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Nitrogenase Activity and Nitrate Respiration in *Azospirillum* spp.

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Abstract. The interaction between nitrate respiration and nitrogen fixation in *Azospirillum lipoferum* and *A. brasilense* was studied. All strains examined were capable of nitrogen fixation (acetylene reduction) under conditions of severe oxygen limitation in the presence of nitrate. A lag phase of about 1 h was observed for both nitrate reduction and nitrogenase activity corresponding to the period of induction of the dissimilatory nitrate reductase. Nitrogenase activity ceased when nitrate was exhausted suggesting that the reduction of nitrate to nitrite, rather than denitrification (the further reduction of nitrite to gas) is coupled to nitrogen fixation. The addition of nitrate to nitrate reductase negative mutants (nr^-) of *Azospirillum* did not stimulate nitrogenase activity. Under oxygen-limited conditions *A. brasilense* and *A. lipoferum* were also shown to reduce nitrate to ammonia, which accumulated in the medium. Both species, including strains of *A. brasilense* which do not possess a dissimilatory nitrite reductase (nir^-) were also capable of reducing nitrous oxide to N_2.

Key words: *Azospirillum* – Denitrification – Nitrogen fixation.

Spirillum lipoferum Beijerinck, a microaerophilic nitrogen-fixing bacterium found to be associated with several grain and forage grasses (Day and Döbereiner, 1976; Döbereiner and Day, 1976) is widespread in tropical regions (Döbereiner et al., 1976). On the basis of DNA homology studies, this organism has recently been reclassified into a new genus containing two species, i.e., *Azospirillum brasilense* and *Azospirillum lipoferum* (Krieg, 1977; Tarrand et al., 1978) and henceforth will be described by this nomenclature.

Azospirillum is capable of a number of different nitrogen transformations. Under aerobic conditions, it can utilize both ammonia (Okon et al., 1976, 1977b) and nitrate (Neyra and van Berkum, 1977) for growth, and under microaerophilic conditions it is capable of rapid growth with dinitrogen as the sole source of nitrogen (Okon et al., 1976). Maximum nitrogen fixation (acetylene reduction) occurs at a dissolved oxygen concentration corresponding to a pO_2 of 0.607 kN/m^2 (Okon et al., 1977a, b). Under anaerobic conditions, nitrate is dissimilated and can serve as an alternative electron acceptor (Neyra et al., 1977; Neyra and van Berkum, 1977). Some strains of *A. brasilense* are able to reduce nitrate to nitrite only, while others reduce nitrite to nitrous oxide and nitrogen gas (Neyra et al., 1977). Furthermore, nitrate respiration appears to support nitrogen fixation under anaerobic conditions (Neyra and van Berkum, 1977). Denitrification and nitrate-dependent nitrogenase activity have also been demonstrated in *Rhizobium* (Zablotowicz et al., 1978; Rigaud et al., 1973).

The objectives of this work were to study further the inter-relationship between nitrate reduction and nitrogen fixation in *Azospirillum lipoferum* and *A. brasilense*.

A preliminary report of this work has been presented (Scott and Scott, 1978).

Materials and Methods

Five strains of *Azospirillum brasilense*, Sp 7 (ATCC 29145) Sp 13, Sp 34, Sp 82 and Sp Br 14 and one of *A. lipoferum*, Sp USA 5 were used in these experiments. Strains Sp 7 and Sp 13 are active denitrifiers and reduce nitrate to nitrite which is further reduced to gas when oxygen is limiting (Neyra et al., 1977). Strains Sp 34, Sp 82 and Sp Br 14 reduce nitrate to nitrite but can not reduce nitrite further (nir^- strains). Otherwise they appear to be identical to other strains of *A. brasilense*

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(Tarrand et al., 1978). Nitrate reductase negative mutants (nr⁻) of *A. brasilense* (Sp13 and Sp34) and *A. lipoferum* (Sp USA 5) were obtained by selection for chlorate resistance. The procedure of isolation and the characterization of these mutant was described by Magalhães et al. (1978). Stock cultures of the bacteria were maintained on nutrient agar slopes at 4 C.

Media and Growth Conditions

A. brasilense and *A. lipoferum* were grown in the N-free semi-solid (0.1 % agar) medium described by Okon et al. (1976). However, the high phosphate concentration used by Okon et al. (1976) was found to inhibit growth of *A. lipoferum*, therefore, for this species the phosphate concentrations was reduced to a fifth. Batch cultures (40 ml), inoculated with 2.5 % inoculum, were grown in 200 ml medical flasks at 35 C for 24 h. At the end of this period, the cultures ($O.D._{560}$, 0.20 – 0.25; light path 1 cm) were thoroughly homogenised and aliquots (5 ml) dispensed into McCartney bottles (27 ml) fitted with rubber stoppers. Triplicate samples were used in all experiments. Due to the poor oxygen protection mechanism of this organism nitrogenase activity is lost by disturbing the cultures and protein synthesis is required for recovery of nitrogenase activity (Okon et al., 1976). Therefore, the flasks were left firmly in a wooden rack for 3 h before the cultures were carefully flushed with helium to lower the oxygen tension to a pO_2 of less than 0.202 kN/m² (as determined by gas chromatography) and potassium nitrate (10 mM) and acetylene (10.132 kN/m²) were added. In some experiments, as indicated in the text, the helium atmosphere was replaced by nitrogen. The cultures were incubated at 35 C in a shaking water bath.

Assays

The concentration of nitrite in the culture samples (0.1 ml) was determined by the method of Nicholas and Nason (1957).

Ammonia was determined colorimetrically, using Nessler's reagent, in cell supernatants clarified by centrifugation at 6,000 × g for 10 min.

Protein was estimated by the procedure of Lowry et al. (1951) using bovine serum albumin as the standard.

Acetylene reduction was determined by gas chromatography in a Perkin Elmer gas chromatograph using a Porapak N column (2 m × 3 mm) at 110 C and hydrogen flame ionization detection. Oxygen and nitrous oxide were determined at ambient temperature using a Carle Portable Gas Chromatograph Model 7500 fitted with either a molecular sieve column (1 m × 3 mm) or a Porapak Q column (1 m × 3 mm), respectively and a thermal conductivity detector.

Results

Denitrification Studies

Denitrification in *Azospirillum* is favoured by a neutral pH (Fig. 1). The reduction of nitrate to nitrite proceeded rapidly at both pH 7 and pH 8 but the further reduction of nitrite was more sensitive to pH changes and was maximal at pH 7. Acetylene (10.132 kN/m²), a known inhibitor of nitrous oxide reductase (Yoshinari and Knowles, 1976), was present in all vials. At the end of the experiment levels of 1.02, 2.36, and 0.77 µmol of nitrous oxide per flask were found for cultures incubated in media of pH 6, 7, and 8 respectively.

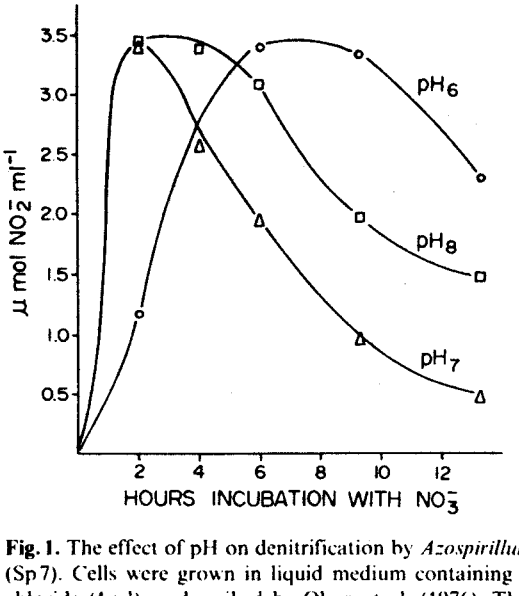

Fig. 1. The effect of pH on denitrification by *Azospirillum brasile* (Sp 7). Cells were grown in liquid medium containing ammoni chloride (1 g/l) as described by Okon et al. (1976). The cells w harvested by centrifugation at 8000 × g and then resuspended in same medium adjusted to pH 6, 7, or 8. All other conditions are described for the nitrate dependent nitrogenase studies (see "*Me ods*")

Table 1. Dissimilation of nitrous oxide by *Azospirillum brasile* (Sp 7 nir⁺; Sp Br 14 nir⁻) under oxygen limited conditions. Cells w grown in liquid media containing ammonium chloride (1 g/l) described by Okon et al. (1976). Aliquots (5 ml) of the culture w then transferred to McCartney bottles (see "*Materials a Methods*") and the atmosphere replaced with nitrogen. Nitrous oxi (0.01 atm) was added to bottles with and without acetyle (10.132 kN/m²) and the cultures were then shaken vigorously at 35

Time (h)	N_2O (µmol flask)			
	$-C_2H_2$		$+C_2H_2$	
	Sp 7	Br 14	Sp 7	Br 14
0	7.6	7.1	7.6	6.8
2	2.4	5.6	7.1	6.8
4	0.9	3.8	5.8	5.7
6	0.4	0.3	5.4	6.4
8	0	0.3	6.7	4.7
22	0	0	6.9	5.3

The time course of nitrous oxide disappearance wi and without acetylene was studied using a denitrifyi strain of *A. brasilense* (Sp 7) and a strain (Sp Br 14) th appears to lack the dissimilatory nitrite reducta (nir⁻). In the absence of acetylene a rapid disa pearance of nitrous oxide was observed with bo strains (Table 1). The experiment was repeated wi strain Sp 82 (nir⁻) with similar results (not shown). *brasilense* nir⁻ strains thus possess the enzyme compl for this step in the denitrification pathway even thou

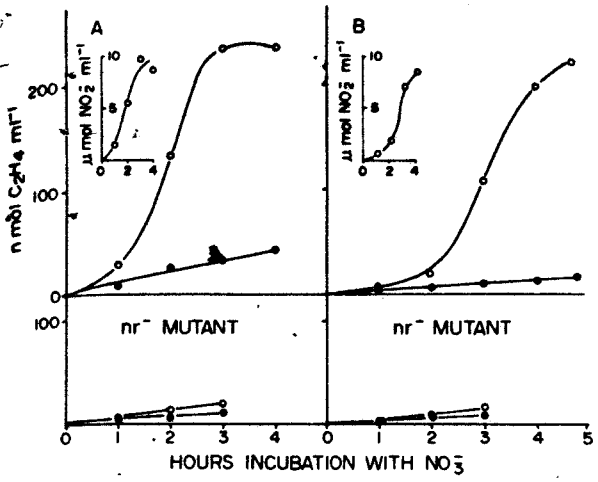

Fig. 2. The effect of nitrate on nitrogenase activity (ethylene accumulation with time) under oxygen limiting conditions in two strains of *A. brasilense* (**A**) Sp13 (nir$^+$) and (**B**) Sp Br82 (nir$^-$) and in nitrate reductase negative mutants. Control (●); plus 10 mM potassium nitrate (○). The inserts show the time course of nitrate reduction. No nitrite was formed with the mutants

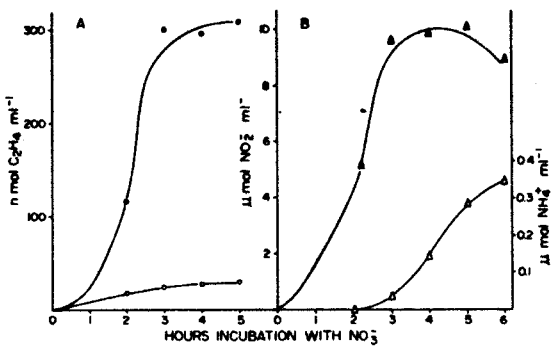

Fig. 3. The effect of nitrate on nitrogenase activity and the reduction of nitrate (10 mM) to nitrite and ammonia under oxygen limiting conditions in *A. lipoferum* (Sp USA 5). Control (○); plus 10 mM potassium nitrate (●); nitrite (▲); ammonia (△)

they cannot reduce nitrite. The inability of these strains to reduce nitrite to nitrous oxide (Tarrand et al., 1978; Neyra et al., 1977) was observed under various growth conditions and seems to be due to a lack of the dissimilatory nitrite reductase. The nitrous oxide reduction observed could not have been catalysed by nitrogenase as the cells had been grown in the presence of ammonium chloride. In the presence of acetylene (10.132 kN/m^2), little loss of substrate occurred during the period examined (Table 1).

Nitrate Dependent Nitrogen Fixation

The results presented in Fig. 2 show that the addition of nitrate to severely oxygen-limited cultures of *A. brasilense* strains Sp13 (nir$^+$) and Sp82 (nir$^-$) resulted in a rapid stimulation of nitrogenase activity. Similar results were obtained with strains Sp7 (nir$^+$) and Sp Br14 (nir$^-$). The time course of nitrite accumulation was coincident with the pattern for nitrogenase activity. An initial lag phase of about 1 h was observed. Addition of chloramphenicol, at a concentration (50 µg/ml) which does not inhibit nitrogenase activity (Okon et al., 1976), resulted in an inhibition of the nitrate-dependent nitrogenase activity (data not presented). Upon exhaustion of nitrate, the nitrogenase activity in oxygen-limited cultures returned to the low basal rate observed without nitrate (Fig. 2). The necessity of a functional nitrate reductase for nitrogenase, activity under oxygen-limited conditions was confirmed by the use of two nitrate reductase negative (nr$^-$ nir$^-$) mutants. These mutants showed no increase in nitrogenase activity and no reduction of nitrate to nitrite in the presence of nitrate (Fig. 2). Under microaerophilic conditions they exhibited high rates of nitrogen fixation, even in the presence of 10 mM nitrate (Magalhães et al., 1978).

Following the confirmation of two distinct species of *Azospirillum* (Tarrand et al., 1978) it was necessary to extend these physiological studies to *A. lipoferum*. Nitrate-dependent nitrogenase activity was also demonstrated in this species (Fig. 3A) and the rates observed seemed higher than those for *A. brasilense* (Fig. 2). In these experiments, as in those described by Neyra and van Berkum (1977) no growth was observed in cultures incubated under anaerobic conditions in the presence of nitrate. Because of this, we examined culture supernatants for ammonia at intervals during nitrogen fixation under a nitrogen atmosphere. Ammonia (0.05 ± 0.01 µmol/ml) was detected in the medium after a period of 3 h (Fig. 3B). However, cultures incubated under an atmosphere of helium had similar levels of ammonia at this time, suggesting that the ammonia was formed by nitrate reduction rather than nitrogen fixation. Furthermore, nitrogen fixation (acetylene reduction) had ceased at this time (Fig. 3A). Ammonia is first detectable at 3 h and increases over a time period corresponding to nitrite disappearance (Fig. 3B). There was no formation of ammonia in controls without nitrate. Similar results were obtained for *A. brasilense* (Sp13) under identical conditions.

Discussion

The results presented here demonstrate that *Azospirillum brasilense* (nir$^+$), *A. brasilense* (nir$^-$) and *A. lipoferum* are all capable of nitrogen fixation under conditions of severe oxygen limitation, using nitrate as an alternative electron acceptor. In the absence of

nitrate, some nitrogenase activity was still detectable. This may be due to trace amounts of oxygen present in the flasks or to the ability of an organic component of the medium to serve as an alternative electron acceptor, since *Azospirillum* does not fix nitrogen under totally anaerobic conditions (Okon et al., 1976). In all experiments, a lag period was observed before onset of nitrogenase activity, which corresponds to the period of induction of the dissimilatory nitrate reductase. In several bacteria, this enzyme is a membrane-bound protein preferentially synthesized under oxygen-limiting conditions in the presence of nitrate (Delwiche and Bryan, 1976; Haddock and Jones, 1977). Mutants of *Azospirillum* lacking the dissimilatory nitrate reductase were unable to fix nitrogen under oxygen-limited conditions, indicating that energy coupling to nitrate respiration is necessary to provide ATP for nitrogenase activity. The nitrogenase activities observed in these experiments are comparable to those observed under microaerophilic conditions (Magalhães et al., 1978) and are substantially higher than the rates observed by Rigaud et al. (1973) in *Rhizobium* bacteroids under similar conditions. These authors attributed the relatively low activities and eventual loss of activity to inhibition by nitrite, which accumulates in the media. However, *Azospirillum* spp. maintained high nitrogenase activities even in the presence of 10 mM nitrite. Nitrogenase in microaerophilic cultures (semi-solid medium) and also in vitro nitrogenase activity of *Azospirillum* is extremely sensitive to nitrite. But when respiring nitrate this organism must possess a mechanism which permits exclusion of nitrite from the cell (Neyra and van Berkum, 1977). Nelson and Knowles (1978) were unable to observe nitrogenase activity in denitrifying *Azospirillum brasilense* continuous cultures probably because under these conditions nitrite was not or incompletely excluded from the cells. The phenotypic expression of nif^{+}_{Kp} genes on a plasmid, introduced into various nitrate reductase defective mutants of *E. coli* was tolerant to 50 mM nitrate in two of the mutants but nitrite almost completely inhibited nitrogenase activity in all mutants and parent strains (Skotnicki and Rolfe, 1977). Nevertheless several mutants presumably defective in nitrate reduction (Chl^{-}) showed inhibition of nitrogenase activity by nitrate. This was interpreted by the authors as effects independent of nitrate reduction. More difficult to understand is the nitrate dependent nitrogenase activity which was observed by the same authors in one *Klebsiella pneumoniae* mutant (Chl^{-}) presumably defective in nitrate reduction. This mutant was also nitrogenase defective and nitrogen fixation had been partially restored by the above mentioned nif^{+}_{Kp} plasmid. The nif^{+} parent strain of *K. pneumoniae* was unable to fix nitrogen in the presence of 50 mM nitrate.

In several strains of *Azospirillum*, the nitrite produced under anaerobic conditions can be further reduced to nitrous oxide (Neyra et al., 1977). In the studies carried out here we have demonstrated that part of the nitrite can be reduced to ammonia. The appearance of ammonia in the medium coincided with nitrite disappearance. Reduction of nitrate to ammonia has also been observed in *Klebsiella* spp. under anaerobic conditions (Hadjipetrou and Stouthamer, 1965; Kleiner, 1976), although this organism does not reduce nitrate to gaseous products. Ammonia is also a major product of nitrate dissimilation in *Clostridium perfringens* (Hasan and Hall, 1975; Woods, 1938).

Azospirillum is considered to be an important nitrogen fixing organism in association with several tropical grain and forage grasses (Döbereiner and Day, 1976). However, under conditions of oxygen limitation, such as occur in waterlogged soils, this organism may contribute to losses through denitrification. The ability of *Azospirillum* to couple nitrate respiration to nitrogen fixation under oxygen limitation as well as to reduce nitrate to ammonia are intriguing processes demanding further study.

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