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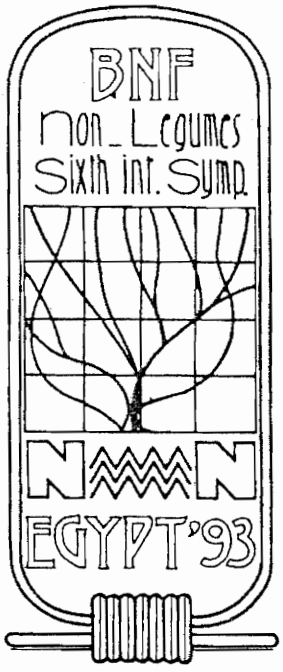

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Azospirillum and Related Organisms: Ecological, Physiological, Biochemical and Genetical Aspects

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Abstract. This article summarizes recent data on the subjects and includes new, unpulished results. Several types of enzymes are involved in the generation and utilization of nitrous oxide by microorganisms. Nitrite produced in nitrate respiration by *Azospirillum* can exert phytohormonal effects similar but not identical to auxin (= indole-3-acetic acid) in several plant assays. When supplied with tryptophan, *Azospirillum* excretes large amounts of auxin into the medium. Evidence is now presented that auxin is mainly synthesized from tryptophan by the pyruvate decarboxylase pathway in *Azospirillum*. DNA amplification fingerprinting using short arbitrary primers is shown to be a useful tool to mark the different *Azospirillum* strains. DNA-DNA hybridization experiments have been started to screen for the distribution of denitrification genes in strains of the genus *Azospirillum*. The preliminary results indicate that the denitrification genes are more widespread in this genus than believed hitherto. *Escherichia coli* is found to reduce nitrous oxide to dinitrogen by an unidentified enzyme.

The formation and utilization of nitrogenous oxides by microorganisms

In bacteria, several pathways are involved in the formation or utilization of the gaseous nitrogenous oxides nitrous oxide (N_2O) and nitric oxide (NO). In contrast, nitrogen dioxide is believed to be formed by the chemical oxidation of nitric oxide.

a) **Dinitrogen fixation.** This reaction is catalyzed by nitrogenase according to the equation: $N_2 + 8\ H^+ + 8\ e^- \Rightarrow 2\ NH_3 + H_2$. This enzyme complex can also catalyze the reduction of several other substrates which have a triple bond in common (Burns and Hardy, 1975; Bothe et al., 1982). Among these, the reduction of N_2O to N_2 should be mentioned in this context. NO is not reduced by nitrogenase.

b) **Denitrification.** In this pathway, nitrate is reduced to nitrite as respiratory electron acceptor alternative to O_2. The product formed, nitrite, is then further converted to the gases NO, N_2O and N_2. Denitrifying bacteria can be distinguished by the possession of their type of nitrite reductase. The best investigated species, *e. g. Pseudomonas stutzeri*, *Paracoccus denitrificans* and *Azospirillum brasilense* possess a cytochrome c,d_1 containing enzyme catalyzing the reduction of nitrite to NO. A separate

enzyme with both heme b and c in the prosthetic group then performs the reduction of NO to N_2O (for the literature see Bothe, 1992). Other bacteria, *e. g. Achromobacter cycloclastes* , *Alcaligenes xylosoxidans, Rhodobacter sphaeroides* f. *denitrificans* (see Coyne and Tiedje, 1990) express a non-heme nitrite reductase with Cu in the prosthetic group. This enzyme apparently reduces NO_2^- directly to N_2O without the formation of free NO. In all organisms N_2O- reductase catalyzing the formation of N_2 is a multi Cu-enzyme which may exceptionally contain additionally cytochrome c as shown for *Wolinella succinogenes* (Zhang et al., 1991). Dissimilatory nitrate reductase contains the molybdopterine cofactor in all denitrifying bacteria investigated thus far. The production of N_2O and NO by nitrifying bacteria probably involves the same enzymes as in denitrifying microorganisms.

c) **Assimilatory and dissimilatory nitrate reduction.** It is known for some time that assimilatory nitrate reductase of plants can reduce NO_2^- to NO and N_2O in a side reaction (Klepper, 1987). This reaction is also performed by nitrate reductase of *E. coli* which is likely a dissimilatory enzyme.The activity in this organism amounts to about 5 % of the rates of either nitrate or nitrite reduction and must be attributed to nitrate reductase as mutants defective in this enzyme do not perform this gas production (Satoh et al., 1983; Smith, 1983). In *E. coli* (Ji and Hollocher, 1988) and *Pseudomonas aeruginosa* (Voßwinkel et al., 1991), the formation of NO and N_2O is affected by the amount of nitrite and the pH in the medium. Lower concentrations of nitrite and neutral pH-values favour N_2O- production whereas NO is formed at high nitrite amounts and at pH-values around 6.0 .

d) **Nitrate ammonification.** This pathway typically occurs in the Enterobacteriaceae. Nitrite is formed from nitrate and then reduced to ammonia without the generation of any free intermediate. Nitrate reduction in *Escherichia coli* and other enteric bacteria serves in the removing of reducing equivalents generated during fermentative growth of these bacteria. *E. coli* and other enteric bacteria were believed not to reduce NO or N_2O until recently (see , however, the end of this article).

The importance of the production of NO_x (NO, NO_2, also N_2O) as of N_2 can be summarized as follows:

(i) The production of gaseous nitrogen by denitrification or nitrification results in a loss of combined nitrogen(nitrate, ammonia) otherwise available for the growth of plants. (ii) NO_x is deleterious to plants, because products formed from it contribute to the acid rain depositions, N_2O is a significant component of the greenhouse effect and NO and NO_2 interfer with the ozon layer of the stratosphere. (iii) Denitrifying bacteria (particularly the aerobically growing ones) may be used in waste water treatments to remove combined nitrogen by the conversion to N_2. (iv) In medicine, NO has been recognized as a signal transmitter in brain.

It is not aimed in the present communication to extensively review the literature relevant in the field. The data obtained from the own investigations on denitrification by *Azospirillum* will be summarized briefly, and it will also be communicated that, unexpectedly, *E. coli* is able to reduce N_2O to N_2.

Summary of the data obtained from *Azospirillum* experiments

a) *Azospirillum* grows anaerobically either with nitrate, nitrite or with nitrous oxide as sole respiratory electron acceptor (Zimmer et al., 1984; Penteado Stephan et al., 1984).

b) *Azospirillum* possesses a cytochrome c,d_1-type nitrite reductase (Danneberg et al., 1986). The occurrence of a NO-reductase is to be expected but has not been investigated yet.

c) Determinations of the maximal molar growth yields and measurements of the H^+-translocation using the oxidant pulse method independently of each other indicated that *Azospirillum* grows as efficiently with N_2O as with O_2 as terminal respiratory electron acceptor. Growth yields were 1/3 lower with nitrite and 2/3 lower when nitrate was the sole electron acceptor in respiration (Danneberg et al., 1989).

d) Experiments performed with a model system using germinated wheat seeds and *Azospirillum* showed that the bacterium performs either denitrification or nitrogen fixation in the association, depending on the availability of combined nitrogen in the medium. In such experiments performed for one week the bacteria had lived exclusively from the organic carbon supplied by the plant roots (Neuer et al., 1985).

e) *Azospirillum* can perform nitrate dependent N_2-fixation, when nitrate is added to N_2-fixing cells under anaerobic growth conditions. This is, however, only a transitory reaction proceeding only as long as the enzymes of assimilatory nitrate reduction are expressed by the cells (Bothe et al., 1981).

f) The bacterium excretes nitrite up to 3 mM in liquid cultures grown anaerobically with nitrate as respiratory electron acceptor. The nitrite formed exerts phytohormal effects, similar but not identical to auxin (indole-3 acetic acid) in several phytohormone assays. Nitrite probably acts together with ascorbic acid in plant cells (Bothe et al., 1992). Nitrite particularly induces the formation of additional lateral roots which can also be achieved with *Azospirillum* but not with auxin. This is a new avenue to explain the interactions between *Azospirillum* and plants (Zimmer et al., 1988).

g) *Azospirillum* excretes indole acetic acid into the medium in dependence of the amount of tryptophan supplied. Other phytohormones are not excreted into the medium (or at least not in such amounts which could affect plant growth) (Zimmer and Bothe, 1988).

h) *Azospirillum* forms and utilizes NO only with low activities in contrast to a newly isolated strain of *Pseudomonas aeruginosa*. Both *Azospirillum* and *P. aeruginosa* do not grow and do not form an H^+-gradient with NO as the sole respiratory electron acceptor. In contrast, *Paracoccus denitrificans* forms a gradient of 3.5 H^+/NO in oxidant pulse experiments. This bacterium, however, does not grow with NO, probably due to the toxicity of this compound to the *Paracoccus* cells (Voßwinkel et al., 1991).

The biosynthesis of auxin by *Azospirillum brasilense*

The biosynthesis of the auxin indole-3-acetic acid (=IAA) is still an open field in bacteria and also in plants. As reviewed in detail (Elmerich et al., 1992; Hartmann and Zimmer, 1993), two pathways are probably involved in the tryptophan (=Trp)-dependent synthesis of IAA in *A. brasilense*: (i) the pathway involving indole-3-acetamide as an intermediate, (ii) the pathway with the intermediates indole-3-pyruvate (=IPyr) and indole-3-acetaldehyde. There are some actual data suggesting the existence of an indole-3-acetamide pathway for *A. brasilense* Sp7 (Bar and Okon, 1993). However, after an incubation of 4 days, the concentration of IAA formed from indole-3-acetamide amounted to 1/1000 of that produced from Trp under identical assay conditions. The indole-3-acetamide-pathway likely plays at best a minor role in the synthesis of IAA in *Azospirillum*.

Our former studies had shown that *A. brasilense* Sp 7 could neither convert indole-3-acetonitrile and tryptamine, the intermediates of other known biosynthetic pathways for the biosynthesis of IAA, nor significant amounts of indole-3-acetamide compared to Trp (Zimmer and Elmerich, 1991). The bacterium formed IAA when grown in the presence of indole-3-lactate or indole-3-ethanol (Zimmer and Elmerich, 1991). As the latter two indole-derivatives were the reduced compounds of the instable intermediates IPyr and indole-3-acetaldehyde of IAA biosynthesis these findings could be taken as an indirect hint for the existence of the IPyr-pathway in *Azospirillum*.

In order to show that the IPyr-pathway occurs in *Azospirillum*, IPyr or Trp was added to the culture just after the bacteria had reached the stationary phase, and further incubation was limited to 16 h, as IPyr is known to be instable in solution. After the end of the experiment the concentration of IAA was determined by HPLC.

These experiments (Table 1) led to the following conclusions: (i) During the short incubation time of 16h, the IAA formation by *A. brasilense* was strictly dependent on the presence of Trp or IPyr. (ii) Most of the IAA formed in case of the incubation in presence of IPyr was enzyme catalyzed and not due to chemical degradation as shown by the control with boiled samples . (iii) IPyr is directly converted to IAA and not via Trp and then by an other pathway. This can be concluded as the conversion to Trp could not be inhibited by phenyl- or hydroxyphenyl-pyruvate which are competitive inhibitors for the conversion of IPyr to Trp by aromatic aminotransferases. These experiments indicate the existence of an IPyr-decarboxylase in *Azospirillum* as it has been published for *Enterobacter cloacae* (Koga et al., 1991).

In order to isolate the structural and regulatory genes involved in IAA biosynthesis of *A. brasilense* Sp7, recently a segment of the IPyr-decarboxylase was amplified by PCR successfully using primer deduced from the published sequence of *Enterobacter cloacae*. A clone from a Lambda gene bank of the *A. brasilense* genome could be identified by hybridizing with this segment as a probe. The sequencing of this clone is underway.

Table 1: Tryptophan or indole-3-pyruvate dependent formation of indole-3-acetate by cultures of *A. brasilense* Sp7

conditions			concentration of indole-3-acetate in the culture [mg/l]
culture			0.1
culture	+ tryptophan		41.1
culture	+ indole-3-pyruvate		32.5
boiled	+ tryptophan		0.2
boiled	+ indole-3-pyruvate		10.5
culture	+ trytophan	+ phenyl-pyruvate	40.8
culture	+ indole-3-pyruvate	+ phenyl-pyruvate	36.3
culture	+ tryptophan	+ hydroxyphenyl-pyruvate	42.6
culture	+ indole-3-pyruvate	+ hydroxyphenyl-pyruvate	33.7

The cultures were grown aerobically in minimal medium at 30 °C. After 48 h ($O.D._{560nm}$ = 1.6) 40 mg/l tryptophan, indole-3-pyruvate, phenyl-pyruvate or hydroxyphenyl-pyruvate were added and incubation was continued at 30 °C for 16 h. The concentration of indole-3-acetic acid was determined by HPLC (separation on a C-18 column, ethanol/5% acetic acid 30:70 (v/v) as mobil phase, detection at 280 nm).

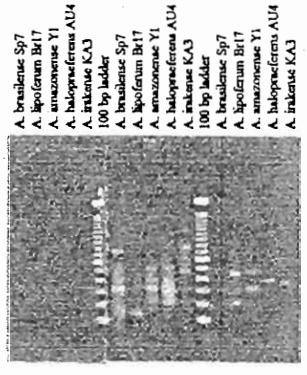
In summary, the existence of the IPyr-decarboxylase pathway in *Azospirillum* is now fairly conclusive. The construction of mutants defective in this pathway will elucidate the biosynthesis of IAA by *Azospirillum* and the role of this phytohormone in the *Azospirillum*-plant association.

DNA-probing to monitor the distribution of denitrification genes within the genus *Azospirillum*

It had been suggested (Krieg and Döbereiner, 1986) that the distribution of enzymes involved in denitrification is a good criterion for the classification of *Azospirillum* strains. Activity measurements (Neyra et al., 1977) indicated that only approximately half of the strains of *A. brasilense* and *A. lipoferum* dissimilate nitrite to nitrous oxide and dinitrogen. Claims were also forwarded that plant roots are infected almost exclusively by non-denitrifying strains (Magelhaes et al., 1978). The experiments with a model *Azospirillum*-wheat association (Neuer et al., 1985), however, already indicated that the capability for denitrification is much more widespread within the genus *Azospirillum* than believed hitherto. This laboratory recently used DNA-probes of dissimilatory nitrite and nitrous oxide reductases to screen for the distribution of denitrifying bacteria in soils by DNA probing. The examination of five different soils showed that denitrifying bacteria pre-

ferentially associated with the roots of plants, whereas nondenitrifying predominantly colonized the bulk, plant-free soil (Linne von Berg and Bothe, 1992). A similar approach is currently underway to monitor the distribution of denitrifying genes within the genus *Azospirillum*. To start, the *Azospirillum* strains available in our laboratory were characterized by DNA amplification fingerprinting using very short arbitrary oligonucleotide primers (see Caetano-Anolles et al., 1991; Carlson et al., 1991). Primers of 8, 9 and 10 nucleotides in length were used to get a DNA banding pattern after amplification of the genomic DNA by the polymerase chain reaction (PCR) and separation of the bands by agarose gel electrophoresis. Fig 1 shows that the 10b primer gave a distinct pattern with each of the DNA templates isolated from five *Azospirillum* strains. The 9b primer could also be used, whereas the 8b oligonucleotide synthesized did not show any amplification product (probably because of its choice which was not optimal). The technique allows to characterize all *Azospirillum* strains investigated by the specific spectrum of their short DNA products (Fig 2).

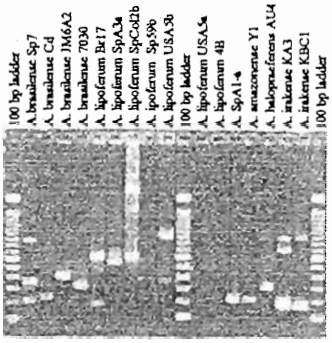
Fig. 1 : The characterization of five *Azospirillum* strains by DNA amplification fingerprinting using short oligonucleotide primers of different length



Genomic DNA (0.1ng) was used from all strains. The arbitrary primers used were : 8b = 5' - GGA AGA GC - 3' ; 9b = GAG GGT GCC - 3'; 10b = 5' -CAA AGC CTC G - 3' . Amplification was performed by standard protocols with an annealing temperature of 42° C.

Southern hybridizations were then performed using the dioxigenin-labeled gene probe (an 1,2 kb fragment) of dissimilatory nitrous oxide reductase from *Pseudomonas stutzeri* (Viebrock and Zumft, 1988) and genomic DNA isolated from the different *Azospirillum* strains (Table 2). DNA-DNA hybridization signals were obtained with all *A. brasilense* and *A. lipoferum* strains tested and also with *A. halopraeferens*. The DNA isolated from *A. amazonense* and *A. irakense*, however, did not give hybridization signals in the stringency range employed. It is noteworthy that *A. brasilense* 245, a strain isolated from wheat roots, was positive in the assays. The results are totally in accord with previous activity measurements with the wheat-*Azospirillum* association (Neuer et al., 1985).

Fig. 2 : The characterization of selected *Azospirillum* strains by DNA amplification fingerprinting



The 10 b primer was used for amplification . The conditions were the same as in Fig.1

Table 2: The distribution of the N_2O-reductase gene within the genus *Azospirillum* monitored by DNA-probing using a gene probe of the enzyme from *Pseudomonas stutzeri*

strain assayed		size of the hybridization signal
Azospirillum brasilense	Sp7	> 20 kb
	Cd	> 20 kb
	JM6A2	> 20 kb
	245	~ 20 kb
Azospirillum lipoferum	Br17	10 kb and >20 kb
	SpA3a	~ 14 kb
	SpCol2b	> 20 kb
	Sp59b	~ 18 kb
	USA5b	> 20 kb
	4B	> 20 kb
Azospirillum amazonense	Y1	none
	Y2	none
	Y6	none
Azospirillum halopraeferens	AU4	~19 kb
Azospirillum irakense	KA3	none
	KBC1	none

Isolated genomic DNA (2 µg) was digested with *Eco*RI, separated by agarose gel electrophoresis and transferred onto nitrocellulose filters. The N_2O-reductase fragment (1.2 kb) from *Pseudomonas stutzeri* (Viebrock and Zumft, 1988) was labeled with digoxigenin by PCR with specific oligonucleotide primers. Hybridization was performed at 68 °C overnight.

Experiments are currently planned with more *Azospirillum* strains, and probes will also be developed for dissimilatory nitrate and nitrite reductases, particularly from *Azospirillum*, to extend this type of investigation. At the current experimental state generalisations are not yet allowed. However, the capability for denitrification is obviously more widespread than believed hitherto.

The occurrence of a nitrous oxide reductase in *Escherichia coli*

In a screening program for the distribution of denitrification activities in microorganisms, we noted, to our surprise, that *E. coli* also is able to reduce N_2O quantitatively to N_2 (for details see Kaldorf et al., 1993). The overall specific rate amounts to approximately 1.9 µmol/ mg protein x h in intact cells and is thus in the range of the activities of other enzymes involved in nitrate ammonification of this bacterium. The reaction was followed by measuring the consumption of N_2O and the formation of N_2 both by gas chromatography. In addition, this enzyme activity was also proven to occur in *E. coli* by monitoring the formation of $^{15}N_2$ from $^{15}N_2O$ in a mass spectrometer. The expression of this activity requires anaerobic conditions and is under control of the *fnr*-system as shown by an experiment with a *fnr*$^-$-mutant. The apparent K_m (about 3 mM) is very high which may explain that the reaction has not been described as yet. Acetylene and nitrite are effective inhibitors whereas azide is ineffective. The reduction of N_2O by *E. coli* is not coupled with the generation of energy as the bacterium does not grow with N_2O as sole respiratory electron acceptor. The reaction is not restricted to *E. coli* but also was demonstrated in *Yersinia kristensenii* and *Buttiauxella agrestis* of the Enterobacteriaceae. The enzyme involved might catalyze something else under physiological conditions and could not be identified as yet. It is, however, neither nitrogenase nor the typical nitrous oxide reductase found in denitrifying bacteria. Thus microorganisms have now been described to possess three different types of enzymes catalyzing the reduction of N_2O to N_2.

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