PHYSIOLOGY, BIOCHEMISTRY, AND GENETICS OF *AZOSPIRILLUM* AND OTHER ROOT-ASSOCIATED NITROGEN-FIXING BACTERIA

Author: **Fábio de Oliveira Pedrosa**
Department of Biochemistry
Universidade Federal do Paraná
Curitiba, Paraná, Brazil

Referee: M. G. Yates
A.R.C. Unit of Nitrogen Fixation
University of Sussex
Falmer, Brighton, Sussex, England

I. INTRODUCTION

The advent of the acetylene reduction assay[1,2] greatly facilitated the discovery and study of new root-associated nitrogen-fixing bacteria.

Nitrogen-fixing organisms or diazotrophs[3] found associated with the roots of plants belong to several genera and display varied physiological behavior. The association between a diazotroph and a plant root is called "diazotrophic rhizocoenosis",[4] which means "associative nitrogen fixation". In diazotrophic rhizocoenosis, there is none or only small morphological and physiological modification of each partner, although some mutual benefit can be demonstrated.[5-10]

Diazotrophs found associated with plant roots are organisms of typical free-living behavior[11] and, as such, seem to retain their incapacity for excreting the product of nitrogen fixation, NH_4^+, directly to the host or the environment. This may explain, provided an ample supply of carbon substrate is available, the low levels of ^{15}N-isotope incorporated immediately into plant biomass.[5,12-14] No evidence was found for direct transport of fixed nitrogen from the diazotroph *Azospirillum brasilense* to the host *Setaria italica*. Instead a slow transfer occurred, suggesting a gradual death of bacteria and a subsequent assimilation of their mineralized nitrogen by the host.[14] This situation contrasts with the observed, immediate excretion of NH_4^+ by symbiotic diazotrophs such as rhizobia.[15]

In several diazotrophic rhizocoenosis, however, the ^{15}N-isotope dilution technique, which measures long-term incorporation, has indicated that up to 30% of the total nitrogen may be derived from biological nitrogen fixation.[8,16-19]

Several authors believe that the stimulatory effects of associated diazotrophs, *Azospirillum* and *Azotobacter*, on plant growth and yield are not due exclusively to nitrogen fixation but to other factors such as phytohormones[20] and pectinolytic enzymes[21,22] produced by the bacteria. For review and discussion, see References 6, 9, 10, and 23.

II. DIAZOTROPHS FOUND ASSOCIATED WITH PLANT ROOTS

The great majority of N_2-fixing bacteria found associated with plant roots and rhizosphere soil belongs to the families Azotobacteriacea, Bacillaceae, Enterobacteriaceae, Pseudomonadaceae, and Spirillaceae. Most species of diazotrophs have been found associated with roots of grasses (Gramineae),[5-7,24,25] although representatives have been isolated from the surfaces of nodules and roots of legumes[26] and from a variety of other plants species, including mono- and dicotyledoneae.[27-29] These root associations between diazotrophs and plant roots have also been described as associative symbiosis,[30] nonsymbiotic N_2-fixing associations,[6,31] and diazotrophic rhizocoenosis.[4] Usually more than one diazotroph can be found associated

with the same plant,[6] and the predominant organism may reflect trends imposed by the composition of the enrichment-selection media and other experimental conditions used in the isolation procedure.[31] A procedure called the "spermosphere model" uses exudates from germinating seeds as nonbiased carbon sources for enrichment, and isolation of the most probable rhizocoenotic diazotrophs has been proposed to alleviate part of these problems.[32] In this system, exudate composition will probably define which organisms will have their growth favored.

A. Azotobacteraceae

These strict aerobes fix nitrogen under air, although they may benefit from low oxygen tensions.[3]

The classical example of a highly specific diazotrophic rhizocoenosis is that involving *Azotobacter paspali* and tetraploid cultivars of *Paspalum notatum*.[33,34] ^{15}N incorporation studies have demonstrated unequivocal N_2-fixation by this association.[12] Recent estimates, based on the ^{15}N-isotope dilution method, suggest that up to 10% (or 20 kg N/ha/year) of the total N incorporated by *P. notatum* may be derived from root-associated nitrogen fixation.[8] Other less well-characterized possible rhizocoenosis between azotobacters and wheat, cotton, and legumes;[35,36] *Azotobacter chroococum* and *Eragrostis ferruginea*,[37] maize and sorghum;[38,39] and *A. vinelandii* and *Melilotus officinales, Elmyus angustus, Agropyron trichophorum*, maize, and sugar cane[40,41] have been reported. The presence of *A. vinelandii* in sugar cane roots has been dismissed, although it may occur in rhizosphere soil.[42]

Beijerinckia spp. have been reported to be preferentially enriched in the rhizosphere of sugar cane in Brazil.[43,44] The organisms were found to be mainly associated with the root surfaces.[43] Other researchers were unable to find *Beijerinckia* spp. associated with sugar cane roots in Egypt and also in Brazil.[42] These organisms also occur in greater numbers in the rhizosphere of forage grasses.[45,46]

Derxia gummosa has been frequently isolated from grass roots in the tropics.[47,48] This organism, in contrast to the other azotobacters, has the ability to grow autotrophically using H_2 as an energy source.[49] The extent and importance of these rhizocoenosis to tropical agriculture is not known.

B. Bacillaceae

These include facultative anaerobes (*Bacillus*) and anaerobes (*Clostridium*), all expressing diazotrophy under strict anaerobic conditions.[3] *Bacillus polymyxa, Bacillus* spp., and *B. macerans* have been isolated from the rhizosphere of several gramineae, including forage and grain crops.[18,42,50-54] Rennie and Larson[18] reported strong evidence for a diazotrophic rhizocoenosis between a *Bacillus* spp. and spring wheat cultivars resistant to root rot. A new species, *B. azotofixans*, has been isolated from soil and unsterilized or surface-sterilized macerated roots of different gramineae, including wheat, sugar cane, and *Brachiaria ruziziensis*.[55] The original *B. polymyxa* strain Hino appears to be a variant of a *B. azotofixans*.[56]

Obligate diazotrophic anaerobes, such as *Clostridium*, have not been commonly identified from the rhizosphere. There are, however, early reports on the establishment of these organisms in the root zone of wheat, maize, tomato, and lucerne.[24] *Clostridium*-like anaerobes, however, have been shown to be stimulated by the rhizosphere of several wheat varieties, although they also occurred in those of maize and sorghum.[38] Further studies are required to evaluate the significance of these apparent associations. A nitrogen-fixing organism identified as *Clostridium butyricum* was isolated from tussock-grassland soils dominated by *Festuca novae-zelandiae*.[54]

C. Enterobacteriaceae

Diazotrophic strains of *Enterobacter cloacae, E. agglomerans, Erwinia herbicola, Klebsiella pneumoniae, K. planticola, K. terrigena*, and *K. oxytoca* and strains of unidentified

Enterobacteriaceae have been isolated from the roots and rhizosphere soils of several plants including forage grasses, wheat, sorghum, rice, sugar cane, legumes, and various other species.[26,32,41,42,51,53,54,57-67] The actual sites where these diazotrophs associate to the root system and their significance remain to be determined.

Recently, evidence for micronodule formation by *K. oxytoca* and two strains of *Klebsiella* spp. on rice seedling roots was obtained by scanning electron micrography.[67] The authors claimed that the micronodule consisted of aggregated bacteria surrounded by a membrane structure and was found to be strongly associated with the roots.[67]

K. oxytoca is a common organism in the rhizosphere of rice[32,68] and, according to Postgate[3] and Ladha et al,[59] is the species to which *K. pneumoniae* strain M5al, the best-studied diazotroph, should belong. The recent demonstration that the nitrogen fixation (*nif*) genes of *K. oxytoca* NG13 and those of *K. pneumoniae* M5al show only a 4% difference in their base sequence[68] supports the above classification. *K. pneumoniae* M5al may eventually prove to be a rhizocoenotic diazotroph.

D. Pseudomonadaceae

Several strains of *Pseudomonas* spp. capable of fixing nitrogen have been isolated from rice,[65,69] wild rice (*Oriza punctata* and *O. australiensis*), an aquatic weed (*Monochoria vaginalis*),[70] and from *Deschampia ceaespitosa*.[71] The earlier belief that *Pseudomonas* spp. were unable to fix nitrogen but able to simulate it by behaving as efficient, fixed-nitrogen scavengers[72] may have to be abandoned, especially now with the recent demonstration of nitrogen fixation by a certified strain, *Pseudomonas saccharophila* Doudoroff ATCC 15946,[73] and the studies on lipopolysaccharide composition on the N_2-fixing *Pseudomonas* of *D. caespitosa*.[71] These organisms are microaerophiles when fixing nitrogen in N-free semisolid medium.[71,73] Diazotrophic growth may require small amounts of yeast extract.[73]

E. Spirillaceae

Three genera of microaerophilic diazotrophs, *Azospirillum*, *Herbaspirillum*, and *Campylobacter* are found intimately associated with plant roots. The association between *Azospirillum* spp. and roots of several gramineae species is the best known of all diazotrophic rhizocoenosis. Aspects of microbiology, ecology, plant infection, specificity, inoculation, and agronomy of this rhizocoenosis are reviewed elsewhere.[4-6,9,10,25,74-79]

Four species of *Azospirillum*, *A. brasilense*, *A. lipoferum*,[76] *A. amazonense*,[77] and *A. halopraeferans*,[78] have been found in rhizosphere soil and also closely associated with roots of gramineae, including forage grasses, sorghum, wheat, maize, corn, and rice, from both temperate and tropical regions.[79] These organisms are typical aerobes capable of fixing nitrogen only under microaerobic conditions.[76,80,81]

Herbaspirillum seropedicae represents a new genus of microaerobic diazotroph found in rhizosphere soil and roots of gramineae.[82] DNA and rRNA homology studies have confirmed morphological and biochemical differences between *H. seropedicae* and *Azospirillum* spp.[82-84]

A microaerophylic nitrogen-fixing organism was found associated with the roots of cord grass (*Spartina alterniflora* Loisel), identified as *Campylobacter* sp.,[85] and assigned to a new species *Campylobacter nitrofigilis*.[86]

III. PHYSIOLOGY AND BIOCHEMISTRY

The ability to fix nitrogen and grow diazotrophically is found in organisms of most diverse physiological behavior. This capacity is found among anaerobes, facultative anaerobes, obligate aerobes, autotrophs, heterotrophs, mixotrophs, photoautotrophs, photoorganotrophs, hydrogen oxidizers, and methanotrophs.[3]

In this section, physiological aspects related to the expression and functioning of nitrogen fixation in rhizocoenatic diazotrophs are covered. These organisms are considered to be free-living diazotrophs since they express nitrogen fixation in culture or independently of a host.[5]

In order to fix nitrogen, an organism must be present in a favorable environment. This environment should supply nutrients and conditions capable of promoting and supporting synthesis and activity of the nitrogen-fixing complex, nitrogenase. Factors essential for nitrogen fixation to occur include sources of energy (ATP and reductant), molybdenum, iron, and sulfur, the absence of oxygen and hydrogen at the nitrogenase site, absence of fixed nitrogen (NH_4^+), appropriate temperature, and of course the presence of dinitrogen (N_2) and nitrogenase. Furthermore, a system for ammonia assimilation must be present in order to incorporate newly fixed nitrogen (NH_3) into biomolecules. The present day consensus is that the following stoichiometry for the nitrogenase reaction exists:

$$N_2 + 8e^- + 8H^+ + 16ATP \rightarrow 2NH_3 + 16ADP + 16\ PO_4^{3-} + H_2$$

Nitrogen fixation is an energy-demanding process, expending 4 electron pairs and 16 ATP to reduce N_2 and NH_3 with concomitant production of H_2.[3]

Oxygen is detrimental to nitrogen fixation by (1) irreversibly inactivating the enzyme, (2) reversibly inactivating nitrogenase by discharging the electron donors to nitrogenase (autoxidation of flavodoxins and ferredoxins) or by promoting conformational changes in nitrogenase, and (3) by repressing nitrogenase synthesis. Aerobic diazotrophs have developed mechanisms to exclude O_2 from the nitrogenase site.[87-89]

Molybdenum, sulfur, and iron are elements essential to nitrogen fixation since they participate in the electron transfer metal-sulfur clusters of nitrogenase and ferredoxins.[3]

Hydrogen competitively inhibits nitrogen reduction by nitrogenase, and a buildup of H_2 at the nitrogenase site may severely depress N_2 reduction.[87] Although highly diffusible and considered unlikely to accumulate at the nitrogenase site,[90] most aerobic diazotrophs, nevertheless, possess a safeguard enzyme, H_2-uptake hydrogenase, to reoxidize the H_2 produced by nitrogenase activity.[87,91]

Fixed nitrogen, mainly NH_4^+, effectively repressess nitrogen fixation in free-living diazotrophs.[92] In photosynthetic bacteria such as *Rhodospirillum rubrum, Rhodopseudomonas capsulata,* and *R. palustris*, addition of NH_4^+ to actively nitrogen-fixing cultures promotes immediate, reversible inactivation of nitrogenase by a process called nitrogenase "switch-off".[92,93] *A. brasilense* and *A. lipoferum* behave similarly.[94,95]

A. Energy Metabolism

Root-associated nitrogen-fixing bacteria obtain the energy required for growth and nitrogen fixation from the oxidation of carbon compounds or hydrogen. These oxidations supply nitrogenase with reducing power and ATP.

1. Carbon Metabolism

a. Carbon Sources

Azospirillum spp. are typical aerobes with weak or no fermentative ability.[76] Organic acids (malate, lactate, pyruvate, and succinate) are the preferred carbon sources for all four species.[76-78,80,81]

Carbohydrates are utilized in a species-specific manner: *A. amazonense* is able to grow diazotrophically on disaccharides such as sucrose,[77] which is not utilized by the other species. *A. lipoferum* grows on D-glucose and D-fructose while *A. brasilense*,[76] *A. halopraeferans*[78] do not utilize D-glucose. Additional informations on carbon sources for diazotrophic growth of *Azospirillum* spp., *E. agglomerans, K. pneumoniae, Pseudomonas* sp. DC, and *H. seropedicae* are found elsewhere.[30,49,62,76,77,80,82,96-101]

Table 1
PATHWAYS FOR CARBOHYDRATE METABOLISM BY *AZOSPIRILLUM* spp.

	Carbon source			
	D-Glucose	D-Fructose	D-Gluconate	Sucrose[a]
A. brasilense	(−)	EMP	ED	(−)
A. lipoferum	EMP ED	EMP ED	ED	(−)
A. amazonense	ED	ED	ND	ED
A. halopraeferans	ND	ND	ND	(−)

Note: EMP = Embden-Meyerhof-Parnas or glycolytic pathway; ED = Entner-Doudoroff pathway; (−) = no growth; ND = not determined.

[a] After hydrolysis to D-glucose and D-fructose by β-D-fructofuranosidase.

All metabolizable carbon sources are capable of supporting diazotrophic growth. The apparent exception is α-ketoglutarate, which, according to Das and Mishra,[102] will only support *A. brasilense* growth in the presence of NH_4^+. This suggests that a symport-like system may operate in the simultaneous transport of α-ketoglutarate and NH_4^+ in *A. brasilense*. However, these results seem to contradict those of Martinez-Drets et al.,[99] who found that cells of *A. brasilense* grown on NH_4^+ plus D-fructose and α-ketoglutarate failed to assimilate ^{14}C-α-ketoglutarate. At least one point requires clarification: D-fructose may have had a repressible effect on the induction of the α-ketoglutarate transport system.

b. Metabolism of Mono-, Di-, and Polysaccharides

Pathways for the metabolism of D-glucose, D-fructose, D-gluconate, L-arabinose, and sucrose have been elucidated in *Azospirillum* spp. *A. brasilense* and *A. lipoferum* possess all enzymes of the Embden-Meyerhof-Parnas (EMP) pathway and *A. lipoferum*, furthermore, has all the enzymes of the Entner-Doudoroff (ED) pathway (Table 1).[97,99,103] *A. amazonense* metabolizes sucrose, D-fructose, and D-glucose via the ED pathway but lacks the EMP pathway.[100] The oxidative branch of the hexose monophosphate pathway (HMP) is regarded as being absent in Azospirilla since the above species lack $NADP^+$-6-phosphogluconate dehydrogenase.[97,99,100,103] The enzymes of the isomerization branch of the latter pathway have not been determined but must be present to supply the cells with pentoses for nucleic acid synthesis.

An interesting observation is evident when one compares the effect of glycolytic and gluconeogenic subtrates on the levels of the enzymes of the EMP pathway in *A. brasilense*. D-Fructose, a glycolytic substrate, induced 6-phosphofructokinase,[99,103] a key irreversible enzyme of the catabolic EMP pathway,[99,103] while repressing fructose 1,6 bisphosphatase,[97] a key irreversible enzyme of the anabolic pathway. Conversely, the gluconeogenic substrates glycerol, lactate, malate, and D-gluconate induced the anabolic phosphatase and repressed the catabolic kinase.[97,99] Unfortunately, these experiments were carried out by three different groups, none of them incorporating all the substrates or measuring both enzymes.

Metabolism via HMP or ED involves initial phosphorylation of the hexose. Glucokinase is present in *A. brasilense*, *A. lipoferum*, and *A. amazonense*.[99,100,103] Hexokinase occurs only in *A. lipoferum*, while fructokinase is restricted to *A. amazonense*.[99,100,103] Since only glucokinase, which is specific for D-glucose, is present in *A. brasilense*, D-fructose phosphorylation was found to occur in this organism via an inducible phosphoenol pyruvate-phosphotransferase system (PEP-PTS),[103,104] which also occurs in *A. lipoferum*.[103] The product of this activity, D-fructose-1-phosphate is further phosphorylated by 1-phosphofructo-

kinase, and enzyme also inducible by D-fructose and found in high levels in *A. brasilense* and in *A. lipoferum*.[99,103] *A. amazonense* cells grown on sucrose lack 1-phosphofructokinase,[100] which suggests that the D-fructose PEP-PTS system may also be absent. This conclusion is, however, premature until this system is investigated using D-fructose grown cells. The inducible D-fructose PEP-PTS system of *A. brasilense* requires a soluble, fructose-specific (Enzyme I, $M_r \sim 80,000$) and a membrane bound (Enzyme II) fraction.[104] This system is specific for D-fructose not acting on D-glucose.[103,104] A similar system for D-glucose is also absent from *A. brasilense* and *A. lipoferum*.[103,104] *A. brasilense*, although possessing pathways capable of metabolizing D-glucose, fails to do so due to its inability to transport ^{14}C-D-glucose.[99,103] *A. brasilense* may be regarded as cryptic for D-glucose utilization. Poor growth on D-glucose by strains of *A. brasilense* may have been due to membrane leakiness.[76,105]

A. brasilense and *A. lipoferum* metabolize D-gluconate via the ED pathway producing pyruvate and glyceraldehyde-3-phosphate.[97,99,103] The enzymes involved, gluconokinase-6-phosphogluconate dehydratase and 6-phospho-2-keto-3-deoxygluconate aldolase, are constitutive in *A. lipoferum* cells grown on D-glucose and D-fructose[99,103] and in *A. amazonense* cells grown on D-sucrose,[100] whereas in *A. brasilense*, the former two enzymes are inducible by D-gluconate.[97,99] *A. amazonense*, although possessing the enzymes for D-gluconate metabolism, fails to grow on this substrate, possibly for the lack of a specific transport system.[100] This remains to be elucidated.

The absence of the enzyme NAD(P)-D-glucose-6-phosphate dehydrogenase activity in *A. brasilense* cells grown on D-fructose prevents the catabolism of this sugar by the ED pathway.[97,99,103]

Lactate dehydrogenase, pyruvate dehydrogenase, and malic enzyme are required for growth on lactate, pyruvate, or tricarboxylic acid cycle (TCA) intermediates and have been demonstrated in *A. brasilense* and *A. lipoferum*.[97,99,103] In addition, NAD(P)-β-hydroxy-butyrate dehydrogenase activity increased under diazotrophic conditions[106] and followed the content of poly-β-hydroxybutyrate (PHB) of cells.[107]

The products of the EMP pathway (pyruvate), the ED pathway (pyruvate and glyceraldehyde-3-phosphate), and of the depolymerization of PHB after conversion to acetyl-Coenzyme-A are further oxidized by the citric acid cycle which is present in most aerobes and facultative anaerobes.[108]

L-Arabinose is metabolized by *A. brasilense* through an inducible, nonphosphorylated pathway involving the enzymes L-arabinose dehydrogenase, L-arabinolactonase, L-arabinonate dehydratase, 2-keto-3-deoxy-L-arabinonante dehydratase, and 2-keto-glutarate semialdehyde dehydrogenase, giving α-ketoglutarate as a final product.[109] This pathway also operates in *P. fragi* and *P. saccharophila*[110-112] and in fast growing rhizobia.[113]

L-Arabinose dehydrogenase was partially purified from L-arabinose grown *A. brasilense*.[114] It had a molecular weight of 175,000 dalton and was specific for substrates having L-arabino configuration at carbons 2, 3, and 4 such as L-arabinose, D-galactose, and D-fucose. The enzyme was inactive on D-ribose, L-fuctose, L-rhamnose, D-mannose, and D-glucose and accepted NAD^+ or $NADP^+$ as co-enzymes.[114]

A. brasilense and *A. lipoferum* can use straw and xylan for diazotrophic growth with ^{15}N incorporation.[101] Both species were found to produce and excrete xylanase.[115] Another extracellular enzyme, pectic lyase or polygalacturonic acid transeliminase capable of degrading pectins, was found to be produced by several *Azospirillum* strains.[22] *Azospirilla* can thus, degrade two major components of plant residues, xylans and pectins, an ability which may play an important role in the survival, nitrogen fixation, and in the establishment of rhizocoenosis.[22,116] *K. pneumoniae*[117,118] and *B. polymyxa*[119] also synthesize and excrete polygalacturonic acid transeliminase and have pectinolytic activity.

Cellulolytic activity is rare among diazotrophs,[3] the only example being that of a marine

spiral-like bacteria found in the Deshayes gland of shipworms.[120] Although unable to decompose cellulose by themselves, diazotrophs including *A. brasilense*, *A. lipoferum*, and *B. macerans* will fix nitrogen and grow on cellulose or wheat straw in combination with *Cellulomonas gelida*, a cellulose decomposer bacteria.[115,121,122] Previously, a strain of *Azospirillum* had been isolated from cellulolytic N_2-fixing mixed cultures.[123] Syntrophic associations between nitrogen fixers and cellulose decomposers with mutual benefits have been known for a long time and have involved *Azotobacter*, *Clostridium*, *Klebsiella*, and *Enterobacter* (see References 121 to 123).

c. Metabolism of PHB

PHB is a reserve polymer commonly found in prokaryotes. Species of *Azotobacter*, *Beijerinckia* and *Derxia*,[124] *Azospirillum*,[76,77,106] *Pseudomonas* sp.,[69] *Bacillus*, *Rhizobium* (*Bradyrhyzobium*), and members of *Chromatiaceae* and *Rhodospirillaceae*[125] have been shown to accumulate PHB. In *A. beijerinckii*, PHB accumulation occurs under conditions of nutrient imbalance,[126] and this accumulation has been proposed as a mechanism whereby NAD(P)H is reoxidized under conditions of O_2-limitation.[127,128]

A. brasilense and *A. lipoferum* accumulate large amounts of PHB under diazotrophic conditions, i.e. grown on N_2 at low dissolved O_2 tension (DOT),[106,129] but less than 1% when grown on NH_4^+ and under air.[106] It was later found that PHB also accumulated in the presence of NH_4^+ provided the oxygen tensions were kept low (0.007 atm).[130,131] These observations were extended by Tal and Okon,[107] who found that two factors, oxygen-limitation in chemostat cultures and a high C/N ratio in late log phase batch cultures, favored PHB synthesis in *A. brasilense*. The authors proposed that there are two different or complementary modes of regulation of PHB synthesis in *Azospirillum*: one is regulated by pO_2 and the other by the C/N ratio exposed to air.[107] Although exposed to air, batch cultures at the end of the exponential growth phase tend to be microaerobic due to the intense respiratory activity of the cell population. This is evidenced by derepression of nitrogenase in late log phase cultures of *A. brasilense* batch-grown aerobically with glutamate.[94] Therefore, although carbon availability is essential, oxygen-limitation seems to be the primary factor in PHB accumulation in *A. brasilense*. In *Azotobacter beijerinckii* chemostat studies, it was found that O_2-limitation triggered PHB accumulation.[126,132] The depression effect of NH_4^+ on PHB accumulation by *A. brasilense*[106,107] is probably due to the conversion of the carbon source to nitrogenous compounds instead of PHB,[107] as previously suggested for *A. vinelandii*.[133]

PHB is a source of carbon and energy which is mobilized under conditions of starvation.[127] Tal and Okon[107] identified several carbon- and energy-demanding processes which could be supported by the metabolization of PHB in *A. brasilense*. These include respiration, cryptic growth, nitrogen fixation, survival, and aerotaxis.[107] PHB-rich cells survived better with stresses caused by desiccation, osmotic shock and pressure, and UV radiation.[107] The role of PHB in supporting nitrogenase activity in *A. brasilense* was first demonstrated by Vargas.[134] The demonstration of H_2-dependent nitrogenase activity in *A. brasilense* was only possible after starving the cells in order to diminish the level of the endogenous energy source[135] or immediately after nitrogenase derepression in cells previously grown on NH_4^+.[136]

d. Tricarboxylic Acid Cycle (Krebs Cycle)

The enzymes of this cycle TCA[108] are present in *Azospirillum* spp.,[97,99,100,103] and are functional as inferred from the utilization of cycle intermediates as sole carbon sources for growth[80] and by ^{14}C-respirometry.[137] This cycle is probably present in the newly discovered rhizocoenotic diazotrophs *H. seropedicae*,[82] *Pseudomonas* sp.,[62,69,71] *A. halopraeferans*,[78] *K. pneumonia*, *E. agglomerans*, *Enterobacter* sp.,[71] and *B. azotofixans*[55] as suggested by their utilization of citric acid-cycle intermediates as sole carbon source.

e. Glyoxylic Acid Cycle

In order to grow on 2-carbon substrates, e.g., ethanol and acetate, organisms require a functional glyoxylic acid cycle.[138] The two key enzymes of this cycle, isocitrate lyase and malate synthase, are found in *A. brasilense*, *A. lipoferum*, and *A. amazonense*,[99,100,103] which are capable of growth on ethanol and acetate.[97,99,100,103]

The diazotrophic strains of *Pseudomonas*[62,65,69] have not yet been studied with respect to their carbon and energy metabolism, although they probably utilize the pathways described for *P. saccharophila*.[139]

f. Carbon Dioxide Utilization and Methanotrophy

A. lipoferum, *D. gummosa*,[49,96,140] *Pseudomonas* sp.[69] are facultative chemolithotrophs capable of growing on CO_2 plus H_2 as sole carbon energy sources. Ribulose bisphosphate carboxylase and H_2-uptake hydrogenase are present in autotrophically grown *A. lipoferum* and *D. gummosa*, and both species can grow diazotrophically under an atmosphere of H_2, CO_2, N_2, and O_2.[49,96,140] *A. brasilense* is unable to grow chemolithotrophically.[49,140,141] There are no reports of this type of behavior in *A. amazonense*, *A. halopraeferans*, *H. seropedicae*, and *B. azotofixans*.

D. gummosa, *A. brasilense*, and *A. lipoferum* were also shown to grow on methane, methanol, or formate as sole carbon source.[96,142] These results, however, require confirmation.

2. Hydrogen Metabolism

H_2-uptake and evolution by diazotrophs involve three enzymes, an H_2-uptake hydrogenase, a reversible hydrogenase, and nitrogenase. (For reviews see References 87, 91, and 143.) H_2-uptake hydrogenases are present in anaerobes, facultative anaerobes, and aerobes and have an oxidative role in vivo. Electrons removed from H_2 by this enzyme are used in the respiratory chain to drive ATP production. This enzyme is found in most diazotrophs and seems to follow nitrogenase activity. It plays an essential role in the recovery of the energy expended by nitrogenase[90,143] as H_2 compulsorily evolved in the process of N_2 reduction.[144,145] H_2-uptake hydrogenase in diazotrophs may function in

1. Recycling of H_2 evolved by nitrogenase
2. Provision of reducing power for substrate reduction by nitrogenase
3. Support of an H_2-dependent respiratory protection of nitrogenase
4. Elimination of inhibitory H_2 from the vicinity of nitrogenase[90,145]

While no evidence was found for the operation of the latter function in *Azotobacter chroococcum*,[90] the other three seem to operate in most diazotrophs.[87,90] In *A. brasilense*, however, H_2-uptake hydrogenase activity failed to protect nitrogenase against O_2-inactivation.[135]

The reversible hydrogenases present in anaerobes and facultative anaerobes enable them to dispose of excess reductant in the absence of terminal electron acceptors other than protons,[91] conditions that may occur anaerobically.

Nitrogenase is capable of evolving H_2 by its ATP-dependent H_2 evolution activity. In the absence of reducible substrates, all nitrogenase activity is committed into proton reduction and H_2 is evolved at a maximum rate. However, in the presence of saturating or even hyperbaric N_2, nitrogenase ATP-dependent H_2 evolution activity will be depressed to a minimum, but nevertheless about 1 mol H_2 would be evolved per 1 mol N_2 reduced.[145]

The H_2:N_2 ratio reflects the efficiency of energy utilization in nitrogen fixation and is dependent on the interactions of several factors including temperature, the functional ratio of the Mo-Fe/Fe proteins and the energy charge or the ATP/ADP ratio.[147] The H_2:N_2 ratio increased at low temperatures, at high Mo-Fe/Fe-protein ratios, and at low ATP/ADP ratios conditions which affect nitrogenase component protein interaction or function.[147]

In *A. chroococcum* and *K. pneumoniae*, the in vivo ratio H_2:N_2 varies under the influence of factors such as pH, temperature,[148] oxygen, carbon substrates, or the limiting nutrient (Mo, S, or Fe),[149] which may affect the functional Mo-Fe/Fe-protein ratio, the rate of electron flux to nitrogenase, or complex formation between the nitrogenase component proteins.[144] Similar behavior is expected to occur in other diazotrophs.

The H_2-uptake hydrogenase activity present in diazotrophically grown *A. brasilense* and *A. lipoferum* is sufficient to recycle all H_2 produced by nitrogenase[129,136,150-152] so that no H_2 evolves from these cultures.

Ni^{2+} is specifically required for synthesis and/or activity of the H_2-uptake hydrogenases of *A. brasilense*, *A. lipoferum*, and *D. gummosa*,[153] as well as for that of *A. chroococcum*.[154] H_2-evolution by N_2-fixing *Azospirillum brasilense* only occurred when the cells were grown in the presence of compounds capable of chelating Ni^{2+}, such as ethylenediamine tetraacetate (EDTA) or ortho-phenanthroline.[153] EDTA presumably repressed hydrogenase synthesis during growth since the enzyme activity in vitro was not inhibited by 3 m*M* EDTA.[153] H_2 evolution was also observed by direct inhibition of the hydrogenase activity with CO or C_2H_2.[150-152] Other inhibitors of *A. brasilense* H_2-uptake hydrogenase are nitrite and nitric oxide.[155] The requirements for Ni^{2+} and the irreversible inhibition by NO suggests that the hydrogenase of *A. brasilense* is probably of the NiFe-type similar to that of sulfate-reducing bacteria.[156] Hydrogenase activity of intact cells of *A. brasilense*, *A. lipoferum*, and *D. gummosa*, grown diazotrophically, will accept as electron acceptors, O_2 and methylene blue.[129,150-153] In addition, nitrous oxide and nitrite serve as electron acceptors for hydrogenase of *A. brasilense* cells grown anaerobically on nitrate or nitrous oxide.[157]

H_2-uptake hydrogenase synthesis in *A. brasilense* is repressed by O_2 but not NH_4^+.[158] As long as the dissolved oxygen tension is kept low (<9 μM), neither exogenous nor endogenous H_2 produced by nitrogenase is required to induce hydrogenase synthesis in *A. brasilense*, although limitation of carbon substrates or electron donors may enhance activity.[136,158] Hydrogenase activity of batch cultures of *A. brasilense* and *A. lipoferum* grown diazotrophically under low DOT[120,150] or of *A. brasilense* grown anaerobically with N_2O or NO_3^- [157] tends to increase significantly at the end of the exponential phase possibly due to carbon source limitation, as is observed to occur for *A. brasilense* hydrogenase under lactate limitation.[136]

A. brasilense H_2-uptake hydrogenase activity is capable of supporting H_2-dependent acetylene reduction by intact cells starved of carbon metabolites.[135] Carbon starvation was essential to demonstrate H_2-dependent acetylene reduction, although carbon substrates did not inhibit H_2-dependent respiration.[120] Nitrogenase activity was not protected by H_2-dependent respiration because the latter was itself extremely sensitive to O_2 inhibition.[135] Tibelius and Knowles,[158] however, presented results which indicated that the O_2 optimum for the H_2-uptake system was significantly higher than that for nitrogenase. Unfortunately, closer examination reveals that their experiments on the effect of oxygen tension (pO_2) on $^3H_2$-uptake and C_2H_2-reduction activities were carried under different shaking rates, 250 and 300 rpm, respectively. Lower O_2 diffusion rates occur at lower shaking rates and therefore a higher pO_2 optimum is expected to occur under these conditions.

The observation that hydrogenase and nitrogenase activities began to express simultaneously[159] during batch growth of *A. brasilense* in medium, containing glutamate under air,[94] suggests that both systems have similar O_2-sensitivities.

The different response of H_2 and malate-dependent respirations to inhibition by KCN and the lack of inhibition of the H_2-dependent respiration by malate suggest that in *A. brasilense* H_2 and carbon oxidation are linked to independent respiratory chains with no commonly shared intermediates.[135] The H_2-linked respiration appears to have a higher affinity for O_2 but is less efficiently coupled to ATP synthesis than carbon-linked respiration.[135,136]

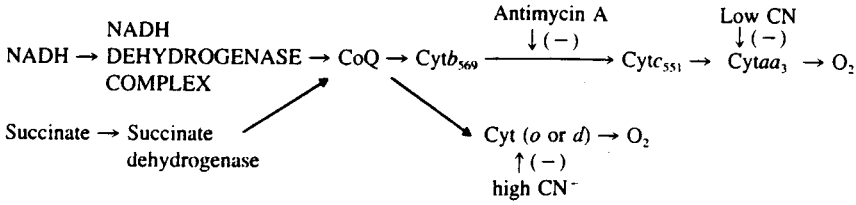
3. Respiratory Chain and Oxidation Phosphorylation

There are few reports on respiratory chains and oxidative phosphorylation of rhizocoenotic

diazotrophs. In *A. brasilense*, the components of the respiratory chain have been determined enzymatically and spectroscopically for cells grown diazotrophically (N_2 plus low DOT) or with NH_4^+ at high DOT.[160-163] Oxygen has a major effect on the composition of the membranes of *A. brasilense*, as revealed by spectroscopic and inhibition studies. *A. brasilense* grown under low DOT contains cytochromes type *b* (λ 559 to 560 nm) and *c* (λ 551 to 553 nm).[160,161] An additional cytochrome, *aa*3 (λ 603 nm), was found in membranes isolated from cells grown at high DOT, which occur only in trace amounts in membranes from low DOT membranes.[161]

Further spectral studies of membrane preparation of *A. brasilense* revealed the presence of two additional terminal oxidases, cytochromes *o* and *d* in cells grown on high and low DOT.[161] The soluble cytochrome *c* of *A. brasilense* has an absorption spectrum with α, β, and γ peaks at 551, 523, and 420 nm, respectively, and its content practically does not change irrespective of the level of oxygen present during growth or whether the strain produces carotenoids.[160,161,164] In addition, various cytochrome *c*-types may be present and show a fused absorbtion maxima at 551 nm.[161] A light membrane fraction from cells grown at high DOT lacks cytochrome *c*.[161] Succinate and NADH oxidases are present in *A. brasilense* membranes and increase with the DOT of the growth media.[130,131]

Oxygen affects the pattern of succinate and NADH oxidation by *A. brasilense* as revealed by inhibition studies.[161] Carbon monoxide inhibited both NADH and succinate oxidase activities of membranes isolated from cells grown at high DOT, but not those from low DOT.[161] Cyanide inhibited both activities irrespective of whether the membrane was from low or high DOT cells, but the inhibition pattern was, however, dependent on the electron donor and the origin of the membrane. NADH oxidase activity showed monophasic inhibition by CN^- for low DOT membranes and biphasic inhibition for high DOT membranes, whereas succinate oxidase activity showed an opposite pattern of inhibition, i.e., biphasic for low DOT membranes and monophasic for high DOT membranes.[161] These results suggest the presence of a branched electron transport chain for NADH oxidation in high DOT membranes and for succinate oxidation in low DOT membranes of *A. brasilense*.[161] Another inhibitor, antimycin A, which blocks electron transfer from cytochrome b_{560} to c_{551}, inhibited NADH oxidase but failed to inhibit succinate oxidase activity in high DOT membranes and had no effect on both activities in low DOT membranes.[161] The authors explain these results assuming that in high DOT membranes NADH and succinate oxidation proceed to oxygen through a branched pathway, with a branch point situated on the substrate site of the antimycin A block (cytochrome $b_{560} \rightarrow$ cyt. c_{551}), and that cytochrome *c* only operates after the antimycin A site on the branch which is sensitive to low CN^- concentrations and terminates with cytochrome *aa*3. The other branch is assumed to be blocked by high CN^- concentration and is the only pathway for succinate oxidation.[161] According to the above results and the probable presence of co-enzyme Q at the branching point, the electron transport chain of *A. brasilense* Cd grown in high DOT membranes should be as follows:



However, there is no experimental evidence to indicate that a carrier-bound electron, located at a branching point would, based only on its origin, follow thereafter a specific branch. Therefore, a possible alternative interpretation is that in high-DOT membranes there are distinct, independent electron transport chains for NADH and succinate oxidation. The

NADH-specific pathway is probably branched and terminates at cytochrome *aa*3 in the branch inhibited by low CN^- concentrations and at cytochromes *o* or *d* for the branch inhibited by high CN^- concentrations. Succinate oxidation would proceed through a pathway possessing antimycin A-insensitive electron carriers and terminate in cytochromes *o* or *d*. Electron carriers, common to both pathways, cannot be shared in order to prevent electron interchanges, which is a precluded state as indicated by the inhibition studies.

For low DOT cells, the biphasic pattern of inhibition by CN^- suggest that there are two branches for electron transport in succinate oxidation.[161] The branch inhibited by high concentration of CN^- is probably that involved in NADH oxidation. Inhibition by antimycin A seems to indicate that NADH and succinate oxidation share common electron carriers, but NADH oxidation has one less terminal oxidase than succinate oxidation, thus suggesting independent pathways as for high DOT membranes. The terminal oxidase for succinate oxidation in low DOT membranes inhibited by low CN^- has an inhibition constant (K_i) similar to that of NADH oxidase inhibition in high DOT membranes.[161] They are apparently the same oxidase cytochrome *aa*3, especially because the other two oxidases cytochromes *o* and *d* are only inhibited by high CN^- concentrations.[162] Further studies are required to complete the picture on electron transport chains in *A. brasilense*.

Kinetic studies on respiration of diazotrophically grown *A. brasilense* disclosed the presence of a very high affinity terminal oxidase with apparent dissociation constant for O_2 (K_s 0.006 μM) regarded as protective oxidase.[165] Two terminal oxidases, K_s 1.5 μM and K_s 28 μM O_2, were found in high DOT membranes of *A. brasilense*.[161] The order of magnitude of these kinetic constants suggests that three terminal oxidases may be present in *A. brasilense*, as indicated by the spectral studies already mentioned.

K. pneumoniae 50231 grown under O_2 limitation, or even when no O_2 was supplied, has a single oxidase showing positive cooperativity and an apparent K_s 0.11 μM O_2.[165] The involvement of cytochrome *o* as an O_2 sensor in *A. brasilense* aerotaxis has been suggested.[162]

There are apparently no estimates of ATP yields by oxidative phosphorylations in *Azospirillum* spp. or any other rhizocoenotic diazotrophs. Some indications of the relative efficiencies for different electron donors or terminal electron acceptors in *A. brasilense* are available. H_2 is less efficient than organic acid as an energy source as reflected by the lower nitrogenase activities at suboptimal O_2-concentrations.[135,136] The terminal electron acceptors O_2 and NO_2^- were three times more efficient than NO_3^- in promoting molar growth yields on malate.[166]

4. Efficiency of Nitrogen Fixation

Nitrogen-fixation efficiency is the amount of nitrogen (milligram) fixed or incorporated into biomass per gram of carbon substrate consumed. It is a complex parameter which gives an idea of the nitrogen-fixation potential of a diazotroph and its usefulness as a biofertilizer. Several factors may affect nitrogen-fixation efficiency of aerobic diazotrophs in general. These are DOT, the carbon source, its availability and preferred metabolic route, temperature and pH, the efficiency of ATP synthesis, the "in vivo" ratio of the nitrogenase proteins (Mo-Fe/Fe protein), the availability of Fe, S, N_2, P, and Mo, the extent of the requirement for maintainance energy, the growth rate, and the presence of hydrogenase.[3,148,149,167] The dissolved oxygen tension affects expression and activity of nitrogenase, the redox state of the electron donors to nitrogenase, the rate of carbon consumption, the chosen respiratory chain for oxidative phosphorylation, the growth rate, and the ratio of functional nitrogenase proteins (Fe/Mo-Fe protein). The energy content of a carbon source depends on its structure and oxidation level and the metabolic routes and their efficiencies in producing ATP and NADH. H_2-uptake by hydrogenase recycles the H_2 compulsorily produced in the nitrogenase-catalyzed reduction of N_2 to NH_3.[144,145] Theoretically, a minimum of 25% of the energy consumed in nitrogen fixation is evolved as H_2[149] and could be recovered by hydrogenase, thus increasing the efficiency of nitrogen fixation.[90,168]

The molar ratio of H_2 evolved to N_2 reduced by chemostat-grown *A. chroococcum* under O_2^- and C-limitation was minimum (ca. 1) under optimum O_2 concentration for nitrogenase activity and increased (up to 8.2) at lower of higher O_2 concentration.[149] The high H_2:N_2 ratios have been interpreted as consistent with a high ratio of functional Mo-Fe/Fe protein (> 1) and low Mg-ATP concentrations.[149]

The functional concentration of the Fe protein is likely to decrease at low O_2-concentrations due to low levels of Mg-ATP and at high O_2 due to "switch-off" in conformational protection or to partial denaturation.[149] Significantly Mo-limited cultures showed no substantial increase in the H_2/N_2 ratio at extreme O_2 levels and these results were interpreted as consistent with an Mo-Fe/Fe-protein ratio <1,[149] a situation where Mo-Fe-protein synthesis is limited while Fe-protein synthesis is unimpaired.[169] The calculated nitrogen fixation efficiencies for the various nutrient-limited chemostat cultures, under optimum O_2 levels, were:

1. O_2-limited: 18 to 32
2. Mo-limited: 25 to 31
3. SO_4^{2-}-limited: 37 to 48
4. C-limited: 49 to 58,

and there was no correlation between efficiency of nitrogen fixation and nitrogenase activity.[149]

Temperature may also affect the functional Mo-Fe/Fe-protein ratio.[147] A high H_2:N_2 ratio implies that less N_2 is actually reduced, and even if an efficient H_2-uptake hydrogenase were present, only a fraction of the energy expended on H_2 evolution would be recovered due to the lower efficiency of H_2-dependent ADP-phosphorylation.[135,168]

Aerobic diazotrophs (aerobic and facultative anaerobes) can metabolize carbon substrates via EMP and/or ED and by TCA and finally oxides NADH and $FADH_2$ produced by the above pathways in an O_2-dependent respiratory chain. Table 2 shows the calculated theoretical maximum energy produced (ATP) and nitrogen-fixation efficiency which could be obtained in the mentioned pathways, assuming that the number of ADP-phosphorylation sites for NADH and $FADH_2$ oxidation were 3 and 2, respectively (colunm A), or 2 and 1 (column B).

The number of ADP-phosphorylation sites present in the respiratory chain is essential in defining the energetic potential of a carbon substrate. In the calculation of the nitrogen-fixation efficiency, the following stoichiometry for the nitrogenase reaction was considered.

$$N_2 + 8e^- + 8H^+ + 16\ ATP \rightarrow 2NH_3 + 16\ ADP + 16\ PO_4^{3-} + H_2$$

Taking 8 electrons as being equivalent to 4 NAD(P)H or 12 ATP, a total of 28 ATP is found to be required. This number is not far from those commonly obtained in in vivo measurements.[3]

These theoretical maximum nitrogenase-fixation efficiency values could be approached if

1. The maintainance energy consumption was kept at a minimum.
2. Most of the catabolic energy was directed toward nitrogenase activity.
3. O_2-dependent carbon consumption was kept at a minimum.
4. All carbon substrates were completely oxidized and very low levels of anabolic activity were present.
5. NH_4^+ assimilation was kept at a level just sufficient to remove inhibitory or repressive levels of NH_4^+ from the system. These conditions could possibly occur under O_2- or C-limitation.

Table 2
THEORETICAL MAXIMUM ATP YIELD AND NITROGEN-FIXATION EFFICIENCY OF VARIOUS CARBON SOURCES

Carbon source	Mol wt	Yield ATP/mol C-substrate		N_2-Fixing efficiency (c) (mg N/g C-substrate)		
		A (a)	B (b)	A (a)	B (b)	
D-Glucose or D-fructose	180	38	26	211	144	(1)
D-Glucose or D-fructose	180	37	25	206	139	(2)
Lactate	90	18	12	200	133	(3)
Pyruvate	88	15	10	170	113	(4)
Succinate	118	20	13	169	110	(5)
Acetate	60	11	7	183	117	(6)
Ethanol	46	17	11	370	239	(7)
Malate	134	18	12	134	90	(5)
α-Ketoglutarate	146	24	16	164	110	(5)
Citrate, isocitrate	195	27	18	141	94	(5)
Oxaloacetate	132	15	10	114	76	(5)

Note: Table calculated assuming that: (a) 1 NADH = 3 ATP and $FADH_2$ = 2 ATP; (b) 1 NADH = 2 ATP and $FADH_2$ = 1 ATP; and (c) 28 ATP equivalents are required to fix 1 mol N_2 (28 g). (1) Metabolized via EMP → pyruvate dehydrogenase complex (PDH) tricarboxylic acid cycle (TCA) → oxidative phosphorylation (OP). (2) Metabolized via ED → EMP → PDH → TCA → OP. (3) Metabolized via lactate dehydrogenase (LDH) → PDH → TCA → OP. (4) Metabolized via PDH → TCA → OP. (5) Metabolized via TCA → malic enzyme → TCA → OP, balanced to complete oxidation. Conversion of any TCA intermediate into acetyl-CoA is assumed to occur via malic enzyme and pyruvate kinase. (6) Metabolized via acetyl-CoA synthetase → TCA → OP. (7) Metabolized via NAD^+-dependent alcohol and acetaldehyde dehydrogenases, and then as in (6).

There has been controversy about the efficiency of nitrogen fixation by *Azospirillum* spp. Initial high values (52 to 116 mg N/g lactate) found for diazotrophs grown in a N-free semisolid medium containing low (0.05%) lactate[30,81] have not been confirmed.[170] The maximal nitrogenase-fixing efficiencies obtained by Okon et al.[80] ranged from 21 to 24 mg N/g carbon for growth on lactate, malate, and succinate, with no marked difference among substrates and their concentrations. Efficiencies in the range of 24 to 29 mg N/g were also found for 17 *Azospirillum* strains grown in malate or succinate both at low, O_2-limiting concentrations (0.05%).[25]

Under similar conditions, *A. amazonense*[77] strains showed efficiencies at about 30 mg N/g sucrose. The ethanol present in NFb medium most probably affected the initial very high nitrogen-fixation efficiencies first observed by Day and Döbereiner.[30,81] Other studies with *A. brasilense* Sp7, grown diazotrophically under controlled DOTs,[170] showed that the oxygen tension had a marked effect on the efficiency as previously shown for *A. chroococcum* and *K. pneumoniae*.[167] A maximum efficiency of about 12 mg N/g carbon occurred at low DOTs (0.002 to 0.003 atm O_2) and decreased to 1.2 to 2.1 at higher DOTs (0.014 to 0.018 atm O_2). At the optimal DOT range for nitrogenase activity and diazotrophic growth (0.005 to 0.007 atm O_2), *A. brasilense* nitrogen-fixation efficiencies were 8 to 10. Extensive studies carried out on the efficiency of nitrogen fixation by *Azospirillum* Sp7 growing in batch and chemostat under controlled O_2 tensions showed that the apparent optimum O_2 tension for

nitrogen fixation was 0.0005 (atm O_2) and gave N_2-fixation efficiencies of 25 to 28.1 mg N fixed per 1 g succinate consumed.[134] These values were similar to those obtained by Neyra and Döbereiner[25] in semisolid medium, which implies that optimum O_2 is reached during growth on N-free semisolid medium with 0.17% agar. Vargas[134] argues that the lower efficiencies found by Okon et al.[170] suggest suboptimal growth conditions, such as high DOTs during growth in semisolid medium containing malate as the carbon substrate.

From Table 2, it is apparent that succinate is theoretically 22 to 26% more energetic than malate, but this difference alone could not explain the higher efficiency with succinate. Low efficiencies (8.8 to 9.4) were found for O_2-limited chemostat cultures of *A. brasilense* Sp7 and *A. lipoferum* Br17 grown with excess lactate and glucose, respectively.[150] However, in batch cultures under controlled dissolved O_2 tensions (0.003 atm) and low carbon concentrations, the efficiencies of N_2-fixation for both species were higher and increased at the end of the exponential phase.[129,150] In *A. lipoferum* Br17 grown on D-glucose (2 g/ℓ), the efficiencies increased from 20 in the early to 48 at the end of the log phase,[129] while in *A. brasilense* grown on L-lactate (1.4 g/ℓ), the efficiency increased from 12 to 92.[150] In both cases, the efficiencies increased at low carbon concentrations as previously suggested.[30,81]

A substantial increase in H_2-uptake hydrogenase activity and in PHB content occurred simultaneously with the increase in N_2-fixing efficiency,[129] indicating that PHB utilization could not explain the high efficiencies observed.

The increase in hydrogenase activity was suggested to explain the increase in N_2-fixing efficiency in *A. lipoferum* and *A. brasilense*.[129,150] This increase, however, as pointed out by Volpon et al.,[129] could not explain the large increase in H_2-fixing efficiencies just by recycling H_2 since even at the lowest hydrogenase levels no H_2 evolved from these batch cultures. In addition, assuming that *Azospirillum* spp. behaves similarly to *A. chroococcum*,[149] the recycling of the H_2 produced by nitrogenase activity would result in the recovery of from 2.5 to 10% of the total energy expenditure of the cell in respiration. Thus only negligible increases in the efficiency of nitrogen fixation would be expected.

The high N_2-fixation efficiencies observed[129,150] are probably the result of tight or preferential coupling of energy-producing pathways to nitrogen fixation under carbon and O_2-limitation. Overall, the efficiencies in the range of 20 to 30 found for the species of *Azospirillum* are in the range usually found for aerobic nitrogen fixers.[11] The nitrogen-fixing efficiency of strain of *H. seropedicae* is in the range of 12 to 15 mg N fixed per 1 gram malate.

Facultative anaerobes and anaerobes fixing nitrogen anaerobically are expected to have lower N_2-fixing efficiencies since they rely on substrate level phospholylation to produce ATP and this seems to be the case. In anaerobic chemostat cultures of *K. pneumoniae*, the introduction of limited O_2 concentrations led to an increase in the molar growth yield and in the efficiency of nitrogen fixation.[167] This has been interpreted as being associated with a general upgrading of the energy status of the cell supported by oxidative phoshorylation.[167,172]

B. Nitrogen Metabolism

Nitrogen metabolism by root-associated diazotrophs is not restricted to N_2 fixation. Diazotrophs are capable of assimilating NH_4^+, and some will assimilate NO_3^- and also denitrify. There are no reports of diazotrophs capable of carrying out nitrification.

1. Nitrogen Fixation

a. Oxygen Protection

Oxygen inactivates and represses nitrogenase synthesis.[92] Aerobic diazotrophs are, therefore faced with the problem of excluding oxygen from the site of nitrogenase and, at the same time, utilizing O_2 as the terminal electron acceptor for ATP production. For this reason, aerobic diazotrophs have evolved mechanisms to protect nitrogenase synthesis and activity

from excess O_2. This subject has been reviewed by several authors.[3,87-89] The suggested protective mechanisms involve: (1) respiratory protection, (2) conformational protection, (3) compartmentalization, (4) enzymatic protection mediated through superoxide dismutase, catalase, and peroxidase, (5) chemical protection mediated by carotenoids,[164] (6) cellular aggregation, (7) extracellular gum protection, and (8) avoidance — passive in anaerobes and active in aerobic diazotrophs mediated by aerotaxis.

Oxygen generally is not a problem for aerobic and facultative anaerobic diazotrophs growing on fixed N, although some microaerophiles such as *Azospirillum* tend to seek regions of decreased O_2 tensions, even when growing in the presence of NH_4^+.[173,174]

The recently described rhizocoenotic diazotrophs include aerobes and facultative anaerobes. Aerobes such as *Azospirillum* spp., *H. seropedicae*, *C. nitrofigilis*, and *Pseudomonas* sp. fix nitrogen under conditions of very low dissolved oxygen concentrations.[30,69,71,77,80-82,86] Actually an essential step in their isolation involves growth on N-free semisolid medium incubated under air or reduced O_2 tension, as for *Azospirillum* spp.[30,81]

Facultative anaerobes (Enterobacteriaceae and Bacillaceae) usually only fix nitrogen anaerobically.[3] However, since these organisms have the potential to respire O_2 and thus increase the efficiency of ATP production, one may expect that under well-controlled low O_2 concentrations they will derepress nitrogenase and grow diazotrophically, as has been demonstrated for *K. pneumoniae*.[167,175] Several strains of facultative anaerobes isolated from plant roots, and belonging to the species of *K. pneumoniae*, *Enterobacter* sp., and *E. agglomerans*, derepressed substantial levels of nitrogenase activity when grown in semisolid N-free medium under air.[61]

Azospirillum spp., among the root-associated diazotrophs, is the best studied in terms of O_2 protection of nitrogenase. Mechanisms that may afford such protection in *Azospirillum* spp. include limited respiration, aerotaxis, conformational change in nitrogenase, and the presence of carotenoids. The respiratory rates of *A. brasilense* are only about 10 to 20% of those of *Azotobacter*. This low respiratory rate suggests that *Azospirillum* spp. cannot effectively remove the excess oxygen present under aerobic conditions, thus their microaerophilic behavior when fixing nitrogen.[30,80,81] Nitrogenase activity of intact cells of *Azospirillum* spp. and *H. seropedicae* show typical bell-shaped curves when measured as a function of oxygen partial pressures(pO_2).[77,80-82,129,135,136]

Optimum pO_2 values are variable, and their comparison should be made carefully since they are affected by factors such as temperature, pressure, flask size and geometry, shaking modes and rates, the ratio of cell suspension volume to the flask volume, which alters O_2-diffusion rates, and also by the cell densities and physiological state (age, respiratory rate).

Diazotrophic growth and nitrogen fixation by *A. brasilense* occur optimally with dissolved O_2 concentrations in the range of 0.6 to 8 μM; concentrations above 11 μM are not permitted.[165,170,178] *A. lipoferum*, *A. amazonense*, and *H. seropedicae* behaved similarly.[77,82,129] These O_2 concentration optima are sufficient to saturate the high affinity terminal oxidases present in *A. brasilense*.[161,165]

Azospirillum spp., grown in N-free semisolid medium, migrates actively to regions of O_2 concentrations compatible with N_2 fixation. This aerotactic response has been quantified and found to depend on O_2 concentrations, age of the culture and incubation time, and to be unaffected by the N source.[173,174] Neither adaptation nor respiratory protection in *A. brasilense* to high O_2 concentration was found,[176] in contrast to those observed in *A. chroococcum*.[177]

Catalase, peroxidase, and superoxide dismutase activities are present in *A. brasilense*.[76,77,131,178] The level of catalase activity was shown to decrease during batch growth of *A. brasilense*; higher oxygenation rates did not reverse this fall.[131,178] That of *A. brasilense* superoxide dismutase, on the contrary, increased with increasing O_2 concentrations.[131,178] It was found in cells grown anaerobically on nitrate or nitrous oxide as terminal electron acceptors and was only slightly affected by the N source.[178] Catalase activity of *A. brasilense*

increased under low O_2 concentrations and was highest in cells grown anaerobically with NO_3^- as the electron acceptor.[178] These results were interpreted as evidence that catalase induction in *A. brasilense* was independent of O_2 and was linked to the synthesis or respiratory proteins (cytochromes and the reductases of nitrogen oxides) required for growth under this condition.[178] One may speculate that under low O_2 tensions catalase may function as an oxygen scavenger capable of recovering minute amounts of O_2 imobilized as H_2O_2.

A. brasilense had very low levels of membrane-bound peroxidase activity (0.03 to 0.08 units/100 mg protein).[178] A single Fe-superoxide dismutase isozyme was reported for *A. brasilense*[178] grown on N_2, NO_3^-, or NH_4^+. Moore et al.,[179] on the contrary, found a single Mn-Fe-superoxide dismutase isozyme in *A. brasilense*, and two Fe-superoxide dismutase isozymes in *A. lipoferum*.

Catalase, superoxide dismutase, and peroxidase may constitute a protective mechanism against oxygen toxicity in *Azospirillum* spp., as in other aerobes.[180,181] However, a specific role of these enzymes in protecting nitrogenase in *Azospirillum* is lacking. Furthermore, a catalase-overproducing mutant of *A. brasilense* Sp7 behaved similarly to the parent strain with respect to nitrogenase inactivation and repression by O_2.[182]

Nur et al.[130,131,164] studied carotenoid composition and synthesis in several strains of *A. brasilense* and concluded that they appear to protect the nitrogenase of high carotenoid strains (Cd and CD-1) from oxidative damage. In order to test this hypothesis, Hartmann et al.[183] isolated carotenoid-negative and carotenoid-overproducing mutants. Several of the latter derepressed nitrogenase activity under air, while *A. brasilense* strain Sp7, which is devoid of carotenoids,[164] did not. A high carotenoid strain C-1 also showed slightly higher pO_2 optima for nitrogenase activity.[183] Some facts, however, argue against a protective role for carotenoids. First, in none of these reports, were the dissolved O_2 concentrations or the respiratory rates of the cultures under nitrogenase derepression determined.[164,183] Second, carotenoid synthesis was stimulated by high O_2 and by NH_4^+ conditions incompatible with nitrogenase derepression and activity.[131,164] Third, complete blockage of carotenoid synthesis in strain Cd still led to nitrogenase derepression in the presence of air.[164] This suggests that a factor distinct from carotenoids is protecting nitrogenase against O_2 damage. Nur et al.[164] observed the formation of cell aggregates by *A. brasilense* Cd under aerobic conditions which, however, permitted nitrogenase derepression and parallel carotenoid synthesis. The authors suggested that cell aggregation may provide a microenvironment for the N_2-fixing system. Bergersen[89] discussed the role of cell aggregation in nitrogenase protection against O_2 damage.

A comparison[184] of the effect of DOT on nitrogenase activity of *Azospirillum* spp. revealed that *A. amazonense* Y1 was more tolerant than *A. lipoferum* RG20a which, in turn, was less sensitive than *A. brasilense* Sp7. The higher tolerance of *A. amazonense* Y1 nitrogenase to O_2 contrasts with that previously reported.[77] In the latter report, however, nitrogenase activities of culture suspensions, adjusted to the same turbidity, were determined as a function of O_2 partial pressure (gas phase) and not as dissolved O_2. Cultures of the same density do not necessarily respond similarly to O_2. Evidence has also been found for an oxygen-mediated, partially reversible "switch-off" of *Azospirillum* spp. nitrogenase,[184] similar to that described for Azotobacteriaceae.[3]

b. Nitrogenase

The nitrogenase complex consists of two iron-sulfur proteins, a Mo-Fe protein (dinitrogenase) and a Fe protein (dinitrogenase reductase). Both are required for activity and are extremely O_2 sensitive.[3,185-189] The best studied nitrogenases are from *K. pneumoniae*, *A. vinelandii*, *A. chroococcum*, and *C. pasteurianum*. Reports on nitrogenases of the new rhizocoenotic diazotrophs are scarce. Active nitrogenase was detected in crude extracts of *E. cloacae*,[57] and component proteins were purified from *A. brasilense* and *A. amazonense*

nitrogenases.[94,170,190,191] The nitrogenase of *Azopirillum* spp. conforms with the binary protein system[3] described for other diazotrophs, except that purified Fe protein of *A. brasilense* and *A. lipoferum* requires activation by an activating enzyme similar to that described for the photosynthetic diazotroph *R. rubrum*.[170,190,192-194]

The activating factor, however, does not participate in the catalytic cycle of nitrogenase since once activated the Fe protein remains active and capable of transferring electrons to the Mo-Fe protein in the absence of the activating factor.[92,196] The Fe protein of *A. amazonense* is active as isolated and therefore does not seem to require activation.[191]

Highly purified nitrogenase components were obtained from *A. amazonense* and characterized.[191] Fe- and Mo-Fe-protein preparations were devoid of residual activity when assayed alone and reached activities of the order of 1800 and 2400 nmol C_2H_4 produced per 1 min per 1 mg protein.[190] The optimal combination of Fe- and Mo-Fe proteins for maximum nitrogenase activity of the Fe protein was at a molar ratio of 2 Mo-Fe proteins per 1 ℓ Fe protein, and for that of the Mo-Fe protein the ratio was 120 Fe proteins per 1 ℓ Mo-Fe-protein.[191] The molecular weights of the dimeric Fe and of the tetrameric Mo-Fe proteins of *A. amazonense* were 66,000 and 210,000 daltons, respectively.[191] The Fe protein was found to be composed of two unequal monomers of apparent molecular weights of 31,000 and 35,000, whereas the Mo-Fe protein was an $\alpha_2\beta_2$-type tetramer with subunits of 50,000 and 55,000.[191] The metal content of both proteins was 2 g atom Mo, 24 g atom Fe and 28 g atom acid labile sulfide for the Mo-Fe protein, and 4 g atom Fe and 6 g atom acid labile sulfide for the Fe protein.[191] The amino acid composition and the isoelectric points (4.6 for the Fe protein and 5.2 for the Mo-Fe protein) were determined.[191] The molecular weights of the subunits of the Fe and Mo-Fe proteins of *A. brasilense* Sp7 were found to be 60,000 and 64,000 for the Mo-Fe protein and 33,000 and 36,000 for the Fe protein.[195]

Fe and Mo-Fe proteins of *A. brasilense* and *A. amazonense* were capable of reconstituting active nitrogenase with the complementary proteins of other diazotrophs. The nitrogenase components of *A. amazonense* had high cross reactivities with the Fe or Mo-Fe proteins of *A. vinelandii*, *K. pneumoniae*, and *R. rubrum*, but were inactive in the presence of the nitrogenase components of *C. pasteurianum*.[191] Similar observations were previously made with the nitrogenase components of *Spirillum lipoferum*,[196] now *A. brasilense* strain Sp7.[76] Nitrite and azaserine have been reported as inhibitors of nitrogenase activity of *A. brasilense* in vivo.[192,199,200]

In recent years, physiological, biochemical, and genetic evidence indicate that *Azotobacter* spp. contain an alternative nitrogenase in which vanadium replaces molybdenum.[194a] The V-nitrogenase purified from *A. chroococcum* strain MCD 1155, in which the structural genes for the conventional Mo nitrogenase are deleted, also contains two iron sulfur proteins, a tetrameric V.Fe-protein (Acl*), analogous to the Mo-Fe protein, and a dimeric Fe protein (Ac2*), analogous to the Fe protein (Acl), with molecular weights of 210,000 (subunit mol wt: 50,000 and 55,000), and 60,000 (subunit mol wt: 31,500), respectively.[194a] Acetylene is a poor substrate for the V-nitrogenase.[194a] The presence of similar V-nitrogenases in other organisms has been anticipated.[194a] Attempts to demonstrate in vivo V-dependent nitrogenase activity have so far failed.[194b]

c. Regulation of Nitrogenase Activity

Nitrogenase activity was found to be regulated by NH_4^+ in photosynthetic bacteria such as *R. rubrum* and *Rhodopseudomonas capsulata*, and in *Azospirillum* spp.[92-94,184,192] Addition of NH_4^+ to cultures actively fixing nitrogen caused a reversible inactivation of nitrogenase by a process called "NH_4^+-switch-off". This involves covalent modification of the Fe protein by reversible attachment of an ADP-ribosyl group to a specific arginine residue.[197] NAD^+ is the donor of the modifying group in a reaction catalyzed by an "inactivating enzyme".[197a] Activation of the Fe-protein requires removal of the ADP-ribosyl moiety by a membrane bound, constitutive "activating enzyme".[194]

Nitrogenases of *A. brasilense*, *A. lipoferum*, and *A. amazonense* were found to be inactivated in vivo by externally added NH_4^+ or glutamine.[94,192] The molecular mechanisms involved in the inactivation process were, however, found to be different among the *Azospirillum* species.[192] In *A. brasilense* and *A. lipoferum*, nitrogenase switch-off by NH_4^+, but not by glutamine, was prevented or reversed by methionine sulfoximine (MSX), an inhibitor of glutamine synthetase.[192] MSX did not act on the partially inactivated nitrogenase of *A. amazonense*.[192] In addition to NH_4^+, anaerobiosis was also capable of triggering ADP-ribosylation of the Fe protein of *A. brasilense* and *A. lipoferum*.[184] The Fe protein of *A. amazonense* was not found to be modified by ADP-ribosylation in vivo[184] or in vitro.[191]

Recent evidence suggests that regulation of nitrogenase activity by "NH_4^+-switch-off" may take place in salt marsh sediments and roots ot the grass *S. alterniflora*.[198] The diazotrophs involved have not been identified.

d. Regulation of Nitrogenase Synthesis

NH_4^+ amino-acids, nitrate, nitrite, and carbamoyl phosphate are known to repress nitrogenase synthesis.[3,92] In all free-living diazotrophs, high concentrations of NH_4^+ effectively represses nitrogenase synthesis,[3,92] and this possibly holds for all newly discovered diazotrophs.

Repression of *A. brasilense* nitrogenase by NH_4^+ ions was abolished by MSX or methionine sulfone, both inhibitors of glutamine synthetase (GS).[106] Similar results have been observed with other diazotrophs, including *A. vinelandii* and *K. pneumoniae*.[201] The possible involvement of GS in the regulation of nitrogenase synthesis in *A. brasilense* was suggested.[106] The effect of other nitrogen compounds on nitrogenase activity of *A. brasilense* has been determined.[94,184,200,202-204]

A. brasilense grew and derepressed nitrogenase in a liquid medium containing glutamate under air.[94] This procedure allowed for the screening of hundreds of putative nitrogen-fixation-deficient mutants.[94,193] Asparagine, aspartic acid, lysine, and tyrosine (8 m*M*), added to N-free semisolid media, also had no repressible effect on nitrogenase synthesis by *A. brasilense*, whereas alanine, glutamate, glutamine, glycine, and cysteine repressed.[203] Hartmann et al.[184] studied the effect of several amino acids on nitrogen fixation and NH_4^+ excretion by different strains of *Azospirillum* spp. grown in semisolid media. *A. brasilense* strains had high nitrogenase activities in the presence of proline, histidine, and glutamate (10 m*M*), whereas zero or very low activities were found in *A. lipoferum* and *A. amazonense* strains.[184] However, at the time of assay, all *A. lipoferum* cultures were overgrown and nitrogenase was possibly inactive.

Nitrate repressed nitrogenase activity in *A. brasilense* and *A. lipoferum*, and this repression was dependent on nitrate reduction.[200] Inhibition and repression of *A. brasilense* and *A. lipoferum* nitrogenases by nitrate occurred after a lag period required for the induction of nitrate assimilatory enzymes and its conversion into nitrite and ammonia.[199,200,203,204] Nitrite was an effective inhibitor of nitrogenase activity and a repressor of its synthesis.[199,200,204] These effects do not seem to require conversion to NH_4^+.[200] Nitrate reductase-negative mutants of *A. brasilense* and *A. lipoferum* are unable to grow on NO_3^- derepressed nitrogenase.[200] Nitrogenase activity was not repressed by high NO_3^- concentrations in *B. azotofixans*, *Azotobacter paspali*, *Beijerinckia fluminensis*, but was repressed in *A. vinelandii*, *D. gummosa*, and *B. indica* after a lag period of 3 hr.[55,205] The capacity to fix nitrogen in the presence of high concentrations of mineral nitrogen (NO_3^+ and NH_4^+) may be of ecological importance.[55]

2. NH_4^+ Assimilation

Enzymes involved in the assimilation of NH_4^+ in bacteria are glutamate dehydrogenase (GDH), GS, and glutamate synthase (GOGAT).[3] They are present in *Azospirillum brasilense* and *A. lipoferum*,[94,106,193,206,207] and most probably in all new rhizocoenotic diazotrophs. In

A. brasilense, the levels of GS and GOGAT were higher in cells grown in N_2 than in NH_4^+, while GDH levels were higher in cells grown in NH_4^+.[106] These results suggested that N_2-grown *A. brasilense* assimilates NH_4^+ primarily via GOGAT and GS, as found for other diazotrophs.[3] The inability of GS- and GOGAT-negative mutants of *A. brasilense* to grow diazotrophically supports this hypothesis.[206,207] In *A. brasilense*, GS activity is regulated by adenylylation promoted by NH_4^+.[106,206]

The effect of glutamate on the levels of NH_4^+ assimilatory enzymes of *Azospirillum* spp. was studied.[184] *A. lipoferum* and *A. amazonense* had higher GDH and glutamate uptake activities than *A. brasilense*.[184] In contrast, *A. brasilense* had active glutamine synthetase and NH_4^+ transport activities, indicating that the cells were N-limited.[184] Glutamate (1 m*M*) partially switches-off nitrogenase activity of *A. lipoferum* and *A. amazonense* but had no effect on nitrogenase activity of *A. brasilense*.[184]

NH_4^+ uptake by *A. brasilense* and *A. lipoferum* occurs via an energy-dependent transport system.[208] This system is present in N_2-grown cells but absent in those grown on NH_4^+.[208] Aspartate and glutamate allow for the synthesis of the NH_4^+-transport system in *A. brasilense* but not in *A. lipoferum*.[208]

A NAD-dependent dehydrogenase was found in *A. brasilense*.[106] A NAD(P)H-dependent cold labile GDH of *A. brasilense* has been purified and characterized.[209] The GOGAT purified from *A. brasilense* was found to be a NADP-dependent, iron-sulfur flavoprotein.[210]

3. Nitrate and Nitrite Assimilation

Nitrate-dependent growth requires the presence of assimilatory nitrate and nitrite reductases.[211] *Azospirillum* spp.,[76-78,199,200,212,213] and *H. seropedicae*[82] are capable of NO_3^--dependent growth under air or microaerobically (semisolid medium).

Bacillus azotofixans and *Pseudomonas* sp. Dc[71] failed to reduce nitrate. The same happened with *Beijerinckia fluminensis* and *A. paspali*.[205] NO_3^--dependent anaerobic growth of *A. brasilense* and *A. lipoferum* requires active assimilatory nitrate and nitrite reductase and also the enzymes for NO_3^- dissimilation.[176,199,212,213] *A. amazonense*[200] and *H. seropedicae*[82] are unable to carry out NO_3-dependent anaerobic growth.

4. Denitrification

Under conditions of severe O_2-limitation (microaerophilically or anaerobically), NO_3^- is utilized as the terminal respiratory electron acceptor by most strains of *A. brasilense* and *A. lipoferum*.[163,176,199,200,212-214] Production of gas bubbles and nitrite disappearance in cultures grown in semisolid medium containing NH_4NO_3 was taken as presumptive evidence for denitrification to yield volatile products.[213] Based on these criteria, it was found that less than one half of the *A. brasilense* strains and several *A. lipoferum* strains were true denitrifiers,[213] as was *A. halopraeferans*.[78] N_2 was found to be the final product of denitrification by *A. brasilense* Cd,[163] Sp7, and many others. By the same criteria, *A. amazonense*[77] and *H. seropedicae*[82] were unable to denitrify. In *H. seropedicae*, however, some N_2O may accumulate under C_2H_2.[82] *Pseudomonas* sp. DC,[71] *Pseudomonas* sp.,[69] and *B. azotofixans*[55] are apparently unable to denitrify. *A. brasilense* is capable of anaerobic growth dependent on NO_3^-,[157,176,199] NO_2^-,[215] and N_2O[157,216] as the terminal respiratory electron acceptors. This implies that all steps of denitrification are linked to ATP production and coupled to an anaerobic respiratory chain. In *A. brasilense*, the relative efficiencies in energy production indicate that NO_2^- is equivalent to O_2, while NO_3^- is only one third as efficient.[217]

Dissimilatory nitrate, nitrite, and nitrous oxide reductase are found in *A. brasilense* and *A. lipoferum* and follow complex patterns of regulation involving O_2, NO_3^-, NO_2^-, N_2O, and NH_4^+ concentrations and pH.[176,199,200,212-215] *A. brasilense*[215] grown anaerobically at neutral pH on NO_3^-, NO_2^-, or N_2O induced similar levels of nitrate reductase activity (12.3 to 17.8 μmol NO_3^- utilized per 1 hr per 1 mg protein). Cells grown on NO_3^- had very low

levels of NO_2^- and N_2O reductase activities.[215] Growth on NO_2^- or N_2O induced high levels of N_2O and NO_3^- reductase and low levels of NO_2^- reductase.[215] High pH (7.5) was required for full induction of nitrite reductase by nitrite (10 m*M*).[125] These results suggest a pattern of sequential induction of these enzymes. Furthermore, it was observed[200,212] that the first step in NO_3^- dissimilation by *A. brasilense* and *A. lipoferum* occurred with NO_2^- accumulation.[200,212,213] The second step, NO_2^- reduction to gaseous products, occurred only after near complete exhaustion of NO_3^-.[176,200,212,213] At this stage, nitrite reductase and nitrous oxide reductases were presumably induced by NO_2^-. N_2O, which is usually only detected following inhibition of nitrous oxide reductase by acetylene, is then reduced to N_2.[212,213,216] This sequential type of control had been proposed to operate in *A. brasilense* by Nelson and Knowles.[176]

Dissimilatory NO_2^- reductase of *A. brasilense* probably contains cytochromes *c* and *d*, although an additional Cu-containing enzyme may also be present.[166] Cell-free preparations of *A. brasilense* grown anaerobically in NO_2^- produced large quantities of nitric oxide. This was assumed to be an artifact activity of solubilized dissimilatory nitrate reductase.[166]

5. Nitrate-Dependent Anaerobic Nitrogen Fixation

NO_3^- has been shown to support anaerobic nitrogenase activity (C_2H_2 reduction) in *A. brasilense* and *A. lipoferum*.[212-214] These results were not confirmed by Nelson and Knowles.[176] However, short-term nitrate-dependent acetylene reduction was demonstrated in *A. brasilense* and *A. lipoferum* strains grown under N_2-fixing conditions but not in cultures grown in NO_3^-.[199] The activity lasted for 3 to 4 hr or until the enzymes of nitrate assimilation were synthesized, and the accumulated nitrite (1 m*M*) inhibited nitrogenase activity.[199]

C. Temperature

Rhizocoenotic diazotrophs have been isolated in tropical, temperate, and cold climatic regions. These organisms generally grow and fix nitrogen in the mesophilic range of temperature (25 to 45°C); no truly psychrophilic species have been isolated from temperate or cold climates or any thermophilic species from subtropical or tropical regions.[3,217]

There is, however, a tendency for individual species to distribute themselves in the mesophilic range of temperatures according to their climatic region of origin. Thus, diazotrophs isolated from tropical regions such as *Azospirillum* spp., *D. gummosa*, and *A. paspali* have temperature optima for diazotrophic growth in the upper range (32 to 40°C).[218] *K. pneumoniae* M5al is less tolerant to high temperature when fixing nitrogen than growing on NH_4^+.[219] *K. pneumoniae* M5al grows at 37°C when supplied by NH_4^+ but not when depending on N_2 fixation.[219,220] This phenomenon is related to the thermolability of the *nif* A gene product of *K. pneumoniae*.[221]

Tropical strains of *A. brasilense* and *A. lipoferum* grow diazotrophically in the range of 32 to 40°C, with an optimum at about 35 to 37°C, and no growth occurs below 14°C or above 40°C.[30,81] *A. amazonense* optimum temperature for growth on N-free medium was 35°C,[77] while it was 41°C for *A. halopraeferans*.[78] *H. seropedicae* optimum temperature for diazotrophic growth was 34°C, with no growth at 22 or 38°C.[82] Several cold-climate strains of root-associated diazotrophs including *K. pneumoniae, K. terrigena, E. agglomerans, A. lipoferum*, and *Pseudomonas* sp. Dc were isolated at 14 to 37°C and tested for diazotrophic growth in a range of temperatures.[217] All strains failed to grow at 4 or 42°C. *K. pneumoniae* and *A. lipoferum* strains were the only species capable of fixing N_2 at 37°C, the optimum temperature for the latter species. *K. pneunomiae* had maximal nitrogenase activity at 20 to 28°C, whereas that of *K. terrigena* and *E. agglomerans* was at 14 to 28°C. Optimum temperature for nitrogenase activity in *Pseudomonas* sp. Dc was 20°C. At 14°C, *E. agglomerans* and *K. terrigena* fully derepressed nitrogenase activity, while *A. lipoferum* and *K. pneumoniae* strains exhibited zero or less than 25% of maximal activity.[217] Although

each species had a preferred optimal temperature range, no significant differences in the temperature range between strains isolated at different temperatures or locations were found. Temperate climate strains of *A. lipoferum*, *Klebsiella* sp., and *Enterobacter* sp., isolated from cereal roots, had maximal nitrogenase activities at 25 to 30°C, but *A. lipoferum*, surprisingly, failed to grow and derepress nitrogenase activity at 35°C in a N-free semisolid medium.[222] A strain of *E. cloacea* isolated from the rhizosphere of rice in the subtropical region of China was capable of diazotrophic growth at 39°C.[223] Apparently, tropical and subtropical regions select for organisms capable of growing diazotrophically above 32°C, while temperate and cold climate regions do not appear to impose any selective pressure in terms of temperature since species capable of fixing N_2 at high temperatures are found as well in cold climate soils.

IV. GENETICS

A. Introduction

Most genetic studies in rhizocoenotic diazotrophs are on *A. brasilense*. There are fewer reports on the genetics of nitrogen fixation of *A. lipoferum*,[224] *K. oxytoca* NG13,[68,225,226] *E. agglomerans*,[63,227,228] *E. cloacae*,[223] and *Pseudomonas* sp.[229] There are no reports on *A. amazonense*, *A. halopraeferans*, *B. azotofixans*, or *C. nitrofigilis*. Among the Azotobacteriaceae, *A. vinelandii* and *A. chroococcum* have been studied with respect to the genetics of nitrogen fixation, and great developments are in progress.[230,232] *A. paspali*, *D. gummosa*, and *Beijerinckia* spp. have apparently not yet been studied.

K. pneumoniae M5al is the best-studied diazotroph. The structural organization and regulation of the nitrogen fixation (*nif*) genes are well known and taken as models for *nif* genetic studies in other diazotrophs. Since there are reasons to belive that *K. pneumoniae* M5al is actually a strain of *K. oxytoca*[3] and that *K. oxytoca* NG13 is reputed as an efficient nitrogen-fixing organism found in the rice rhizosphere, *K. pneumoniae* M5al can be considered a potential rhizocoenotic diazotroph. The basic developments of the genetics of nitrogen fixation concerning this organism will be discussed. For authoritative reviews on the genetics of nitrogen fixation by *K. pneumoniae*, see References 231 to 234.

B. Genetics of Nitrogen Fixation

1. The nif Genes: Location, Structural Organization, Function, and Regulation of Expression

a. K. pneumoniae M5al

A combination of classical genetics and recombinant DNA studies have established that expression of nitrogen fixation in *K. pneumoniae* M5al requires 17 contiguous *nif* genes. These are organized in seven or eight operons located on the chromosome between the *his* operon and the *shi* A gene and spans about 23 kbp. The order of the nif genes is as follows:

hisD . . . nifQB.AL.F.M.VSU.XNE.YKDH.J . . . *shi*A

Transcription is from right to left, except for operons *nif*F and *nif*J. The relative molecular masses and functions of the *nif* genes are presented in Table 3.

The operon *nif*HDKY encodes, respectively, the polypeptides of the Fe protein (*nif*H), and the α (*nif*D) and β (*nif*K) subunits of the Mo-Fe protein of nitrogenase; the product of *nif*Y has a molecular weight of about 24 kdaltons of unknown function. Further processing of these gene products, and the synthesis and insertion of the Fe-Mo co-factor into the Mo-Fe protein, is required to produce fully active nitrogenase. The product of *nif*M is involved

Table 3
SIZE AND FUNCTION OF THE *nif* GENE PRODUCTS OF *K. PNEUMONIAE* M5a1

Operon	Gene	Polypeptide mol wt (kdaltons)	Function
BQ →	B	49	Synthesis of Fe-Mo-cofactor
	Q	?	Molybdenum transport
LA →	L	45	Repressor of *nif* transcription
	A	57	Activator of *nif* transcription
F ←	F	19	Flavodoxin-electron transport to nitrogenase
M →	M	28	Processing of Fe protein
USV →	U	25	Processing of Mo-Fe protein
	S	45	Processing of Mo-Fe protein
	V	42	Synthesis of Fe-Mo-cofactor
ENX →	E	40	Synthesis of Fe-Mo-cofactor
	N	50	Synthesis of Fe-Mo-cofactor
	X	18	Unknown
HDKY ←	H	35	Subunit of Fe protein
	D	56	α-Subunit of Mo-Fe protein
	K	60	β-Subunit of Mo-Fe protein
	Y	24	Unknown
J ←	J	120	Pyruvate-fladoxin oxidoreductase

Note: Data compiled from Cannon et al.[233] where original references are to be found. The *nif* operons are transcribed in the directions indicated by the arrows. *Nif*M can be transcribed from the *nif*U promoter.

in post-translational modification of the Fe protein whereas those of *nif*U and *nif*S are involved in post-translational modification of the Mo-Fe protein. Synthesis of Fe-Mo co-factor, an essential metal cluster of active Mo-Fe protein, requires the products of *nif*B, V, N, and E. Molybdenum uptake is the probable function of the *nif*Q gene product. Electron transfer to nitrogenase requires the product of *nif*F, a flavodoxin, and the product of *nif*J, a pyruvate-flavodoxin oxidoreductase. The functions of *nif*X and *nif*Y gene products are unknown. Regulation of expression of nitrogen fixation requires the products of the *nif*LA operon; the *nif*A product functions as a transcriptional activator of all other *nif* transcription. Expression of the *nif*LA operon is, in turn, under control of the general nitrogen source utilization system (*ntr*).

***Nif*-expression** — Excess NH_4^+, O_2, or temperatures above 35°C repress nitrogenase synthesis in *K. pneumoniae*. The molecular basis of these repressions rests in the control of expression of the *nif*LA operon, in the activities of the *nif*L and *nif*A gene products, and in the thermolability of the *nif*A gene product. Synthesis of nitrogenase in *K. pneumoniae* is under control of the general nitrogen utilization system and of the *nif* specific regulatory system encoded by the operon *nif*LA. The model proposed by Merrick[235] integrates both systems and has been confirmed (Figure 1). Transcription of the *nif* operons is activated by the *nif*A gene product in the presence of an inactive *nif*L gene product. Under conditions of intermediate NH_4^+ (>4 m*M*) or O_2 (>0.1 μ*M*) levels, the *nif*L protein is activated and blocks *nif* transcription activated by the *nif*A protein. The *nif*A protein is thermolabile, being inactive at temperatures above 35°C, thus explaining the absence of nitrogenase synthesis above this temperature.

Activation by the *nif*A protein requires the product of *ntr*A, a σ factor which probably modifies RNA polymerase specificity towards *nif* and *ntr* promoters. Under low NH_4^+ (<4

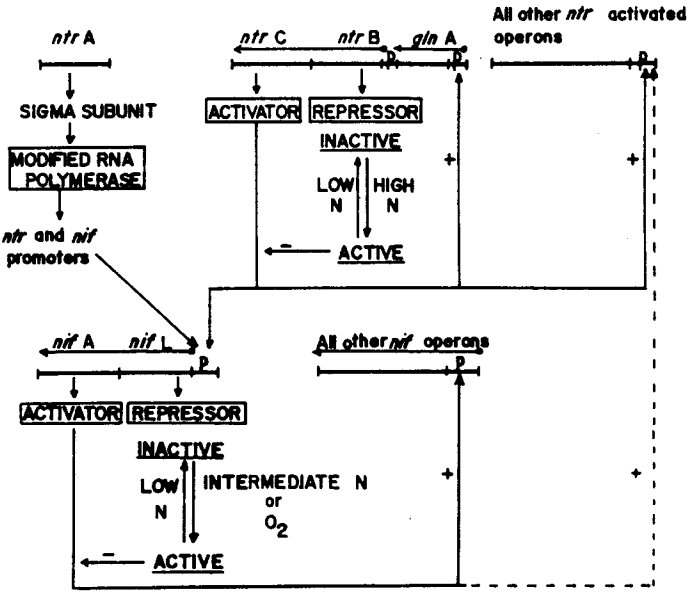


FIGURE 1. Current model for *nif* regulation in *K. pneumoniae*.[233,235]

m*M*), *ntr*C protein activates transcription of the *nif*LA operon and the *ntr* operons by the *ntr*A protein-modified RNA polymerase. At high levels of NH_4^+ (~20 m*M*), transcription of *nif* LA and other *ntr* operons mediated by the *ntr*C protein is repressed by the *ntr*B gene product by a mechanism possibly analogous to that of *nif*L. Therefore, NH_4^+ repression of *nif* is first sensed by the *nif*LA control system and then, at high NH_4^+ levels, by the *ntr* system. The *ntr* system is apparently insensitive to O_2 repression.[3,231-235] *K. pneumoniae* *ntr*A$^-$, *ntr*C$^-$, and *nif*A$^-$ mutants are unable to derepress nitrogenase and constitute a class of regulatory mutants. Due to structural and functional similarities between *ntr*C and *nif*A gene products, *ntr*C$^-$ mutants can be complemented by *nif*A. Besides activating the *nif*LA operon, the *ntr*C gene product weakly activates the *nif*H promoter.[235a] Constitutively expressed *nif*A will overcome NH_4^+ and O_2 repression, possibly due to the titration of *nif*L gene product by excess *nif*A gene product.[221,231-235]

The *nif* promoters of *K. pneumoniae* lack the canonical −10 and −35 elements present in *Escherichia coli* promoters.[233] They have, instead, conserved sequences[233] around −12 and −24 and, in addition, an essential sequence at about −135 from the transcription initiation site.[235b] This site is required for *nif*A- but not *ntr*C-mediated transcription activation and also for inhibition of chromosomal *nif* expression observed in cells harboring multicopy plasmids containing certain *nif* promoters.[235b]

b. K. oxytoca NG13

Nif genes, homologous to all 17 of those of *K. pneumoniae*, were found in the chromosome *K. oxytoca* strains NG13, NSG3, and X3d.[68] The restriction enzyme patterns showed that the *nif* genes of *K. oxytoca* are very similar to those of *K. pneumoniae* M5al. The authors concluded that the organization of the *nif* gene cluster of *K. oxytoca* strains is essentially the same as that of *K. pneumoniae* M5al, although some differences were found in the restriction pattern.[68] The whole *nif* cluster of *K. oxytoca* strain NG13 was cloned in plasmid

pBR322. Initially, two contiguous HindIII fragments of the *nif* cluster were cloned into plasmids pNOCl1A (*nif*Q-K) and pNOCl4A (*nif*DHJ). Later, a recombinant of these two plasmids was constructed, and the resulting plasmid pNOW24 contained the entire *nif* cluster (*nif*Q-J). Plasmid pNOW25 was capable of conferring nitrogen-fixing capacity to *E. coli* strain KO60.[68] Restriction maps of the cloned *nif* genes of *K. oxytoca* NG13 were the same as those of *K. pneumoniae* M5al for *Hind*III, *Xho*I, *Eco*RI, and *Bam*HI, differed slightly with respect to *Pst*1, *Sal*I, and *Bg*III sites.[68] Quantitative data suggest that there is about a 4% difference in the base sequence of the *nif* gene cluster of *K. oxytoca* NG13 and *K. pneumoniae* M5al.[68]

A 2.9 kb *Sal*I fragment containing *K. oxytoca* NG13 *nif*A was subcloned into plasmid pNOW7A.[226] The cloned *nif*A gene was expressed constitutively from the tetracycline promoter of pACYC184.[226] Plasmid pNOW7A (*nif*A^c) complemented *K. pneumoniae* UNF714 *nif*A$^-$ mutant and eliminated NH_4^+ repression in this strain and in *K. oxytoca* NG13.[226]

Recombinant plasmid pNT11, a cointegrate of pNOW7A (*nif*A^c) and plasmid RSF1010, was transferred to *A. lipoferum* FS by transformation.[226] *A. lipoferum* FS (pNT11) transconjugants expressed nitrogenase activity in the presence of NH_4^+ (5 m*M*). Constitutive expression of nitrogenase in the presence of NH_4^+ by *A. brasilense* transconjugants carrying *K. pneumoniae nif*A expressed constitutively had been demonstrated previously.[94,193]

c. *E. agglomerans*

E. agglomerans,[63] *Lignobacter*,[236] and fast growing rhizobia[237-242] are the only diazotrophs where the *nif* genes are located on plasmids. Five strains of nitrogen-fixing *E. agglomerans* from wheat rhizosphere were found to contain one or two plasmids with molecular weights of 70 to 100 Mdalton.[63] Each strain had a plasmid capable of hybridizing with a ^{32}P-labeled 6.2-kb EcoRI fragment of pSA30 containing the *nif*HDK genes of *K. pneumoniae* M5al.[63] Strains cured of plasmids lost their capacity to fix nitrogen.[63]

Plasmid pEA3 (*nif*) from *E. agglomerans* was purified and shown to hybridize with the purified *K. pneumoniae nif*HDK region.[63] These results are strong evidence for a plasmid location of the structural nitrogenase genes of *E. agglomerans*.[63] Plasmid pEA3 has been cloned into plasmid pHC79.[227] The *nif* genes of *E. agglomerans* showed extensive homology with those of *K. pneumoniae* but had a different restriction pattern.[227] Similar to the *K. pneumoniae nif* genes, the plasmid-borne *nif* genes of *E. agglomerans* were found to be clustered and present in single copies.[277] Possibly relevant to the discussion of a plasmid location for *nif* genes is the high instability of diazotrophy generally observed during the isolation of putative diazotrophs from mixed populations.[66] This instability suggests that *nif* plasmids, if they occur in these initially diazotrophic populations, are highly unstable under nonselective conditions[66] and also implies that strong selective pressures for *nif* prevails in the rhizosphere. Alternatively, syntrophic interactions among bacterial strains or chemical signals of plant origin may be required for *nif* expressions.[66] Postgate[3] suggested that the *nif* gene cluster may constitute a complex transposon, autonomous to some extent, which during evolution has distributed itself laterally among prokaryotes transported by conjugative plasmids. Klingmuller et al.[257] demonstrated that the *nif* plasmid pRD1[3] can be forced to integrate in the chromosome of a nondiazotrophic strain of *E. cloacae* upon chlorate selection. The integration inactivated the nitrate reductase gene and caused *E. cloacae* to express nitrogenase activity in the presence of nitrate.[257]

The ability of plasmids of the incP and incF groups to mobilize and transfer the entire *nif* gene cluster of *K. pneumoniae* M5al to several nondiazotrophic enterobacteria[3] and the presence of natural *nif* plasmids[63,236-242] may explain the apparently random distribution of diazotrophy among Enterobacteriaceae[3] and other bacterial families.

d. E. cloacae

Total DNA from a subtropical strain of *E. cloacae* was found to hybridize with ^{32}P-labeled *K. pneumoniae* M5al *nif* genes.[223] *E. cloacae* DNA was cleaved with *Hind*III or *Bam*HI and ligated into pBR322.[223] Two recombinant plasmids, pSTll4O and pSTll3O, were identified by hybridization with *K. pneumoniae nif* genes. Plasmid pSTll4O contained the sequences homologous to *K. pneumoniae nif*BALFMSUXNEY, and plasmid pSTll3O contained sequences homologous to *nif*YKDHJ genes.[223] The *nif* gene order in *E. cloacae* was concluded to be similar to that in *K. pneumoniae*.[223] *E. cloacae nif*A was subcloned and shown to activate *K. pneumoniae* and *Rhizobium meliloti nif*H::*lac* fusions in a strain of *E. coli*.[223] *E. cloacae nif*A gene product-mediated activation was less temperature sensitive than that by *K. pneumoniae nif*A gene product.[223]

E. cloacae does not express nitrogenase activity in the presence of high NH_4^+ concentrations.[223] However, an *E. cloacae* strain harboring a constitutive *nif*A gene from *K. pneumoniae* synthesized nitrogenase mRNA and reduced acetylene in the presence of 15 m*M* at 30°C, but not at 39°C,[223] confirming the thermolability of *K. pneumoniae nif*A gene product.[221] Similar results were described for *A. brasilense* strains.[94,193] Regulatory systems for *nif* expression similar to those described for *K. pneumoniae* thus seem to operate in *E. cloacae*. However, in contrast to *K. pneumoniae*, the *nif*A gene product of *E. cloacae* is thermostable at 39°C.[223]

e. Pseudomonas sp.

Genetic studies with strains H8 and KLH76 of *Pseudomonas* sp., a diazotroph found in the rhizosphere of rice in the Philippines,[69] have begun.[229] The organisms were shown to undergo plasmid transfer by conjugation.[229] Six putative mutants deficient in nitrogen fixation (Nif$^-$) were isolated following Tn5 transposon mutagenesis.[229]

A partial Sau3A genomic library of strain H8 was constructed in the vector EMBL3 and screened by hybridization with a clone containing a Tn5 insertion in *Pseudomonas* sp. *nif* D or K.[229] A restriction map of the 24-kb region of the chromosome was deduced from two overlapping clones.[229] Homology studies showed that a number of noncontiguous restriction fragments of this region showed homology to a *nif*HDK probe.[229]

f. Azospirillum spp.

Genetic studies of these organisms are progressing faster than in other bacteria of this class. Several genes including *nif*,[224,243] *glnA*,[244] restriction endonuclease,[245] and *his*[246] have been cloned, *nif* specific mutants isolated,[94,193,247] and finally the regulatory mechanisms for *nif* expression approached.[94,193,206,207,244,247] Other developments concern the identification in *Azospirillum* spp. of genes homologous to *Rhizobium nod* and *fix* genes[249] and to *Agrobacterium* chromosomal virulence genes (*chvA* and *chvB*[250]). These findings open interesting perspectives to the understanding of the interactions between *Azospirillum* spp. and the associated plants.

Location of *nif* genes — There is strong evidence that the *nif* genes of *A. brasilense* and *A. lipoferum* are located in the chromosome.[251,252] There are, however, two reports indicating that in certain strains the *nif* genes may be plasmid borne.[253,254]

Mutants deficient in nitrogen fixation — Regulatory and structural nitrogenase mutants of *A. brasilense* have been isolated following chemical[94,193,247] or site-directed transposon mutagenesis.[255,256]

Several classes of *Azospirillum* mutants can be isolated easily following chemical mutagenesis. However, *nif*-specific mutants have been very difficult to isolate probably due to non-distinctive characteristics of Nif$^-$ colonies in N-free media. Most of the putative nitrogen-fixation deficient (*nif*) mutants, isolated as small colonies from N-free plates, were

indeed mutated outside *nif*. Some Nif$^-$ mutants of *A. brasilense* proved to be NH_4^+ assimilatory mutants and GS[206] or GOGAT[207] mutants.

The discovery that *A. brasilense* grows and derepresses nitrogenase under air in liquid medium supplemented by glutamate[193] has greatly facilitated the screening for Nif$^-$ mutants. Glutamate supports aerobic growth until the respiratory activity of the culture is high enough to decrease the dissolved oxygen concentrations to levels compatible with nitrogenase derepression.

Few *nif* structural mutants of *A. brasilense* strain Sp7, *nif*D$^-$ or *nif*K$^-$ were isolated following chemical mutagenesis and characterized biochemically[193,247] and genetically.[247] Recently, a large collection of mutant Tn5 insertions in the *nif* cluster of *A. brasilense* Sp7 were obtained by site-directed mutagenesis.[255] A fine map of the *nif*HDK and *nif*NE regions was obtained.[255] Other insertion mutants in the *nif*HDK operon of *A. brasilense* have also been obtained by site-directed transposon mutagenesis.[256]

Regulatory mutants — Nitrogen fixation in *Azospirillum* spp. is subjected to repression by NH_4^+ or O_2 and is "switched-off" by NH_4^+ (see Section III.B.1.a., b., and c.)

In *K. pneumoniae*, *nif* expression involves transcriptional regulation mediated by the products of the *nif*LA and *ntr*BC genes (see Section IV.B.1. a). In *A. brasilense*, genetic complementation studies strongly suggest that an analogous system is operative. Three *nif*$^-$ mutants of *A. brasilense* Sp7 (FP8, 9, and 10), obtained after chemical mutagenesis, were complemented for nitrogen fixation by plasmid pCK3, which carries *K. pneumoniae nif*A gene expressed constitutively from a tetracycline promoter.[193] *Nif* expression in these transconjugants was constitutive in the presence of NH_4^+ and, due to the thermolability of the *K. pneumoniae nif*A gene product,[221] occurred at 30 but not 37°C.[193] Since *A. brasilense* strain Sp7 has an optimum temperature for nitrogen fixation at 35 to 37°C, the results suggest that the *nif*A gene product of this strain is thermostable at 37°C. The presence of a *nif*L gene in *A. brasilense* strain Sp7 can be inferred from the constitutive expression of *nif* in the presence of high NH_4^+ levels by pCK3 transconjugants of the wild-type strain.[193]

Evidence for the operation of an *ntr*-like system arose from the demonstration that two Nif$^-$ mutants (FP8 and 9), previously complemented by *nif*A, were also complemented by *K. pneumoniae ntr*C[193] of plasmid pGEl0.[259] FP8 and are probably *ntr*C$^-$ because, as in *K. pneumoniae*, they can be corrected by *nif*A [221,235,260] and because they also show low levels of GS activity (<30% of the wild type), a characteristic of *ntr*C$^-$ mutants.[259]

Another characteristic of these *ntr*C$^-$ mutants of *A. brasilense* was discovered in the transconjugants of plasmid pCK3. While the addition of 1 m*M* NH_4Cl completely switched-off nitrogenase in vivo in the wild-type FP2 and transconjugants FP2(pCK3) and FP10(pCK3), in the FP8 and 9(pCK3) transconjugants, only partial inhibition (25 to 29%) was observed.[94,193] This failure to switch-off could be related to the low levels of glutamine synthetase activity.[193]

A regulatory mutation which simultaneously affects *nif* expression and histidine transport in *A. brasilense* has been described.[261] The mutants had five- to sixfold higher nitrogenase activities than the wild type and were partially insensitive to NH_4^+ repression.[261] The mutants were not characterized genetically.

Activation of *nif* promoters by the *nif*A gene product, and of the *nif*L promoter by the *ntr*C gene product, implies that a *ntr*A-like gene is present in *A. brasilense*.[193] The presence of a *ntr*A-like gene in *A. brasilense* was also supported by the expression of kanamycin resistance,[193] which from plasmid pCKl (*K. pneumoniae nif*A^c) only occurs in a *ntr*A background.[262] These results indicated that a two-tier system for *nif* regulation, similar to that of *K. pneumoniae*, exists in *A. brasilense*.[193] *A. brasilense* strain Sp245 yielded *nif*A$^-$-type mutants which behaved in all respects similarly to those of *A. brasilense* strain Sp7.[263] *A. lipoferum* also seems to be regulated by *nif*A activation.[226]

Two classes of *A. brasilense* strain Sp7 mutants deficient in GS were found.[206] One class

(mutant 7028) has low levels of GS activity and derepressed nitrogenase in the presence of 20 m*M* NH_4Cl and are therefore mutants constitutive for nif expression (Nif^c). The second class (mutant 7029) was affected in GS activity and could not synthesize nitrogenase. The introduction of plasmids carrying the structural gene for GS (*gln* A) from *A. brasilense* or *K. pneumoniae* into mutant 7028 (Gln^- Nif^c) restored nitrogenase repression by NH_4^+.[244,264a] The same plasmid restored nitrogenase activity in mutant 7029 (Gln^- Nif^c).[244] The authors concluded that strains 7028 and 7029 are likely to be *gln*A mutants and that a direct involvement of *Azospirillum* GS in the regulation of nitrogen fixation cannot be ruled out.[244,264a]

*A. brasilense ntr*C mutant FP9 was not complemented by plasmid pAB44, which was capable of complementing the glutamine auxotrophy of mutant 7029 (Gln^- Nif^-) and possibly also carried the equivalent to *ntr*BC genes.[264] This lack of complementation raises the question of the location of *ntr*BC genes in *Azospirillum*.[264] It is interesting to note that in *R. capsulata*, which shares with *A. brasilense* the mechanism of nitrogenase switch-off by NH_4^+ and Fe protein-activating enzyme (see Section III.B.1), *gln*A and *ntr*C genes are unlinked.[265]

g. Homology of Azospirillum spp. DNA with K. pneumoniae and Rhizobium trifolii nif Genes

K. pneumoniae nif fragments, covering the entire *nif* cluster, and *R. trifolii nif*HD genes, were used as hybridization probes for the presence of homologous genes in total DNA of several *Azospirillum* strains. Homology was detected for the structural nitrogenase genes *nif* HDK in *A. brasilense* and *A. lipoferum*.[195,225,243,251,266] Weak homology was found between total DNA from both species and plasmids pPC936 (*K. pneumoniae nif*FLABQ) and pMC71A (*K. pneumoniae nif*A).[195] Homology was detected between *K. pneumoniae nif*J and *A. lipoferum* DNA but not *A. brasilense* strain Sp7 DNA.[195] Finally, no homologous genes to *nif*XUSVM or to *nif*EN was found in *A. brasilense* and *A. lipoferum*.[195] The lack of homology with most of the *nif* genes of *K. pneumoniae* seems surprising since according to Robson et al.[231] there is probably a basic set of *nif* genes (those involved in the synthesis of an active nitrogenase) which is common to all diazotrophs and is apparently absent in *Azospirillum*.

h. Cloning of Azospirillum nif Genes

Genomic libraries of several *A. brasilense* and *A. lipoferum* strains have been constructed in a variety of vectors.[224,243-246,267,268] The nitrogenase structural genes *nif*HDK of *A. brasilense*[243] strain Sp7 and of *A. lipoferum*[224] strains RG20a and RG6xx have been cloned and analyzed. A 6.7-kb *Eco* RI fragment from total DNA of *A. brasilense* strain Sp7 homologous to the *nif*HDK cluster of *K. pneumoniae* was cloned.[224] Heteroduplex analysis of the clone fragments revealed that the *nif*HDK genes of *A. brasilense* are organized in a single operon transcribed in the same direction as in *K. pneumoniae*.[243] These conclusions are supported by transcriptional and translational analysis and the genetic complementation of *A. brasilense* strain Sp7 Tn5 insertion mutants.[255]

The cloned *nif*HDK genes of *A. lipoferum* strains RG20a and RG6xx were found to be clustered and organized in the same order as in *K. pneumoniae*.[224] A 6-kb DNA fragment adjacent to *nif*K of the *nif*HDK cluster of *A. brasilense* strain Sp7 has been cloned.[264] Hybridization studies suggest that this region contains a gene homologous to *nif*E.[264] The physical maps of the *nif* E-*nif*HDK cluster of *A. brasilense* strain Sp7[243,255] and of the *nif*HDK of *A. lipoferum*[224] are available. There are no reports on cloning of *Azospirillum nif*A or *nif*J, despite their identification by hybridization[195] and function.[193]

The understanding of the organization and regulation of *nif* expression and of the mechanism of NH_4 transport and assimilation eventually allow for the construction of new rhizocoenotic diazotrophs capable of excreting NH_4^+ for immediate plant benefit.

i. Homology of Azospirillum DNA with Genes Relevant to Nitrogen Fixation and Plant Interaction

*R. japonicum fix*A and *fix*BC are, in some manner, involved in symbiotic nitrogen fixation. A probe containing *fix*A genes was shown to hybridize total DNA from *A. brasilense* strain Sp7 and R07 and from *A. lipoferum* strain Br17 and S28.[249,264] Homology to common nodulation genes (*nod*) and to host plant specificity genes (*hsn*) of *R. meliloti* and *Azospirillum* DNA has been reported.[249] The chromosomal virulence genes (*chv*A and *chv*B) of *Agrobacterium tumefaciens* was found to have homologous counterparts in *A. brasilense* strain Sp7 and *A. lipoferum* strain Br17.[250] DNA sequences from *A. brasilense* homologous to *Rhizobium nod* and to *Agrobacterium chv* genes have been cloned and analyzed.[268]

The presence of *fix*, *nod*, *hsn*, and *chv* genes in *Azospirillum* spp. does not imply that they are functional.[264] However, it may be significant that genes involved in symbiotic nitrogen fixation, nodulation, host specificity, and virulence are apparently present in *Azospirillum* spp. These reports constitute potentially important developments toward the understanding of the interactions observed between *Azospirillum* spp. and plant roots.

C. *Azospirillum* General Genetics

The genetics of *Azospirillum* spp. have been reviewed by Elmerich et al.[252,264] and Döbereiner and Pedrosa,[75] and only a few aspects are covered herein.

1. Mutation

The collection of *A. brasilense* and *A. lipoferum* mutants is still small, although most classes of mutants can be easily isolated.[252] Spontaneous, chemical, or radiation-induced mutants and also insertional mutants have been obtained. Del Gallo et al.[269] analyzed the effect of several mutagens on the mutational rate of *A. brasilense* and concluded that this species lacks an SOS-DNA repair-like mechanism. Classes of *Azospirillum* spp. mutants include auxotrophs,[248,270-273] those resistant to antibiotic,[193,247,270,272] antimetabolites,[183,248,269,274,275] and hydrogen peroxide,[182] and also mutants deficient in NH_4 assimilation,[206,207,248] nitrate assimilation and denitrification,[200] and nitrogen fixation (see Section IV.B).

2. Gene Transfer and Mapping of the Chromosome of A. brasilense

Conjugation[270-272,276,277] is the most efficient system for gene transfer among *A. brasilense* and *A. lipoferum*. Plasmids of the incPl group (RP4, R68.45, and derivatives) have been transferred from *E. coli* or *P. aeruginosa* to *A. brasilense* at frequencies of 10^{-5}, 10^{-6}, and 10^{-7}, respectively. Among *Azospirillum* species, transfer of incPl plasmids can be as high as 10^{-1} to 10^{-2}.[270-272] Plasmids RP4[277] and R68.45[272] were shown to display chromosome mobilization ability in *A. brasilense* and in *A. lipoferum*. It is relevant that plasmids of the P1 incompatibility group, but not those of Q and W, are stably maintained in *Azospirillum*.[193,272,278]

3. Transformation

A. brasilense and *A. lipoferum* have been transformed with chromosomal[273,279] and plasmid DNA.[226,280] In the procedure utilized to transform *A. lipoferum* with plasmid DNA,[226] early log phase cells (O.D. 0.25 to 0.30) were treated with $CaCl_2$, followed by a heat-shock and a 2-hr expression period. In that utilized to transform *A. brasilense* with plasmid DNA,[280] no competent cells were obtained following calcium treatment or by freezing in ethanol-solid CO_2. However, Ca^{2+}, Mn^{2+}, and K^+ were found to be essential for competence and the efficiency of tranformation on the length and temperature of the heat-pulse.[280] For both *Azospirillum* spp., the efficiency of transformation ranged from 200 to 1000 transformants per 1 μg plasmid DNA.[226,280]

4. Transduction

Although most *A. brasilense* and *A. lipoferum* strains were found to be lysogenic[105] and bacteriophages have been reported,[281,282] no transducing ability has been demonstrated.

5. Mapping of the Chromosome of A. brasilense

The chromosome-mobilizing ability of plasmid R68.45 has been utilized to determine the genetic map of the chromosome of *A. brasilense*.[271,272] Plasmid R68.45 was transferred between *A. brasilense* strains at frequencies of 10^{-2} per recipient, whereas selected markers were transferred at 10^{-6} per recipient, independent of the marker selected.[272] According to Franche et al.,[272] plasmid R68.45 appears to promote a bidirectional, nonpolar chromosomal gene transfer in *A. brasilense* Sp7, suggesting the presence of multiple chromosomal sites for plasmid integration. Similar observations were made by Bazzicalupo and Gallori[271] in *A. brasilense* strain Sp6. Map distances and the tentative gene order have been obtained for several markers.[271,272] The data, however, are not sufficient to establish an unambiguous rough genetic map of the chromosome of *A. brasilense*.

6. Plasmids: Numbers, Sizes, and Functions

Plasmids occur in various numbers and sizes in *A. brasilense* and *A. lipoferum* strains.[251,272,273,276,283] The plasmid pattern is strain specific, and an individual strain can have none or several plasmids of molecular weights ranging from 3.5 to 370 Mdaltons. Variations in plasmid pattern of specific strains have been attributed to plasmid instability, methodology, or to strain misnaming.[252] There are, as yet, no reports on plasmids in *A. amazonense* and *A. halopraeferans*, but they are anticipated. No specific function has been conclusively demonstrated to be plasmid borne in *Azospirillum* spp.

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