2+}$ uptake is inhibited significantly by $Zn^{2+}$ and by $Cu^{2+}$, and to a lesser extent by $Co^{2+}$, $Mg^{2+}$ and $Mn^{2+}$. The nickel uptake system in *B. japonicum*, therefore, does not seem to occur via a $Mg^{2+}$ uptake system as occurs in some other bacteria [106]. Nickel uptake is believed to be energy-dependent in *Methanobacterium bryantii* [103], but not in *Azotobacter chroococcum* [168]. The addition of respiratory inhibitors or uncouplers of ATP synthesis has little effect on $Ni^{2+}$ uptake in cultured cells of *B. japonicum* [205], and thus influx of this metal is not energy-dependent.

*VB-4. Ammonium*

Ammonium-ion transport is obviously important in symbiotic nitrogen-fixers, since fixed nitrogen is exported to the host plant. Methylammonium is often used as an ammonium analog, and in cases where $NH_4^+$ has been shown to compete with methylammonium, the use of the analog is valid [80,123]. There is also a methylammonia permease that does not transport $NH_4^+$ [77]. The ammonium uptake system is induced under N-limited conditions in *R. leguminosarum* [77] and in *Bradyrhizobium* sp. strain 32H1 under conditions where nitrogenase activity is induced [80,83]. Dilworth and Glenn [55] could not find an ammonium uptake system in *R. leguminosarum* bacteroids, and thus argued that this system is probably important for the accumulation of $NH_4^+$ in the soil, but not in the nodule. The induction of the methylammonium transport system in *Bradyrhizobium* sp. strain 32H1, however, coincides with the expression of numerous traits associated with bacteroids [83], which hints at the idea that this transport system has significance symbiotically. The methylammonium uptake system is energy-dependent as seen by the inhibition of influx by metabolic inhibitors and uncouplers [80]. *R. leguminosarum* bacteroids loaded with methylammonium effluxed the $NH_4^+$ analog in response to $O_2$ pulses [123], indicating that $NH_4^+$ export is also energy-dependent in this bacterium.

## VI. Hydrogen oxidation

A hydrogen uptake hydrogenase has been identified in some strains of *B. japonicum, Bradyrhizobium* sp. (mungbean and cowpea), *R. meliloti* (very poor activity), *R. leguminosarum* [38], and in the stem-nodulating bacterium *Azorhizobium caulinodans* strain ORS571 [203]. The source of $H_2$ for hydrogenase is from the nitrogenase enzyme; $H^+$ is reduced to $H_2$ as $N_2$ is fixed to $NH_3$. $H_2$ evolution by nitrogenase is obligatory, and it cannot be eliminated even under experimental circumstances which heavily favor $N_2$ reduction over $H_2$ production [177,199]. Under these conditions approx. 25% of the energy required for nitrogen fixation is committed to $H_2$ production [199], and under physiological conditions in root nodules 40–60% of the energy input to nitrogen fixation is 'lost' to $H_2$ evolution [195]. Thermodynamic considerations mandate that the energy obtained from $H_2$ oxidation cannot completely compensate for the cost of $H_2$ production, but it is plausible that $H_2$ oxidation via hydrogenase yields energy that would otherwise not be available to the cell [56]. The hydrogen uptake (Hup) phenotype of *B. japonicum* has been credited with conferring superior traits on soybean [2,68], and with maintaining a higher steady-state intracellular ATP level in isolated bacteroids [66]. Soybeans grown in an aerated liquid medium, however, showed no differences when inoculated with a $Hup^-$ mutant of *B. japonicum* compared with those inoculated with a $Hup^+$ revertant strain [59]. Those authors argued that $O_2$ is limiting and thus the alleged lower ATP/O ratio with hydrogen as the substrate compared with carbon sources has a deleterious effect on the energy status of the cell. Other studies have shown that varying the amount of $O_2$ available to soybean roots does not have a long-term effect on nitrogen fixation [46,144], indicating that $O_2$ is not limiting in nodules. For the most part, the literature addressing the *R. leguminosarum* hydrogenase does not make a convincing case for $H_2$ oxidation being a beneficial trait in this organism. Only 5 of 14 $Hup^+$ strains of *R. leguminosarum* show $H_2$-dependent ATP synthesis [153] in isolated bacteroids, and hydrogenase activity does not increase symbiotic nitrogen fixation [47,218].

*VIA. Regulation*

Hydrogenase expression in *Rhizobium* is regulated by factors associated with its symbiosis with the plant, and cultured cells do not normally synthesize the enzyme. These factors not only include plant compounds, but also the nodule environment itself, since hydrogenase activity can be induced in culture under certain conditions [132,134,141,143]. To a certain extent hydrogenase derepression conditions mimic the nodule environment, and regulation at this level is much better understood than regulation by specific plant products. It has long been thought that organic carbon represses ex planta hydrogenase expression in *B. japonicum* [134], but recent experiments show that hydrogenase can be expressed when cells are grown heterotrophically in the presence of $H_2$ and under low $O_2$ concentrations [222]. Data indicate that carbon repression of hydrogenase in culture is probably caused by acidification and $O_2$ depletion of the growth medium, and precautions taken to buffer the cultures relieves repression by organic carbon substrates [224]. However, spontaneous mutants of *B. japonicum* which express hydrogenase constitutively also show constitutive expression of ribulose-1,5-bisphosphate carboxylase activity [142], and thus the regulation of hydrogenase and carbon metabolism are linked.

Hydrogenase expression is regulated by $O_2$, and a low $O_2$ tension is required for activity in cultured cells [132,134]. *B. japonicum* mutants have been obtained which are either hypersensitive [135] or insensitive [141,143] to $O_2$. The nature of the genetic lesions in the $O_2$ regulatory mutants are not yet known, nor is it known how $O_2$ regulates hydrogenase expression. However, Novak and Maier [157] found that *B. japonicum* hydrogenase activity is repressed in cultured cells when the DNA gyrase inhibitors coumermycin, novobiocin or nalidixic acid are added to the derepression medium. These repressed cells do not synthesize the hydrogenase

subunits, but protein synthesis in general is not affected [157]. Hydrogenase is therefore regulated by DNA topology; DNA gyrase activity has been shown to affect bacterial genes that are regulated by $O_2$ [18,114,233], including nitrogen fixation genes in *Rhodobacter capsulatus* and *Klebsiella pneumoniae* [114]. Hydrogenase-constitutive mutants of *B. japonicum* do not appear to have greater gyrase activity than does the wild type, but hydrogenase synthesis is not repressed by gyrase inhibitors in the constitutive strains [156].

The expression of hydrogenase is also regulated by the plant host in a manner which is poorly understood. Several *B. japonicum* strains have hydrogenase activity in cowpea (*Vigna unguiculata*) nodules, but have no activity in symbiosis with soybean [111]. Bacterial protein (*B. japonicum* strain USDA 61) from those $Hup^+$ cowpea nodules do not cross-react with antihydrogenase antibody from *B. japonicum* strain SR [223] which suggests either that the enzyme from the 'host control' strain is antigenically different from the conventional enzyme, or that the hydrogenase protein concentration was too low to be detected antigenically. Host control of hydrogenase has also been observed between different cultivars of the same pea (*Pisum sativum*) strain [22–24]. Grafting experiments showed that a transmissible shoot factor controls the expression of hydrogenase activity in the different pea cultivars [23]. The grafting data also showed that the plant host affected the $H_2/N_2$ ratio in nodules formed from a $Hup^-$ strain, and thus the host can influence the efficiency of nitrogen fixation [23]. The fact that high electron flux through purified nitrogenase favors $N_2$ reduction over $H_2$ production [89] leaves open the possibility that host control of $H_2$ metabolism is at the level of photosynthate supply to the nodule bacteroids.

Nickel is part of the hydrogenase enzyme in *B. japonicum* (subsection VIB), and it has been implicated as a regulator of hydrogenase expression [208]. Hydrogenase protein and activity both increase as a function of nickel concentration; this enhancement can be eliminated by the addition of rifampicin, which suggests that nickel regulates hydrogenase at the transcriptional level [208]. A similar phenomenon also occurs in *Alcaligenes latus* [58], but nickel is not required for hydrogenase apoprotein synthesis in *Methylosinus trichosporium* [44] or for carbon monoxide dehydrogenase apoprotein synthesis in *Rhodospirillum rubrum* [37]. In those nickel enzymes not regulated by the metal, enzyme activity can be demonstrated by the addition of nickel to the pre-formed protein.

### *VIB. Components of $H_2$ oxidation*

The *B. japonicum* hydrogenase enzyme has been purified from bacteroids [14], heterotrophically grown cells [206,207], and chemolithotrophically grown cells [90]. The enzyme is a dimer with subunits of approx. 65 kDa and 33–35 kDa in size, and it contains nickel. Nickel has been found in hydrogenases from many organisms, but the actual role of the metal is not known for certain. $H_2$ can react with nickel to form nickel hydrides [210], and $H_2$ apparently binds to nickel in the hydrogenase of *Desulfovibrio gigas* [124]. However, the hydrogenase of *Clostridium pasteurianum* [1] and *D. vulgaris* [97] are very active, yet they do not contain nickel, and thus it is premature to make generalizations as to the specific role of nickel in the *B. japonicum* hydrogenase.

Although hydrogenases are treated as $O_2$-labile enzymes, the *B. japonicum* enzyme has been successfully purified aerobically by affinity chromatography, and these preparations have superior methylene blue-dependent $H_2$ uptake activity [206]. *B. japonicum* hydrogenase purified anaerobically can be inactivated by $O_2$ in both a reversible and irreversible manner [196]. The reversible inactivation requires the presence of an electron acceptor from hydrogenase and high pH [196]. Moshiri and Maier [147] found that *B. japonicum* hydrogenase in membrane vesicles is susceptible to proteases and membrane-impermeant protein modification reagents only when the enzyme is reduced, and not in the oxidized form. The inactivation of activity by these compounds is attributed specifically to the degradation or modification of the approx. 33 kDa subunit [147]. These data indicate that hydrogenase undergoes a conformational change in the membrane which is redox-dependent.

Hydrogen oxidation yields energy via electron transport, and cytochromes and ubiquinone has been directly [160–163] and indirectly [61–63] identified which are reducible by $H_2$ in *B. japonicum* cells under various growth conditions. In addition, the Evans laboratory has claimed that there is a low-potential *b*-type cytochrome, called component 559-$H_2$, in bacteroids and in chemolithotrophically grown cells that is specifically involved in $H_2$ oxidation, but not in the oxidation of other substrates [62,63]. O'Brian and Maier have provided evidence that this component does not exist in bacteroids [165] or in hydrogenase-constitutive cultured cells possessing vigorous hydrogen uptake activity [162,163]. Furthermore, it is the reviewers' opinion that the experimental evidence provided by the Evans group [62,63] in support of component 559-$H_2$ cannot withstand scrutiny, and that their conclusions are actually contradictory to their own data. Firstly, the *b*-type cytochrome assigned 'component 559-$H_2$' [62] is not solely reducible by $H_2$, but also by endogenous substrate [62,63], and thus it is not unique to $H_2$ oxidation. The fact that some cytochrome *b* is reduced faster by $H_2$ plus endogenous substrate than by endogenous substrate alone is not relevant, since this was found to occur with the *c*-type cytochrome as well [63]. Secondly,

the claim that component 559-$H_2$ has a low redox potential and accepts electrons from hydrogenase [62] is contrary to the observation that the component remains oxidized when other cytochromes alleged to be part of the same branch of the electron-transport system are reduced [62,63]. Thirdly, the assertion that component 559-$H_2$ is present in bacteroids [62] is inconsistent with the data of Eisbrenner and Evans [61] which show this component to be absent in those cells. Finally, the method of quantifying component 559-$H_2$ must be incorrect, since Eisbrenner and Evans claim to measure $H_2$-dependent component 559-$H_2$ reduction spectrophotometrically in *B. japonicum* strains which cannot use $H_2$ as a reductant [62]. In a recent review, Evans et al. [69] recalculated the correlation between hydrogenase activity and component 559-$H_2$ expression (from Ref. 62) by omitting the data from $Hup^-$ strains, and they still found a high correlation between the two parameters. This correction, however, does not rectify the error in the method of quantifying component 559-$H_2$, and thus the new correlation data are still meaningless.

*VIC. Genetics*

Genes involved in $H_2$ oxidation are plasmid-borne in *R. leguminosarum*, and are co-transferred with nodulation genes [39]. *B. japonicum hup* genes are presumably on the chromosome as no plasmids have been identified in $Hup^+$ strains of this organism. The structural genes of the *B. japonicum* hydrogenase have been identified and subcloned from a 31 kb DNA fragment which complements $Hup^-$ mutants [234]. DNA fragments of *B. japonicum* containing the hydrogenase structural genes have been used to complement $hup^-$ mutants of *R. leguminosarum* [105], and have also been used as a probe to isolate *R. leguminosarum hup* genes [128]. The hydrogenases of *B. japonicum* and *R. leguminosarum* are therefore genetically similar, and may have been derived from a common ancestor.

In addition to the structural genes, mutant analyses indicate that there are other genes essential for $H_2$ oxidation in *B. japonicum*. Hom et al. [93] complemented a *B. japonicum* point mutant deficient in both hydrogenase and nitrogenase activities with an approx. 23 kb DNA fragment cloned into a broad host range vector. This recombinant plasmid, pSH22, is very likely homologous to pHU1, which contains the hydrogenase structural genes [234], as judged from the similar restriction maps [92,94] and from the fact that the hydrogenase structural genes obtained from a gene expression library hybridize to pSH22 (Moshiri and Maier, unpublished results). $Hup^-$ mutants not complemented by pSH22 have been isolated [93,94], and thus there are *hup*-related genes outside the DNA region contained on pSH22. The regulation of *hup* genes at the molecular level is an important topic of which almost nothing is known. Mutants have been isolated which affect the regulation of Hup expression [131,135,141–143] whose defective gene has not been located or characterized.

**Note added in proof** (received 28 March 1989)

Since the acceptance of this review, numerous important findings have been published which pertain to *rhizobial* heme and leghemoglobin [237–240], hydrogen oxidation [241], ion transport [242] and energetics [243]. In addition, the proceedings of the Seventh International Congress on Nitrogen Fixation have been published in book form [244].

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