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REVIEW ARTICLE

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The "oxygen paradox" of dinitrogen-fixing bacteria

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Abstract N_2 fixation by aerobic bacteria is a very energy demanding process, requiring efficient oxidative phosphorylation, while O_2 is toxic for the nitrogenase complex. N_2-fixing bacteria have evolved a variety of strategies to cope with this apparent "O_2 paradox". This review compares strategies that azospirilla and other well-known N_2-fixing soil bacteria use to overcome this O_2 paradox. Attention will be given to the relationships between the natural habitat of these soil bacteria and their prevailing adaptations. In view of this knowledge the following questions will be addressed: are the specific adaptations observed in azospirilla sufficient to allow optimal proliferation and N_2 fixation in their natural habitat? Could improving the O_2 tolerance of the N_2-fixing process contribute to the development of more efficient strains for the inoculation of plants?

Key words *Azospirillum* species · Oxygen paradox · Nitrogen fixation · Rhizosphere · Nitrogenase complex

Introduction

Azospirillum species are free-living N_2-fixing plant-growth-promoting bacteria that live in close association with roots of cereals. The beneficial effects of *Azospirillum* species on plant development are considered to be mainly due to the production of phytohormones (Okon 1985; Vande Broek and Vanderleyden 1995; Bashan and Holguin 1997). The beneficial impact of bacterial N_2 fixation on plant growth appears to be less significant than that of the rhizobia-legume symbiosis (Okon 1985; Okon and Labandera-Gonzalez 1994; Bashan and Holguin 1997; Okon and Vanderleyden 1997; Holguin et al. 1999). However, N_2 fixation remains important for bacterial survival in N-poor soils and possibly in the root environment. Improved N_2 fixation might therefore result in increased bacterial populations on roots and consequently in increased plant-growth promotion.

Most obligate or facultative aerobic N_2-fixing bacteria have to deal with an "O_2 paradox". During O_2-dependent growth, a minimal O_2 concentration is necessary to support aerobic respiration and ATP synthesis to meet the high energy demand of nitrogenase. However, when present in too high a concentration O_2 can lead to irreversible damage of nitrogenase. A large number of the physiological and morphological characteristics of diazotrophs are devoted to reconciling this O_2 paradox. For a complete description of these adaptations in eubacteria and cyanobacteria we refer the reader to reviews of Gallon (1992) and Fay (1992).

Dedicated to Prof. K. Vlassak on the occasion of his 65th birthday

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O_2 paradox: general aspects

The Mo-containing nitrogenase is the most widely distributed nitrogenase. The alternative nitrogenases containing either V or Fe alone are restricted to some organisms (Burris 1991) and do not occur in azospirilla (Hartmann and Zimmer 1994). The Mo-nitrogenase consists of two proteins: the Fe protein and the Mo-Fe protein (Burris 1991; Kim and Rees 1994; Peters et al. 1995). The Fe protein (dinitrogenase reductase, Fe protein) (*nif*H) is a homodimer which contains a single 4Fe-4S cluster and two binding sites for MgATP (Georgiadis et al. 1992; Peters et al. 1995). The Mo-Fe protein (dinitrogenase, MoFe protein) (*nif*DK) is a $\alpha_2\beta_2$ tetramer (Kim and Rees 1992) that consists of two [8Fe-7S] clusters (P-clusters) and two FeMo cofactors (FeMo

cofactor) (Peters et al. 1997). The reduction of N_2 to NH_4^+ requires eight electrons. As a consequence, a minimum of 16 ATP molecules is required to reduce one molecule of atmospheric N_2. Moreover, the reduction of N_2 is always coupled to the reduction of H^+ to H_2. This H^+ reduction accounts for 25% of the electron dissipation (Burris 1991; Peters et al. 1995). Under normal physiological conditions the ATP requirement for N_2 reduction is closer to 20–30 molecules of MgATP than 16 molecules of MgATP. Consequently, N_2 fixation can consume a significant fraction of the total cellular ATP pool (Burris 1991; Peters et al. 1995). ATP is efficiently provided by aerobic respiration. Therefore, aerobic N_2 fixation requires a minimal concentration of O_2. Even facultative anaerobic N_2-fixing bacteria such as *Klebsiella pneumoniae* show an increased N_2-fixing capacity in the presence of a limiting amount of O_2 (Poole and Hill 1997).

Purified nitrogenase, regardless of its source, is extremely rapidly and irreversibly inactivated by O_2 (Gallon 1992). The Fe protein is much more sensitive to O_2 than the MoFe protein (Wang et al. 1984). The Fe protein and MoFe protein exhibit typical half-lives in air of respectively 45 s and 10 min (Robson and Postgate 1980; Wang et al. 1984). As mentioned earlier, N_2 fixation is coupled to the obligatory production of H_2 (Burris 1991). This nitrogenase-dependent production of H_2 causes a significant waste of ATP and reducing equivalents. Furthermore, high levels of H_2 inhibit nitrogenase activity. Some diazotrophic bacteria express an uptake hydrogenase under N_2-fixing conditions. This hydrogenase can relieve the inhibitory effect of H_2 on nitrogenase and concomitantly provide reducing equivalents to support the respiratory protection by oxidizing the H_2 formed. In some bacteria the coupling of H_2 oxidation to respiration leads to ATP synthesis (Robson and Postgate 1980). An uptake hydrogenase can therefore act at both levels of the O_2 paradox (O_2 protection or energy production).

Notwithstanding the wide range of strategies that N_2-fixing bacteria have developed to cope with this so-called O_2 paradox, the detrimental effect of O_2 on nitrogenase restricts N_2 fixation in most species of the eubacteria to anaerobic or microaerobic conditions. *Azotobacter vinelandii,* equipped with a well-integrated O_2-protection system, is the only described exception, being able to fix N_2 under fully aerated conditions (Poole and Hill 1997). Recently, however, the occurrence of an O_2-tolerant N_2-fixing system was described in *Streptomyces thermoautotrophicus*. In this species the reduction of N_2 is coupled to the oxidation of CO to peroxide. A Mn-containing superoxide oxidoreductase converts the formed $O_2^{\cdot-}$ to O_2 while simultaneously transferring the electrons to a MoFeS-dinitrogenase for the reduction of N_2. The structure of this newly discovered nitrogenase appears to be very different from those of the common Mo-containing nitrogenases (Ribbe et al. 1997), and the physiological relevance of this system needs further validation.

O_2 paradox in symbiotic microorganisms

Rhizobial bacteroids have resolved the O_2 paradox by combining the microaerobic environment of a nodule with a very efficient mode of respiration. The variable diffusion barrier of nodules restricts the O_2 concentration in the infection zone, where the bacteroids reside, to nearly anaerobic levels (5–50 nM) (Witty and Minchin 1998; Kuzma et al. 1999). The mechanisms by which this barrier readily (minutes to hours) responds to fluctuating O_2 concentrations are not clear, but presumably control of the size and distribution of intracellular spaces by osmocontraction and by secretion of occluding glycoproteins within the intracellular spaces might regulate the entry of O_2 (Witty et al. 1986; Minchin 1997). Metabolic activities such as high respiratory activity and concomitantly high levels of ascorbate peroxidases in the endodermis of the nodule have been postulated to be involved in the long-term (days) adaptation to elevated O_2 levels (Dalton et al. 1998). Albeit protective, this O_2 barrier makes bacteroids the primary site of O_2 limitation in the nodules (Kuzma et al. 1999). Despite the low O_2 concentrations in the nodule, respiration by a cyt*cbb*$_3$ (*fix*NOQP) terminal oxidase allows bacteroids to produce sufficient energy to support N_2 fixation (Preisig et al. 1993; Vargas et al. 1996). This oxidase, characterized by an extremely high O_2 affinity [with a K_M of 7 nM (Preisig et al. 1996)], is able to consume the O_2 delivered by the nodule leghaemoglobin (Appleby 1984; Poole 1994). Notwithstanding the presence of this high-affinity respiratory chain and the O_2-delivering leghaemoglobin, N_2 fixation in legume nodules remains limited by O_2 and subsequently by energy (Kuzma et al. 1999). The Fix^- phenotype observed in the absence of a cyt*cbb*$_3$ oxidase in all obligate symbiotic N_2 fixers (Preisig et al. 1993; Schlüter et al. 1997) and the enhanced N_2 fixation capacity observed at increasing rates of respiration in the bacteroids (Soberón et al. 1989; Yurgel et al. 1998), supports this conclusion.

The tight O_2-dependent regulation confines expression of rhizobial *nif* genes to extremely microaerobic conditions (Fischer 1994). Although expression of rhizobial *nif* genes is not restricted to symbiotic conditions, nitrogenase activity under free-living conditions has, to our knowledge, never been conclusively demonstrated. The lack of sufficient energy to support nitrogenase activity under these conditions might, at least to some extent, explain the absence of N_2 fixation by free-living rhizobia. De-repression of *nif* genes under free-living conditions probably occurs only at dissolved-O_2 tensions similar to these present in the nodules. In the absence of leghaemoglobin-facilitated O_2 transport, the high-affinity terminal oxidase might not operate sufficiently at these low O_2 concentrations. Although this is a highly speculative hypothesis, some circumstantial evidence has been reported. It was demonstrated that expression of the bacterial haemoglobin *vhb* from *Vi-*

treoscilla in the strain *Rhizobium etli* altered the expression of *nif* genes ex planta (Ramírez et al. 1995; Minchin 1997). Rhizobia have adapted their N_2-fixing mechanism to extremely low O_2 concentrations, and consequently make any additional nitrogenase-protection system somehow superfluous. For rhizobia, the most important aspect of the O_2 paradox probably is that of energy limitation.

In view of this energy limitation in the nodules, a contribution of hydrogenase to recycling energy seems reasonable (Evans and Burris 1992; Maier and Triplett 1996). It should be noted, however, that in this case energy limitation is caused by the lack of sufficient C-source rather than by the limited availability of O_2. A role of the uptake hydrogenase in energy production during O_2 limitation is less likely since respiration driven by H_2 is less efficient than when other electron donors such as e.g. NADH are used. However, since only few *Rhizobium leguminosarum* strains have been shown to possess H_2-oxidation activities coupled to ATP production (Nelson and Salminen 1982), the energy coupling of the system might rather be an ancillary than an essential function. Hydrogenase activities coupled to the respiratory chain might also function as an additional respiratory protection system by providing reducing power to consume the ambient O_2 (Nelson and Salminen 1982). Indeed, an O_2-sensitive mutant of *Azorhizobium caulinodans* with impaired N_2-fixation ability exhibited a defective uptake hydrogenase during symbiosis (Das and Lodha 1998). Since the structural barrier in rhizobial nodules limits the diffusion of free gases, the predominant role of a hydrogenase in nodules might be the removal of the excess H_2 (Van Soom et al. 1993; Witty and Minchin 1998). *hup* expression in *R. leguminosarum* bv. *viciae* is, in contrast to that in *Bradyrhizobium japonicum*, coregulated with *nif*-and *fix* gene expression in a NifA-dependent, HoxA-independent way, and is restricted to the H_2-rich environment in the nodules. At least in this particular *Rhizobium* strain, this coregulation, which is believed to be an evolved trait, suggests the importance of an uptake hydrogenase in the nodule (Brito et al. 1997).

O_2 paradox in free-living N_2-fixing eubacteria

Several lines of evidence indicate that, in contrast to rhizobia, free-living soil bacteria under N_2-fixing conditions (high C/N ratio) exhibit a maximal growth rate which is limited by the low availability of fixed N rather than by a shortage of energy. In contrast, in the presence of a readily available N source, growth and biomass production of both rhizobia and free-living N_2-fixing microorganisms is presumably limited by energy availability. A first line of evidence for energy not limiting N_2 fixation but rather N-supplement growth was obtained from batch growth experiments with *Azospirillum brasilense* strain Sp7 and a *cyt*N mutant. The lack of a microaerobically expressed cyt*cbb*$_3$ terminal oxidase in the *A. brasilense cyt*N mutant barely affected its growth under N_2-fixing and non-C-source-limited conditions, but drastically decreased growth under N-supplemented conditions as compared to the wild type (Marchal et al. 1998). Secondly, the growth of N_2-fixing *A. vinelandii* cells under non-C-limiting conditions was in line with the conclusion that the high energy demand of its nitrogenase does not limit growth under N_2 fixation. Batch cultures of *A. vinelandii* cells grown on glucose showed a lower yield of biomass, higher respiration rate and higher glycolytic rate compared to growth on galactose. The lower levels of the energy-coupled cyt*o* pathway, present in glucose-grown cells, were compensated for by increased levels of the partially uncoupled cyt*bd* respiratory pathway (Liu et al. 1995). The specific activities of nitrogenase, similar in both cultures, gave rise to a fixed NH_4^+ output. Since in *A. vinelandii* glucose is more rapidly converted to CO_2 than galactose, the uncoupled respiratory chain is believed to remove excess reducing equivalents which cannot be used for protein synthesis because the fixed N output cannot meet the high rate of production of reducing equivalents and ATP produced by glucose-grown cells (Liu et al. 1995). Thirdly, some free-living N_2-fixing bacteria, such as *A. brasilense,* tend to accumulate poly-hydroxy butyrate (PHB) at low dissolved-O_2 tensions or at high C/N ratios, i.e. under N_2-fixing conditions (Nur et al. 1982; Tal and Okon 1985). The production of PHB constitutes an electron sink to remove excess reducing equivalents and requires ATP. In *Azotobacter* species low O_2 concentrations trigger PHB accumulation (Senior et al. 1972; Segura and Espin 1998). An additional electron sink might be essential under conditions where the uncoupled cyt *bd* pathway is not expressed (Poole and Hill 1997; Wu et al. 1997). It is difficult to imagine why energy-consuming processes such as PHB production and futile electron transport to remove excess reductant should operate if growth were limited by energy. Energy limitation during N-supplemented growth might be the cause of the low nitrogenase activity detected in an *A. brasilense dra*T-*nif*A double mutant in the presence of NH_4^+. As explained by the authors, the energy limitation of growth in NH_4^+ would result in a decreased energy supply to nitrogenase (Arsène et al. 1996).

Implications for the regulation of N_2 fixation

The fundamental difference between symbiotic rhizobia, fixing N_2 at nanomolar O_2 concentrations in nodules, and free-living diazotrophs, showing N_2 fixation at more elevated O_2 levels (μM range), is reflected by the differences in regulation of the *nif* genes (genes responsible for N_2 fixation). Transcription of *nif* genes is induced by NifA, an ubiquitous transcriptional activator which usually acts in concert with the alternative σ^{54} (RpoN) factor (Fischer 1994; Merrick and Edwards 1995). The mode of action of the regulator protein

NifA seems to have been conserved, though its regulation shows significant differences among different diazotrophs. Regulation of NifA in response to alterations in NH_4^+ concentrations and/or O_2 levels can occur either at the transcriptional level, or posttranslational level, or at both levels (Fischer 1994; Merrick and Edwards 1995). An additional posttranslational NH_4^+-mediated switching-off of nitrogenase in some free-living N_2 fixers has been described (Ludden 1994; Merrick and Edwards 1995). For a detailed description of the different regulatory systems, we refer the reader to reviews by Fischer (1994) and Merrick and Edwards (1995). However, in the scope of this review, it is remarkable that obligate symbiotic rhizobia seem to regulate their *nif* genes only in response to dissolved-O_2 tension, while N regulation seems to be absent. In contrast, in free-living N_2-fixing microorganisms (including *A. caulinodans*), very tight N regulation of nitrogenase (at the transcriptional and posttranslational level) seems to exist (Fischer 1994; Merrick and Edwards 1995). In some species this regulation is exerted at the level of transcription of *nif*A and is dependent on NtrBC (Fischer 1994; Merrick and Edwards 1995). In addition, posttranslational N regulation of NifA mediated by a P_{II}-like protein seems to be widespread (Arsène et al. 1996; Jack et al. 1999; Souza et al. 1999). In most rhizobia, the NifA regulatory domain which interacts with P_{II} is still present, but is believed to be either vestigial or has an alternative function (Souza et al. 1999). For several species, direct (Ludden 1994; Zhang et al. 1995) or indirect (Munoz-Centeno et al. 1996) evidence exists for the presence of an ADP-ribosylation system, responsible for the NH_4^+-mediated posttranslational inactivation of the nitrogenase itself. In contrast, O_2 regulation of *nif* gene expression in free-living diazotrophs occurs only at the posttranslational level of NifA, and the type of regulation depends on the species (Krey et al. 1992; Macheroux et al. 1998). Although regulatory cascades leading to *nif* gene expression are not yet fully characterized, the difference in N regulation of N_2 fixation between symbiotic and free-living microorganisms corroborates the importance of energy limitation in N-supplemented growth of free-living microorganisms. Tight N regulation allows free-living diazotrophs to readily switch-off ATP-consuming nitrogenase synthesis and activity as soon as growth becomes energy limited. No such regulation is necessary for symbiotic N_2 fixers. Expression of their *nif* genes and of the cyt*cbb*$_3$ oxidase is restricted to extremely low O_2 concentrations, which are very unlikely to be prevalent in their natural free-living environment. Free-living N-supplemented growth of rhizobia probably occurs at more elevated, physiologically relevant O_2 levels, where expression of *nif* genes is completely repressed by O_2 and no wastage of energy due to futile nitrogenase activity in the presence of a readily available N source is likely to occur. In free-living N_2-fixing soil inhabitants, the O_2 concentration allowing maximal N_2 fixation coincides with an O_2 concentration supporting N-supplemented growth. The presence of only O_2-dependent *nif* regulation would be detrimental for energy-limited microaerobic N-supplemented growth. Interestingly, *A. caulinodans*, able to fix N_2 both under symbiotic and under free-living conditions, seems to combine characteristics of free-living microorganisms with typical symbiotic traits. Similar to other rhizobia, its *nif* genes are O_2 regulated (Fischer 1994). However, in contrast to most other symbiotic rhizobia, a second terminal oxidase besides the cyt*cbb*$_3$ oxidase is involved in energy production in the nodule (Kaminski et al. 1996). One can therefore postulate that this alternative cyt*bd* oxidase can potentially support free-living N_2 fixation without the need of leghaemoglobin. If this is correct, the *A. caulinodans* cyt*bd* oxidase is reminiscent of the enteric high-affinity cyt*bd* terminal oxidase (D'mello et al. 1996) which has been shown to support N_2 fixation at nearly anaerobic levels in *K. pneumoniae* (Hill et al. 1990; Juty et al. 1997). However, since under non-N_2-fixing conditions the *A. caulinodans* cyt*bd* oxidase is inactive at dissolved O_2 levels below 3.6 μM, such assumptions are ventured. Alternatively, the expression of *nif* genes in free-living *Azorhizobium* might possibly occur at slightly higher O_2 levels than in most rhizobia, as it has been shown that nitrogenase activity in nodules of *Sesbania rostrata*, the natural host of *A. caulinodans*, occurs at higher O_2 levels than in rhizobial nodules (Gallon 1992). Irrespective of the mechanism, the occurrence of N-supplemented and diazotrophic growth at similar O_2 levels might necessitate and explain the N-mediated control of *nif* genes in *A. caulinodans*. For free-living N_2-fixing bacteria, the most exacting problem of their fixation of N_2 at O_2 concentrations sufficient to support full respiratory activity is the protection of nitrogenase from these elevated O_2 concentrations. As will be illustrated by the following examples, the excess energy present under N_2-fixing conditions can be consumed to provide this protection.

O_2 protection in free-living N_2-fixing *Azotobacter*

A. vinelandii is a free-living N_2 fixer which lives in soil and fresh water. Supplies of C, N, and O_2 are likely to fluctuate in its environment (Poole and Hill 1997). Nitrogenase of *Azotobacter,* however, seems to be adequately protected to be able to cope with highly fluctuating O_2 concentrations. A well-integrated system of protection, comprising conformational protection, respiratory protection, autoprotection and other concomitant morphological and physiological changes, allows *Azotobacter* species to grow under fully aerated conditions (Poole and Hill 1997). This protection system will be described in more detail since it contains all types of protection, some of which can occasionally be encountered in other microaerobic free-living N_2-fixing microorganisms.

In *A. vinelandii* and *Azotobacter chroococcum* a transient O_2 increase leads to a reversible switching-off

of nitrogenase (Poole and Hill 1997). During this switch-off phenomenon a reversible inactivated but protected nitrogenase complex is formed (conformational protection) (Robson 1979; Scherings et al. 1983; Wang et al. 1984; Moshiri et al. 1995). This complex is formed by noncovalent binding of a Fe-S containing redox protein (FeSII or Shethna) to the MoFe- and Fe-nitrogenase proteins (Scherings et al. 1983; Moshiri et al. 1995). Although very circumstantial, evidence is available that the *nif*W gene product might be part of the O_2-protected complex (Kim and Burgess 1996; Lee et al. 1998). The signal which triggers the formation of this complex is unknown, but is very likely to be an alteration in the redox state of the FeSII protein or the dinitrogenase protein (Moshiri et al. 1995). Upon a certain O_2 shift, the more oxidized state of the nitrogenase triggers the formation of the complex. During the switch-off, cells adapt to the higher ambient O_2 concentration by re-allocating their electron flux. At high ambient O_2 concentrations (D'mello et al. 1997; Wu et al. 1997) a partially uncoupled cyt*bd* oxidase (Bertsova et al. 1997; Jünemann 1997; Kolonay and Maier 1997) with low apparent in vivo O_2 affinity is expressed (D'mello et al. 1994; Kolonay et al. 1994; Jünemann et al. 1995). This oxidase probably acts in concert with an uncoupled NADH-dehydrogenase (Bertsova et al. 1998). Electron flow through this uncoupled chain allows high respiration rates and fast consumption of the intracellular O_2 without exhausting the ATP and NADH pools (respiratory protection) (Kelly et al. 1990; D'mello et al. 1997; Poole and Hill 1997; Wu et al. 1997). Besides its assumed role in reducing the cellular O_2 environment, the cyt*bd* oxidase would function as a fast ATP producer (Linkerhägner and Oelze 1995). High amounts of ATP are necessary to support the autoprotection of the nitrogenase. Provided that the amount of nitrogenase is sufficiently high relative to the intracellular O_2 concentration, the dinitrogenase reductase can reduce O_2 to H_2O_2 and possibly to H_2O, thereby reducing the O_2 in the vicinity of the nitrogenase. Moreover, such a constant flow of reducing equivalents through the nitrogenase might help to maintain it in a reduced state (Thorneley and Ashby 1989). This process, called autoprotection, requires high amounts of reducing equivalents and ATP, provided by the cyt*bd* branch of the respiratory chain. Whether the high respiration rates reduce the intracellular O_2 concentration (respiratory protection) (Poole and Hill 1997) or solely function to provide reducing equivalents and ATP to the O_2-consuming nitrogenase (autoprotection) (Dingler et al. 1988; Kuhla and Oelze 1988; Linkerhägner and Oelze 1995; Linkerhägner and Oelze 1997) remains a subject of controversy. Nevertheless, it has been rigorously demonstrated that high respiration rates are essential for O_2-tolerant N_2 fixation in *A. vinelandii*. *A. vinelandii* mutants which lack the cyt*bd* terminal oxidase (Kelly et al. 1990) or *A. vinelandii* cells grown under C-limiting conditions (Wong 1990; Segura and Espin 1998) lose their ability to perform aerobic N_2 fixation. PO_4^{3-} or C-limited cultures of *Azotobacter* benefit from the presence of an uptake hydrogenase (Yates et al. 1997). H_2 recycling can provide electrons and ATP to support respiratory protection and nitrogenase activity during starvation (Yates et al. 1997). The *A. vinelandii* system illustrates that free-living microorganisms can use the surplus ATP and reducing power to prevent O_2-mediated damage to nitrogenase. Besides these well-studied mechanisms, morphological changes (Dingler et al. 1988) and induction of enzymes such as catalase and superoxide dismutase (Dingler and Oelze 1987) have been shown to be involved in O_2 protection, though to a limited extent.

Posttranslational regulation of NifA by O_2

As described above, *Azotobacter* has acquired the unique ability among all N_2-fixing bacteria to fix N_2 aerobically through the interplay of respiratory and/or autoprotection and conformational protection. The difference in posttranslational regulation of NifA between *Azotobacter* species and other free-living N_2 fixers might therefore be not that incidental. NifA of azospirilla, Rhodobacteriaceae and *A. caulinodans* contains conserved cysteine residues in the interdomain linker, which together with cysteine residues from the central domain form a motif resembling metal-binding sites (Fischer 1994). It is assumed that all NifA proteins containing this cysteine-rich motif exhibit O_2-sensitive activity. According to Krey et al. (1992), a change in redox state of the central, bound metal ion causes a conformational change in the nucleotide binding site. Due to this conformational change, subsequent hydrolysis of the nucleotide is blocked so that no open-complex formation can occur (Krey et al. 1992). NifA of γ-proteobacteria such as *Azotobacter* or *Klebsiella* does not contain these conserved residues (Fischer 1994; Dixon 1998). Activity of NifA in these bacteria is inhibited by the flavoprotein NifL (Hill et al. 1996; Dixon 1998; Söderbäck et al. 1998), which is rapidly oxidized in the presence of air (Macheroux et al. 1998). In its oxidized state, NifL forms a complex with NifA, thereby inhibiting NifA activity. By reduction of NifL, the NifA-NifL complex dissociates and the inhibition is relieved. Krey et al. (1992) hypothesized that binding of NifL to NifA would block either nucleotide binding or hydrolysis and would consequently prevent open-complex formation. The relatively high redox potential of NifL necessitates a sufficient amount of reducing equivalents transduced by a specific electron donor to alter the redox state of NifL and relieve the inhibitory effect on NifA (Macheroux et al. 1998). This redox state, necessary to reduce NifL, does not depend on the O_2 level in the cell, but rather on the respiratory activity and on the availability of reducing equivalents (Macheroux et al. 1998). High respiratory activity by the uncoupled respiratory chain makes reduction of NifL compatible with potentially elevated O_2 levels. Regulation by NifL

therefore allows N_2 fixation to occur at high O_2 levels. The redox state of the central, bound metal in cysteine-conserved NifA proteins might be directly responsive to O_2 or to a redox potential different from the one that activates NifL. This difference in redox responsiveness of NifA could allow aerobic nitrogenase expression in *Azotobacter* but exclude it in other N_2-fixing organisms. Alternatively, the respiratory activity in bacteria other than *Azotobacter* might not be sufficient to keep NifA in its reduced state.

O_2 protection in other free-living N_2-fixing bacteria

In other free-living N_2-fixing bacteria, the protection system is not as elaborated as in *Azotobacter*. Studies on *A. caulinodans* indicated that the cyt*aa*$_3$ oxidase might convey some protection by supporting the production of ATP for the autoprotection system (Boogerd et al. 1998). The anoxigenic, phototrophic bacteria *Rhodospirillum rubrum* and *Rhodobacter capsulatus*, characterized by their highly versatile metabolism, are not only able to fix N_2 under anaerobic photosynthetic or chemotrophic conditions, but also under microaerobic conditions (Oelze and Klein 1996). Recent studies on O_2 protection of N_2 fixation have largely ruled out the existence of a well-defined form of protection in these bacteria. In *R. capsulatus*, neither the significant protection of nitrogenase by hydroperoxidase or catalase (Hochman et al. 1992), nor the presence of respiratory protection could be detected (Oelze and Klein 1996). The switching-off of the nitrogenase by ADP-ribosylation in response to NH_4^+ and energy depletion serves only as a regulatory mechanism of nitrogenase activity, and does not confer any O_2 protection. Nevertheless, an ADP-ribosylation-independent partially reversible switch-off, was observed upon O_2 stress in *R. capsulatus* (Oelze and Klein 1996). Similar switch-off phenomena have previously been described for *K. pneumoniae, Rhodopseudomonas capsulata* and *Rhodopseudomonas sphaeroides* (Goldberg et al. 1987). The competition for the available reducing power between respiration and nitrogenase activity was postulated to be the mechanism underlying this phenomenon (Goldberg et al. 1987). At low O_2 stress, respiration is able to partially reduce the O_2 concentration in the vicinity of the nitrogenase. However the re-allocation of electrons from nitrogenase to O_2 consumption results in a simultaneous inhibition of nitrogenase activity. Since a great deal of evidence suggests that energy and reducing power do not limit N_2 fixation in free-living N_2-fixing bacteria, and the mechanism described by Goldberg et al. (1987) excludes the possibility of the well-described system of autoprotection, the physiological relevance of such competition remains questionable. Strains submitted to O_2 stress in the above-mentioned study were grown under anaerobic conditions, and their metabolism may not have been adapted to aerobic C metabolism. Under such conditions a reduction in energy or reducing power is possible, but would reflect only a temporary phenomenon. Indeed, the exact nature of the observed reversible switch-off still needs to be unravelled (Oelze and Klein 1996). Since the *R. capsulatus fdx* gene displays a high degree of similarity with the *fes*II gene of *A. vinelandii*, the formation of an O_2-insensitive, inactivated nitrogenase complex such as that encountered in *Azotobacter,* though unlikely, cannot be excluded (Moshiri et al. 1995).

Bacteria of the genus *Azoarcus* seem to have adapted very well to their endophytic style of growth in the root interior of Kallar grass. At the nanomolar O_2 concentration that probably predominates in the root interior, a hyperinduced state is developed (Hurek et al. 1994, 1995). This hyperinduction comprises formation of special membrane structures referred to as diazozomes, on which nitrogenase is localized. Concomitantly a very efficient respiratory chain terminated by a high-affinity terminal oxidase is induced. This respiratory chain allows, despite the low O_2 concentration, the generation of high ATP levels, supporting an extremely high nitrogenase activity. The high-affinity oxidase might be similar to the rhizobial high-affinity cyt*cbb*$_3$ oxidases (Macht and Reinhold-Hurek 1997). Similar to the situation in symbiotic bacteria, the prevailing feature of the O_2 paradox for endophytic bacteria is the extremely low O_2 concentration in the plant roots.

O_2 protection in azospirilla

Azospirilla are obligate microaerobic N_2-fixing microorganisms which show an optimal O_2 concentration for N_2 fixation at a pO_2 of 0.5–0.7 kPa dissolved O_2 (Okon et al. 1977; Nelson and Knowles 1978; Vande Broek et al. 1996). O_2 concentrations >2 kPa completely prevent N_2 fixation (Hartmann and Burris 1987). A partially reversible switch-off of nitrogenase in response to O_2 stress, independent of the ADP-ribosylation pathway, has been described (Hartmann and Burris 1987). This switch-off phenomenon was attributed to the previously described competition hypothesis of Goldberg et al. (1987). The physiological relevance of this switch-off remains questionable. Nevertheless, some minor protection mechanisms seem to be active in azospirilla. Carotenoids, produced at intermediate O_2 concentrations in N_2-fixing *Azospirillum* cultures, are believed to protect cells against oxidative damage by quenching singlet O_2. Carotenoids might also, by reinforcing the membrane bilayer, reduce O_2 diffusion in the cytoplasm (Nur et al. 1982). Indeed, carotenoids, when overexpressed, could contribute, although to a low extent, to a slightly improved O_2 tolerance of N_2 fixation, but they were not found to increase the optimum O_2 concentration for N_2 fixation (Hartmann and Hurek 1988). As *Azospirillum* is taxonomically closely related to the photosynthetic bacterium *Rhodospirillum rubrum,* carotenoid synthesis is more likely to be a cryptic charac-

teristic conserved from a common ancestor rather than a specific O_2-protection system. Melanin is another pigment produced by azospirilla under some conditions such as desiccation and aging (Sadasivan and Neyra 1987). Although clear proof exists for the role of a melanin-comprising redox buffer in protecting pathogenic fungi against strong oxidants (Jacobson and Hong 1997), melanin production in *Azospirillum* has never been the subject of much research. The production of superoxide dismutase in azospirilla is inversely related to O_2 concentration, and catalase exhibits a higher activity under N-supplemented conditions. Therefore both enzymes are not likely to make any specific contribution to nitrogenase protection (Nur et al. 1982; Clara and Knowles 1983). Indirect evidence based on biochemical assays has indicated the presence of an uptake-hydrogenase system in *Azospirillum* (Chan et al. 1980; Tibelius and Knowles 1983). However, only a limited role of this putative hydrogenase system in the O_2 protection of nitrogenase was suggested (Tibelius and Knowles 1983).

The most important characteristic of the O_2 paradox of azospirilla is probably its extremely pronounced aerotactic response to microaerobic conditions (3–5 μM) (Barak et al. 1982; Zhulin et al. 1996). This preferred range of O_2 concentration coincides with the O_2 concentration allowing generation of a maximal proton motive force (Zhulin et al. 1996). Indeed, in *Azospirillum,* energy conversion into biomass has been reported to be more efficient under microaerobiosis than under aerobiosis (Nur et al. 1981, 1982). Its aerotactic behaviour turns *Azospirillum* into a microaerophilic N_2 fixer which overcomes the critical O_2-paradox by optimizing its energy metabolism to O_2 conditions compatible with nitrogenase activity and conditions present in the rhizosphere. This situation is reminiscent of the hyperinduced state, evolved by *Azoarcus* in microaerobiosis (Hurek et al. 1994). Either the redox potential or the proton motive force, generated by a high-affinity terminal oxidase, are assumed to be the signal regulating positive and negative aerotaxis (Zhulin et al. 1996; Taylor and Zhulin 1998). Indeed, it has been shown that active respiration by a previously described cyt*o* oxidase of *A. brasilense* Cd is necessary to obtain an aerotactic response but not to sustain motility (Reiner and Okon 1986). This signal would be transduced into bacterial motion by a redox-sensitive protein, homologous to the Aer protein of *E. coli* (Zhulin et al. 1997; Taylor and Zhulin 1998). It is noteworthy that the cyt*cbb*$_3$ terminal oxidase (*cyt*NOQP operon) expressed under microaerobic conditions (2.4 μM) could, based on spectral properties, be identified with the previously described cyt*o* oxidase (Reiner and Okon 1986; Marchal et al. 1998). However, a *cyt*N mutant was found to be able to form a sharp aerotactic band (unpublished results, K. Marchal et al.). Azospirilla, being poorly protected against the detrimental effects of O_2 on nitrogenase seems, at first glance, to behave differently from *Azotobacter* species. Nevertheless, similarities are present in the way both organisms cope with the O_2 paradox. Instead of excess respiration, as observed in *Azotobacter*, the surplus energy generated during microaerobic respiration is used for other energy-consuming processes aimed at reconciling the O_2 paradox. Under non-C-limiting conditions, the surplus energy of *Azospirillum* can be used to produce PHB (Nur et al. 1982; Tal and Okon 1985). By sustaining bacterial motion during starvation this reserve C source allows *Azospirillum* to escape from damaging O_2 concentrations to its optimal microaerobic niche (Tal and Okon 1985). Besides its use for the production of PHB, energy could be used for de novo nitrogenase synthesis. An O_2 shift irreversibly damages the nitrogenase and inactivates the NifA protein of *Azospirillum*. However, since transcription of *nif*A is constitutive (Zhang et al. 1997), reduction of O_2 concentrations to levels adequate for nitrogenase activity allows immediate synthesis of nitrogenase. De novo synthesis of nitrogenase might appear a poor adaptation to fluctuating environmental conditions. However, this mechanism is often observed in cyanobacteria (Gallon 1981, 1992). Furthermore, it reflects a difference in ecological niche between azospirilla and *Azotobacter* species. Azospirilla live in close association with respiring plant roots and can avoid high O_2 conditions by aerotaxis. Therefore O_2 stress might usually be temporary, making more permanent adaptations to high O_2 levels, such as those encountered by *Azotobacter,* superfluous.

The morphological differentiation (cyst or floc formation) to which azospirilla are subjected under stress conditions (such as desiccation) or during plant-surface attachment, might serve as an additional protection system, more specific to azospirilla. In a medium containing a high C/N ratio with fructose as a C source, *A. brasilense* cells tend to flocculate (Sadasivan and Neyra 1987; Burdman et al. 1998). After the initial phases of plant-root attachment, cells start to undergo a cyst-like differentiation (De Troch and Vanderleyden 1996; Bashan and Holguin 1997). Both flocculation and encystation comprise a similar differentiation process: cells start to aggregate, cell walls thicken, accumulation of PHB takes place and the production of extracellular polysaccharides starts. Cells progressively lose their motility, become ovoid and are embedded in a dense capsular mesh (Katupitiya et al. 1995). Though the exact nature of this mesh has not yet been defined, it seems to be composed of polysaccharides and proteins (Del Gallo et al. 1989; Bashan and Holguin 1993; Vande Broek and Vanderleyden 1995; De Troch and Vanderleyden 1996). Although a role of bacterial gum in generating a local microaerobic environment has been suggested, some studies refute such a role (Robson and Postgate 1980; Gallon 1992). Floc or cyst formation occurs under various stress conditions and flocs or cysts retain a limited N_2-fixing capacity (Kennedy et al. 1997). We regularly observed floc formation during N_2 fixation at excess O_2 concentrations (Nur et al. 1981; K. Marchal et al., unpublished results). The formation

of cell aggregates might reduce the ambient O_2 concentration. Since the elevated O_2 levels in the vicinity of the flocs could inhibit an optimal N_2-fixing capacity, the output of fixed N_2 must limit the accumulation of biomass. As a result, the excess reducing equivalents will be recycled by PHB synthesis. This might explain the increased PHB formation in the flocs. Interestingly, an increased tendency to form aggregates was observed in a non-motile *fla* mutant (Burdman et al. 1998). Since a non-motile *fla* mutant cannot escape from high dissolved-O_2 tensions, O_2 stress might be more pronounced, resulting in increased aggregate formation. This differentiation process confirms the lack of respiratory protection in azospirilla (Nelson and Knowles 1978). If respiratory protection was active, a more trivial response to a C-rich environment would be increased respiration to reduce intracellular O_2, instead of PHB accumulation. Because a differentiation process similar to floc formation occurs when azospirilla colonize plant roots (Katupitiya et al. 1995), a spontaneous mutant of *A. brasilense* Sp7 which was characterized by a lack of cyst and floc formation, also exhibited an altered colonization pattern on wheat roots (Katupitiya et al. 1995; Pereg-Gerk et al. 1998). Colonizing cells of this mutant did not differentiate but remained in their vegetative form (Katupitiya et al. 1995; Kennedy et al. 1997). Interestingly, no surface colonization was observed and mutant cells resided only in the crevices surrounding the sites of lateral root emergence and in the first few layers of root cortical tissues. Moreover, the nitrogenase activity of seedlings inoculated with the mutant was significantly higher than that of seedlings inoculated with the wild type. It was suggested by the authors that this peculiar difference in N_2 fixation could be attributed to the lower O_2 concentration present in the plant cell layers which the mutants colonized, as compared to that in the environment of the plant root surface where the wild-type cells were present (Katupitiya et al. 1995). This would imply that the O_2 concentration at the surface of the plant root exceeded the optimum for azospirilla, and that azospirilla which lost their motility had to assume the cyst-like form to survive the conditions of O_2 stress. Azospirilla therefore seem to have reconciled the O_2 paradox with respect to their free growth mode but not their associative growth mode.

Future prospects: towards the production of an O_2-tolerant inoculum

The production of an *Azospirillum* strain able to fix N_2 under aerobic conditions necessitates rendering NifA active in an O_2-rich environment and simultaneously protecting nitrogenase against O_2 damage. Although rather ambitious, both goals could potentially be achieved by increasing respiratory activity in *Azospirillum*. Enhanced respiration could, on the one hand, lead to a more reduced intracellular redox state which might revert NifA to its active state. On the other hand, the increased electron flow could possibly contribute to respiratory protection of nitrogenase. However, because of their extreme aerotactic response, azospirilla are generally exposed to microaerobic conditions. Therefore, rather than being able to fix N_2 under fully aerobic conditions, a good inoculum might need to be protected against temporary increased dissolved-O_2 tensions in the soil. Such transient protection could be obtained by heterologous expression of the *A. vinelandii* Shethna protein in *Azospirillum*.

An alternative, even more ambitious, goal of improving biological N_2 fixation in cereal crops is the use of paranodules (Kennedy et al. 1997), whereby the naturally occurring associative colonization by azospirilla is replaced by endophytic colonization. Bacteria enter the plant tissue via the crevices of lateral roots or via dichlorophenoxyacetic acid-induced paranodules. Endophytic colonization is assumed to be more effective than associative colonization: when inside the plant tissue, bacteria might be more protected against O_2, and N transfer to the plant might be higher. However, in the vicinity of the respiring plant tissue O_2 concentrations might become limiting, as occurs in nodules or during endophytic colonization by *Azoarcus* (Hurek et al. 1995; Kuzma et al. 1999). Subsequent decreases in respiration and energy production might inhibit nitrogenase activity. Mimicking the role of leghaemoglobins by expressing a bacterial haemoglobin in the periplasmic space of *Azosprillum* might be a way of overcoming this drawback. It should be noted that the induction of paranodules might be detrimental to plant health.

Alternatively, cocultures of other bacteria with *Azospirillum* might be used. It has previously been shown that in mixed cultures, N_2 fixation by *Azospirillum* exhibits an increased O_2 tolerance (Cacciari et al. 1989; Holguin and Bashan 1996). However, due to excessive respiration of the coculture, O_2 might become limiting. Under such conditions, the presence of an *Azospirillum* strain containing an O_2-buffer such as a bacterial haemoglobin might be advantageous. Finally, the use of a synthetic inoculum might allow for the protection of *Azospirillum* against elevated O_2 levels (Bashan 1998). However, such protection might only last as long as the bacteria are not released from the inoculum into the soil.

The possible expression of the *A. vinelandii cyd*AB (Kelly et al. 1990) operon, the *fes*II gene (Moshiri et al. 1994) and the *Vitreoscilla vhb* gene (Dikshit and Webster 1988) in *Azospirillum* is currently being studied.

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