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Review article

Evolution of the *(Brady)Rhizobium*-Legume Symbiosis: Why Do Bacteroids Fix Nitrogen?

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Abstract

Members of *Rhizobiaceae* have evolved a regulatory system that allows nitrogen fixation to occur in the absence of significant ammonia assimilation. This is achieved by a signal transduction pathway that induces nitrogenase synthesis in response to low oxygen concentration, even when cells have enough nitrogen. This feature of rhizobia may be crucial in their symbiosis with legumes since nitrogen fixation and export of ammonia represent the bacteroid's major metabolic contributions to the symbiosis. But what do bacteroids gain by fixing nitrogen if they are not nitrogen-starved? Several models that attempt to answer this question are summarized here. One model relates nitrogen fixation to oxygen starvation. Others link nitrogen fixation to carbon acquisition from the host and yet other models suggest that nitrogen fixation is a mechanism to subvert host defenses.

Keywords: evolution, *(Brady)rhizobium*, legume, symbiosis, nitrogen fixation

Abbreviations: PBM: peribacteroid membrane, PBS = peribacteroid space

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1. Introduction

Establishment of a nitrogen fixing symbiosis between rhizobia* and legumes is a complex developmental process that involves constant communication between the partners. Legumes respond to bacterial signals, which include plant growth regulators, by producing a specialized organ, the root nodule (Long, 1990; Nap and Bisseling, 1990). Rhizobia invade and colonize the developing nodule tissue and appear to have a number of strategies to either suppress or counteract the normal plant defence response. During nodule formation, rhizobia differentiate into their nitrogen fixing form, the bacteroid. Differentiation of the plant and bacteria involves a change in gene expression in both organisms. In the plant, these changes include the induction of nodulin (nodule specific) gene transcription at various stages of nodule development (Verma et al., 1986; Sanchez et al., 1991). Early nodulins appear to be involved in the infection process. Late nodulins are involved in aspects of nodule metabolism, such as providing reduced carbon for bacteroid nitrogen fixation and assimilating ammonia exported by bacteroids. One late nodulin, leghemoglobin, is largely responsible for transporting oxygen throughout the infected region of the nodule (Appleby, 1984). During nodule development, rhizobia induce the expression of the genes responsible for nodulation (*nod* genes), nitrogen fixation (*nif* and *fix* genes), and microaerobic metabolism (Downie and Johnston, 1986; Kondorosi et al., 1990; Long, 1990, de Bruijn et al., 1990; Gubler and Hennecke, 1986; Earl et al., 1986; Ebeling et al., 1988; Klipp et al., 1988; Bergersen and Turner, 1990).

Surrounding the bacteroids within infected cells of the nodule is a unique plant membrane, the peribacteroid membrane (PBM), that is thought to control metabolite exchange between the plant and bacteria. The PBM is relatively impermeable to various sugars and amino acids that have been tested (Udvardi et al., 1988b; Udvardi et al., 1990) but a dicarboxylate carrier in the PBM facilitates rapid transport of dicarboxylic acids to the bacteroids (Udvardi et al., 1988a; Ou Yang et al., 1990). Nodulin-26 is probably the PBM protein responsible for dicarboxylate transport (Ou Yang et al., 1991). Protein phosphorylation stimulates the rate of malate uptake across the PBM of soybean nodules and this may be important in controlling the bacteroid carbon supply during symbiotic nitrogen fixation (Ou Yang et al., 1991). There is

* In this paper we will use the word rhizobia as a generic term to refer to the three genera: *Azorhizobium*, *Bradyrhizobium* and *Rhizobium*.

also an electrogenic H^+-ATPase in the PBM that could be involved in metabolite transport across this membrane (Udvardi and Day, 1989; Udvardi et al., 1991).

Free-living diazotrophs generally fix nitrogen only under conditions of severe nitrogen limitation. In contrast, nitrogen fixing bacteroids in legume root nodules do not appear to be nitrogen limited. Many of the nitrogen acquisition and assimilation functions that are expressed during nitrogen starvation in rhizobia are not expressed in bacteroids. These include nitrate reductase, glutamine synthetase II, and ammonium transport (Howitt et al., 1986; Carlson et al., 1987; Shatters et al., 1989; de Bruijn et al., 1989). It is intriguing that bacteroids carry out energy-expensive nitrogen fixation despite the apparent absence of nitrogen stress. Uncoupling nitrogen fixation from 'normal' nitrogen metabolism in bacteroids allows most of the ammonia produced by nitrogenase to be exported to the plant. This response is obviously beneficial to the plant. But what do bacteroids gain from this kind of behavior? In other words, why do bacteroids fix nitrogen? In this paper we will consider a number of models that endeavor to answer this question. These models suggest that environmental factors, such as oxygen or carbon deprivation, or pH stress, may have selected for the nitrogen-stress-independent regulation of nitrogen fixation in rhizobia.

2. Regulation of *nif* gene expression in rhizobia

For a free-living diazotroph, such as *Klebsiella pneumoniae*, nitrogen fixation is the organism's ultimate response to nitrogen starvation. We can understand the 'reluctance' of these bacteria to perform nitrogen fixation if we consider the minimum energy requirements of the process:

$$N_2 + 8H^+ + 8e^- + 16ATP \longleftrightarrow 2NH_3 + H_2 + 16ADP + 16Pi$$

In enteric bacteria, regulation of nitrogen metabolism is genetically controlled by a two-component regulatory system encoded by *ntrB* and *ntrC* (Reitzer and Magasanik, 1987). Under conditions of low nitrogen availability, the sensor protein NtrB phosphorylates and thereby activates NtrC, which in turn activates the transcription of several genes involved in nitrogen metabolism (Hirschman et al., 1985; Hunt and Magasanik, 1985). These include the genes encoding glutamine synthetase (GS), nitrate reductase (NR), ammonium transport (Amt), and amino acid uptake and utilization functions (Reitzer and Magasanik, 1987). The nitrogen fixation (*nif*) genes are also under Ntr control

(Gussin et al., 1986). The Ntr system induces *nif* gene expression by promoting the synthesis NifA, which then activates the transcription of the other *nif* genes.

It is not known if rhizobia in nature ever fix nitrogen in the free-living state. However, during symbiosis with legumes, nitrogen fixation is of primary importance. Intriguingly, symbiotic nitrogen-fixing rhizobia do not appear to be nitrogen starved since *ntr*-regulated genes, such as those encoding Amt, NR, and GSII, are not induced in symbiosis (Howitt et al., 1986, Carlson et al, 1987; Shatters et al., 1989; de Bruijn et al., 1989). In bacteroids, the *nif* genes can be induced independently of the Ntr system, a conclusion supported by the observation that *ntrC* mutants are Fix$^+$ (Szeto et al., 1987). This highlights a key difference between rhizobia and free-living diazotrophs. Since nitrogen metabolism cannot keep pace with nitrogen fixation, most of the ammonia produced by nitrogenase is lost to the plant. The ability to induce *nif* genes in an Ntr-independent manner in rhizobia may, in fact, be crucial to the symbiotic existence of the bacteria.

If nitrogen starvation is not the signal that triggers *nif* gene transcription in symbiotic rhizobia, what is? Recent evidence indicates that low oxygen concentrations alone can induce rhizobial *nif* and *fix* genes (Fischer and Hennecke, 1987; Ditta et al., 1987; David et al., 1988; Ratet et al., 1989). Different rhizobia appear to have evolved different mechanisms for inducing *nif* and *fix* gene transcription during microaerobiosis. For example, *Rhizobium meliloti* employs a two-component regulatory system consisting of an oxygen sensor, FixL, and a putative transcriptional activator, FixJ (David et al., 1988). Under conditions of microaerobiosis, FixL phosphorylates and thereby activates FixJ, which in turn activates transcription of the *nif* regulatory gene *nifA*. The NifA protein then induces transcription of all other *nif* and *fix* genes. In *Bradyrhizobium japonicum*, regulation of *nifA* appears to be somewhat different. Although homologs of FixL and FixJ exist, the NifA protein itself appears to be the oxygen-responsive gene regulator (Fischer and Hennecke, 1987). Under microaerobiosis, NifA is active and positively regulates the expression of *nif* and *fix* genes, including *nifA* itself. During aerobiosis NifA is inactive and no NifA-dependent genes are expressed. There is, however, a basal level of *NifA* transcription under these conditions. In *Azorhizobium caulinodans*, the *nifA* promoter is also regulated by oxygen and *NifA*-mediated autoregulation has been suggested (de Bruijn et al., 1988; Ratet et al., 1989). In other respects, however, regulation of *nif* gene expression in *A. caulinodans* appears to be different. Regulation of *nifA* is mediated in part by *ntrB/ntrC* and in part by another two-component regulatory system, *ntrY/ntrX*, that is different from *fixL/fixJ* (Pawlowski et al., 1991).

3. Why Do Bacteroids Fix Nitrogen?

Why do bacteroids fix nitrogen if they do not utilize the ammonia that is produced? A number of models have been proposed in recent years in attempts to answer this question. One model relates nitrogen fixation to oxygen starvation. Others link nitrogen fixation to carbon acquisition from the host and yet other models suggest that nitrogen fixation is not a response to metabolic requirements of the bacteria, but rather a mechanism to subvert host defense responses. We will discuss each of these models in turn.

Regulation of oxygen influx into legume root nodules

An apparent paradox exists in biological nitrogen fixation by obligate aerobes such as rhizobia: oxygen is required for oxidative phosphorylation to produce the ATP needed for the nitrogenase-catalyzed reaction, yet even relatively low concentrations of oxygen inactivate nitrogenase. The dilemma is resolved by carrying out nitrogen fixation in a narrow 'window' of low oxygen concentration within which concentrations of free-oxygen are high enough to support oxidative phosphorylation but are too low to damage nitrogenase.

In legume-*(Brady)rhizobium* symbioses, this 'window' is provided by the interaction of three components of the root nodule: the oxygen sink provided by bacteroid metabolism, leghemoglobin which facilitates the transport of oxygen in the nodule and buffers the free oxygen concentration, and a variable diffusion barrier that controls the entry of oxygen into the nodule. As a result of this interaction, the steady-state concentration of free oxygen in legume root nodules lies within the 3–30 nM range (Layzell et al., 1990). This is approximately five orders of magnitude lower than the ambient oxygen concentration (260 μM).

Legume root nodules possess a layer of closely packed parenchyma cells that surround the infected cells and appear to restrict oxygen diffusion into the regions of the nodule that contain nitrogen-fixing bacteroids (Layzell et al., 1990). This barrier is especially interesting because its resistance to diffusion of gasses can be adjusted (Witty et al., 1984). Treatments that inhibit nitrogenase activity, nitrogen metabolism, or carbon metabolism within the nodule decrease the permeability of the diffusion barrier (Layzell et al., 1990). It has been suggested that the length of the water-filled diffusion pathway around several layers of tightly packed cells determines the resistance to oxygen diffusion into the nodule (Hunt et al., 1988) and that treatments that alter cell turgor may change this resistance. Nitrogen fixation and subsequent nitrogen metabolism may play such a role by increasing the osmotic potential

either directly (by altering the levels of N-containing compounds) or indirectly (by changing metabolic energy available for processes such as starch synthesis from sucrose). Ammonia produced by bacteroid nitrogen fixation may decrease the resistance of the oxygen-diffusion barrier (Layzell et al., 1990; see Fig. 1), resulting in an increased flux of oxygen into the infected zone and,

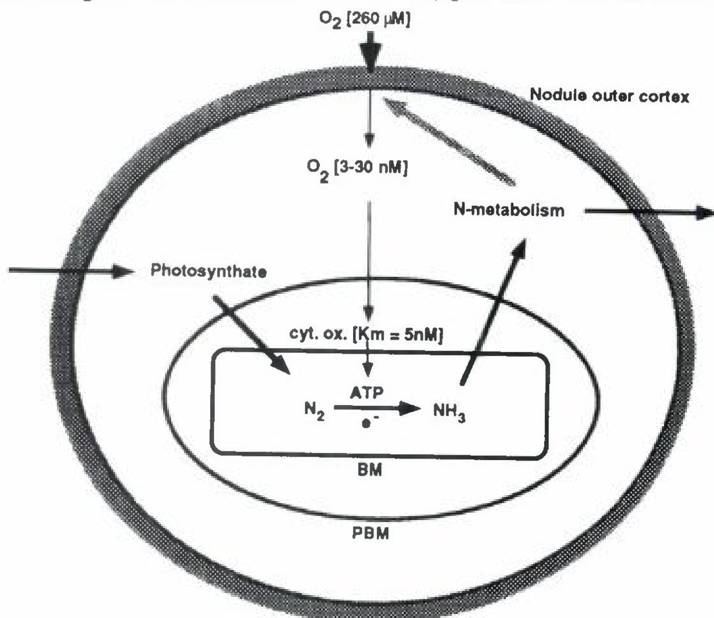

Figure 1. Diagram of a legume root nodule showing how nitrogen fixation by bacteroids may increase the rate of oxygen supply by the plant. The mechanism by which nodule nitrogen metabolism leads to an increase in the flux of oxygen through the nodule outer cortex is unknown.

ultimately, the bacteroids. The release of ammonia by bacteria can influence fungal metabolism (Howell et al., 1988) and it is not difficult to see that it could also affect roots. If rhizobia were oxygen-starved during primitive infections of legume roots, ammonia release as a result of amino acid catabolism or nitrogen fixation might have been an adaptation to increase the availability of oxygen for bacterial metabolism in rudimentary nodules.

The coupling of bacteroid oxygen supply to the export of fixed nitrogen (by the imposition of a variable diffusion barrier in the parenchyma) may represent a plant strategy that prevents bacteroids from becoming parasitic (Layzell et al., 1990).

Nitrogen fixation and the supply of carbon compounds

Other models that attempt to explain why bacteroids fix nitrogen have arisen by considering the symbiosis from the plant's point of view. One of these was proposed by Kahn et al. (1985). They argued that the plant had to protect itself from 'cheaters' – bacteroids that don't fix nitrogen. Such bacteria would arise at significant frequency by mutation and, if they could divert energy from nitrogen fixation to growth, would prosper and ultimately become dominant unless the plant linked nitrogen fixation to the supply of some vital nutrient, such as reduced carbon. In the simplest case, Kahn et al. (1985) proposed that the plant supplies the bacteroid with a compound that contains both N and C, such as the amino acid glutamate. In this way, bacteroid carbon metabolism is coupled to plant nitrogen metabolism (Fig. 2). Since nitrogen is continuously

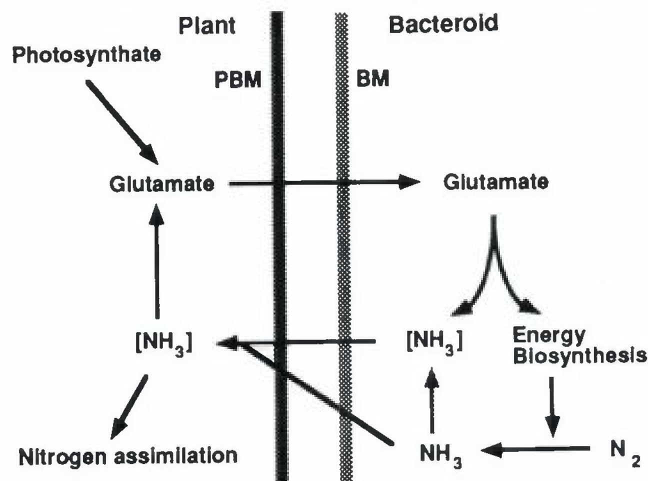

Figure 2. Model of nutrient exchange between plant and bacteroid, proposed by Kahn et al. (1985).

dispatched to the rest of the plant, maintenance of the nodule amino acid pool requires fixed nitrogen from the bacteroid. Thus, the supply of reduced carbon for bacteroid metabolism requires bacteroid nitrogen fixation. A more elaborate version of the model, which involved the operation of a malate-aspartate 'shuttle' was also presented (Kahn et al., 1985). In this model the supply of reducing equivalents (NADH), but not carbon per se, is dependent on bacteroid nitrogen fixation (Fig. 3). Although a number of laboratories have considered aspects of the malate-aspartate shuttle, it is still not clear if the shuttle operates across the symbiotic membranes (the PBM and the bacteroid membrane) of root nodules. Consistent with the model, Rastogi

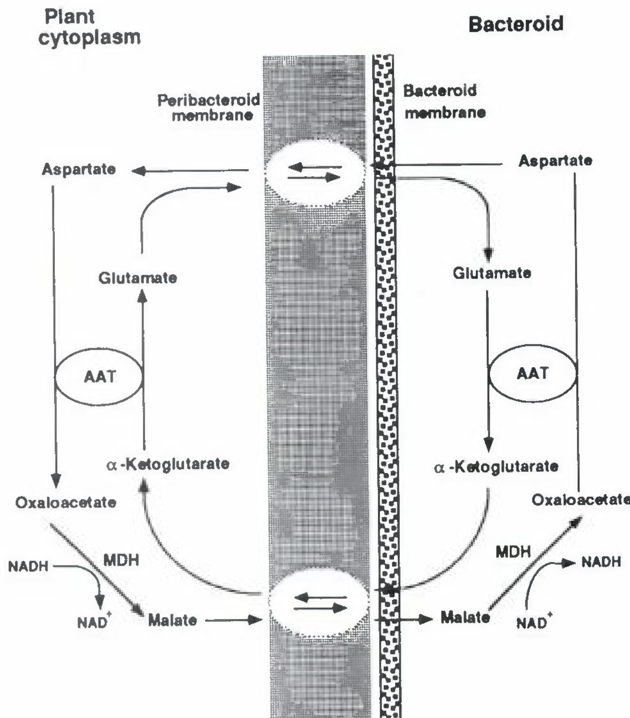

Figure 3. The malate-aspartate shuttle as it may exist in legume root nodules.

and Watson (1991) have recently shown that a bacterial aspartate aminotransferase is necessary for symbiotic nitrogen fixation by *Rhizobium meliloti.* Furthermore, plant aspartate aminotransferases are induced in the developing nodule (Vance et al., 1990). However, the shuttle requires carriers for amino acids in both the PBM and the bacteroid inner membrane. Although such carriers exist in the bacteroid membrane, no such carriers have yet been found in the PBM (Udvardi et al., 1989; Udvardi et al., 1990).

Layzell et al. (1990) proposed a different model for nitrogenase-dependent carbon supply to the bacteroid, based on transport studies of the PBM (Udvardi et al., 1988a; Udvardi and Day, 1989). They argued that if oxygen concentration limited energy metabolism in the plant fraction of the infected zone, then cytosolic ATP concentrations would be relatively low. Udvardi and Day (1989) had previously suggested that the PBM H^+-ATPase, which pumps H^+ into the peribacteroid space (the region between the PBM and the bacteroid membrane), may be required for charge balance during dicarboxylate

anion transport through the PBM to the bacteroid. Elaborating upon this idea, Layzell et al. (1990) proposed that by fixing nitrogen, and thereby increasing both oxygen influx into the nodule (see previous section) and ATP concentration, the bacteriod could assure itself of a reduced carbon source in the form of dicarboxylic acids (Fig. 4). Ou Yang et al. (1991) have recently

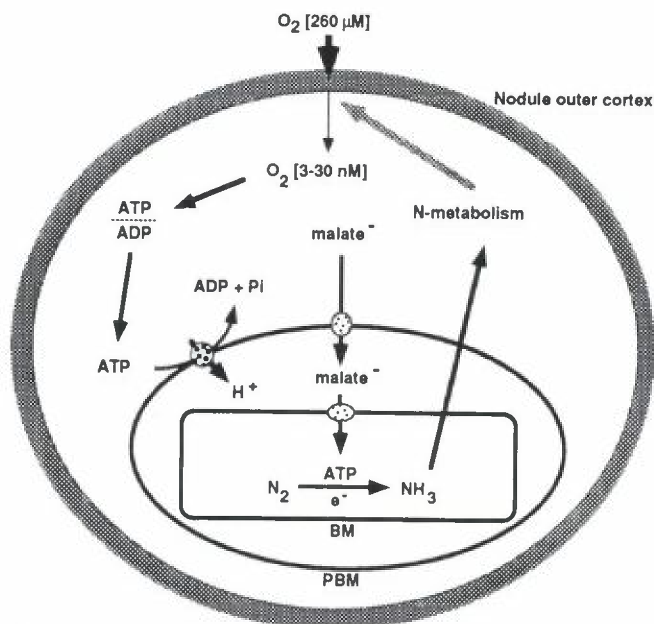

Figure 4. Diagram of a legume root nodule showing how nitrogen fixation by the bacteroid may enhance the rate of dicarboxylate (malate$^-$) transport across the peribacteroid membrane (PBM) by increasing oxygen flux into the nodule and thereby increasing energy charge (ATP concentration) in the plant cytoplasm.

shown that ATP-dependent phosphorylation of PBM proteins stimulates dicarboxylate transport across the PBM of soybean nodules. Therefore, oxidative phosphorylation in the plant fraction may directly control carbon supply to bacteroids by modifying the activity of the PBM dicarboxylate carrier.

We have formulated a third model linking bacteroid nitrogen fixation to carbon metabolism in the plant. Nodule cytosol dark CO_2 fixation, catalyzed by phosphoenol pyruvate (PEP) carboxylase, appears to play a central role in the supply of dicarboxylic acids such as malate, fumarate, and succinate to the bacteroids (Rosendahl et al., 1990). Furthermore, PEP carboxylase activity in legume nodules provides carbon skeletons for ammonia assimilation and export. Addition of ammonia to anaerobic cells of the green alga,

Selenastrum minutum, resulted in a three-fold increase in TCA cycle CO_2 efflux and an eight-fold increase in the rate of CO_2 fixation via PEP carboxylase (Vanlerberghe et al., 1989). Both observations are consistent with increasing carbon flow through TCA cycle enzymes to supply intermediates for amino acid biosynthesis. Much of the CO_2 fixed by PEP carboxylase under these conditions accumulates in succinate. In contrast, under dark aerobic conditions no CO_2 is fixed into succinate or fumarate and only a small amount is fixed into malate. The results obtained under anaerobic conditions suggest the operation of a partial reductive TCA cycle from oxaloacetate to malate, fumarate, and succinate. Such a pathway may contribute redox balance to support partial oxidative TCA cycle activity unde these conditions (Vanlerberghe et al., 1989). If the oxygen-starved, infected cells of legume root nodules behave in the same way as anaerobic *S. minutum* cells, then ammonium production by bacteroids might be expected to stimulate the synthesis of malate, succinate, and fumarate. Since these are the only carbon compounds known to be rapidly transported across the PBM (Day et al., 1990), nitrogen fixation by bacteroids could be seen as an adaptive response by rhizobia to assure an adequate supply of dicarboxylic acids for growth and multiplication.

Coping with an acid environment

Bacteroids in legume root nodules inhibit a compartment (delineated by the PBM) that, in some respects, resembles the interior of a lysozome (Mellor, 1989). A variety of hydrolytic enzymes, including acid proteases, acid trehalase, and α-mannosidase, have been found in the peribacteroid space (PBS), the region between the PGBM and the bacteroid (Mellor, 1989). Furthermore, transport of H^+ (Udvardi et al., 1991) and dicarboxylic acids (Udvardi et al., 1988a) across the PBM has the potential to acidify the PBS. Clearly the plant has raised the menace of acid-activated enzymatic degradation over the potential pathogen. Brewin et al. (1990) have proposed two mechanisms by which bacteroids may counteract acidification of the PBS. First, by taking up dicarboxylic acids and metabolizing them, bacteroids might eliminate that danger. The second mechanism involves NH_3 synthesis by the bacteroids and subsequent export of NH_4^+ across the PBM to the plant cytoplasm (Fig. 5). For this mechanism to operate there has to be an NH_4^+ carrier in the PBM. This does not appear to be the case (Udvardi and Day, 1990). However, the nitrogenase-catalyzed reaction itself consumes at least 8 H^+ per N_2 fixed. Thus, nitrogen fixation per se may be a substantial sink for protons pumped into the PBS by the plant. Whatever the mechanism for removing protons from the PBS, it is

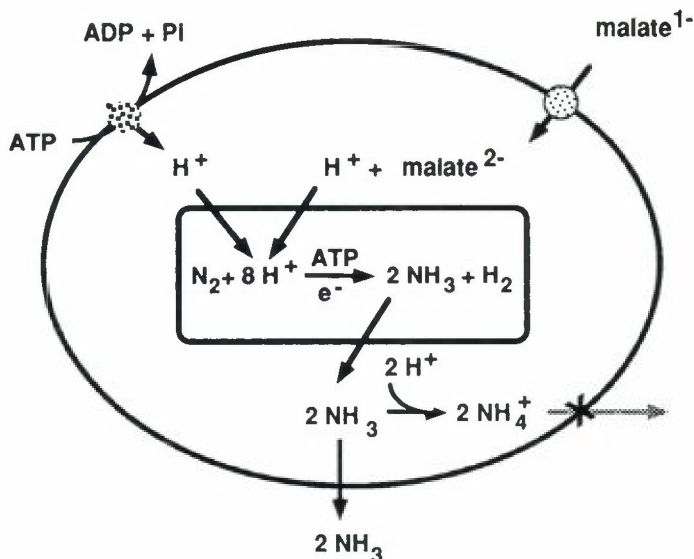

Figure 5. Diagram showing how bacteroid nitrogen fixation and subsequent export of NH_3 may counteract reactions that tend to acidify the peribacteroid space (PBS).

clear that a dynamic equilibrium must exist in order to hold the pH of this compartment at a level suitable for bacteroid survival.

4. Conclusion

The genes for nitrogen fixation (*nif* and *fix*) in rhizobia are regulated differently than those of free-living diazotrophs. The most significant difference is that transcription of the regulatory gene *nifA* in rhizobia can be activated not only by the *ntr* system, as in free-living bacteria, but also by a second mechanism not related directly to nitrogen stress. This second mechanism responds to oxygen starvation and allows nitrogen fixation in the absence of significant nitrogen metabolism. Such 'novel' regulation in rhizobia makes them particularly well suited to perform symbiotic nitrogen fixation. Indeed, we believe that evolution of nitrogen-stress independent activation of *nifA* may have provided the key to a symbiotic existence for these organisms.

A number of models that attempt to explain why bacteroids fix nitrogen, even though they are not nitrogen starved, have been discussed. The predictions made by these models are providing the impetus for some interesting research into the true nature of symbiotic nitrogen fixation.

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