SOME PHYSIOLOGICAL AND BIOCHEMICAL PROPERTIES OF DENITRIFICATION BY AZOSPIRILLUM BRASILENSE

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Summary: Previous experiments from this laboratory showed that *Azospirillum brasilense* Sp 7 can grow anaerobically with either nitrate, nitrite or nitrous oxide as respiratory electron acceptors. Molar growth yield measurements now indicate that *Azospirillum* obtains the same amount of energy with NO_2^- and O_2 as respiratory electron acceptors but only 1/3 of that with NO_3^-. Dissimilatory nitrite reductase catalyzing the reduction of NO_2^- to N_2O is shown to be a cytochrome c, d containing enzyme. A soluble dissimilatory nitrite reductase preparation also catalyzes the formation of nitric oxide which is probably an artifact of the cell-free system. The expression of dissimilatory N_2O-reductase in *Azospirillum* is dependent on Cu in the medium.
Key words: dissimilatory nitrate reduction, nitrite reduction, denitrification, nitrous oxide, nitric oxide, nitrite.

Introduction

The role of *Azospirillum* in enhancing crop productivity has extensively been discussed in the preceeding two workshops on *Azospirillum*. The bacterium may provide excess of fixed nitrogen formed by N_2-fixation to the roots of cereals. Alternatively it could stimulate the growth of the roots and their mineral uptake by excreting phytohormones when living on the surface or inside of the roots (1,2). Generally, *Azospirillum* is only discussed as being beneficial to crop plants. However, the bacterium may cause just the opposite under unfavorable conditions. Similar as *Rhizobium* (3), *Azospirillum* can utilize either nitrate,nitrite or nitrous oxide as respiratory electron acceptor (4,5, 6, 7). The final products of these dissimilatory reductions can be either

Azospirillum III: Genetics, Physiology, Ecology
Ed. by W. Klingmüller
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nitrite or gaseous nitrous oxide or dinitrogen (6,7). Model experiments with wheat-*Azospirillum* associations showed that *Azospirillum*, indeed, performs either N_2-fixation (C_2H_2-reduction) or denitrification (N_2O-formation), depending on the amount of nitrate and/or oxygen available(8). Both activities were strictly dependent on the growth temperature of the association and on the *Azospirillum* strain used (8). The *Azospirillum* strains available are not yet well characterized with respect, to denitrification. Some strains appear to reduce nitrate only to nitrite, whereas others can convert nitrate via nitrite to nitrous oxide and molecular nitrogen (9). The physiological conditions for maximal gas production are partly understood (6,7). Likewise, the biochemical properties of the enzymes involved in denitrification have not yet been elucidated for *Azospirillum*.

Our group is currently trying to characterize the physiological and biochemical properties of denitrification by *Azospirillum*. Some results on nitrate, nitrite and nitrous oxide dissimilation will be presented here.

Materials and Methods

Azospirillum brasilense Sp 7 (ATCC 29145) was used in the present investigation. For growth yield measurements, *Azospirillum* was grown in a chemostatic culture(6) with L- malate limiting. Details are described elsewhere (W. Zimmer, Diplomarbeit, Köln, 1984). For preparing a cell-free preparation performing dissimilatory nitrite reduction, bacteria were used which had been grown anaerobically in continuous culture with nitrite as the respiratory electron acceptor (6) and with DL-malate as the carbon source. The cells were harvested by centrifugation (10 min, 16 000 g), suspended in 10 mM K-phosphate buffer pH 7.5 and broken twice in a French Press at 140 000 kPa. The extract was centrifuged (30 min, 12 000 g) and the supernatant was spun down once more (2h, 100 000 g). The pellet was then suspended in 10 mM K-phosphate buffer pH 7.5 and assayed for activity. All preparations were performed in air. The assays were performed in 7.0 ml Fernbach flasks containing in a final volume of 3 ml: extract with 0.54 mg protein, Na-nitrite 20 µmol, phenazine methosulfate 0.8 µmol, K-phosphate buffer

pH 7.5 500 µmol. The assays were started by adding 50 µmol Na-ascorbate. The reaction was performed for 45 min at 30°C under argon in a shaking water bath. The amount of N_2O formed was determined by gas chromatography. NO formation was followed amperometrically in a separate assay by using a conventional Clark-type-electrode (10). Nitrite was determined colourimetrically with the naphthylamine/sulphanilic acid reagent.

For the growth of _Azospirillum_ under anaerobic conditions with N_2O as the respiratory electron acceptor, a batch culture grown with NH_4Cl (5.0 mM) as the nitrogen source was used as the starting culture. Growth of the cells under different conditions was followed in 100 ml flasks containing _Azospirillum_ NH_4^+-medium, EDTA and the different salts tested as indicated in Table 3 and Fig.2. The flasks were inoculated with 0.1 ml of the starting culture (O.D. 560 nm = 0.06) and evacuated and gassed with argon. The assays were started by adding 20 % (v/v) N_2O, and the cells were grown for 16 h at 30°C in a shaking water bath. Cell growth was monitored by measuring the optical density at 560 nm.

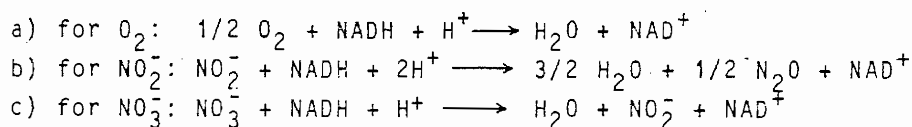
Results and Discussion

Previous results had shown that _Azospirillum_ grows in continuous culture with either O_2, nitrate or nitrite as respiratory electron acceptor (6). Table 1 compares the maximal molar growth yields (Y_S^{max}, determined for infinite dilution rates in continuous cultures) which we now determined for the respiratory electron acceptors O_2,NO_3^- and NO_2^-. The maximal growth yield is 48 g cells/mol malate consumed in the case of O_2 and NO_2^-, but only 15.3 with NO_3^-. Such findings are not surprising, because the free energy change ($\Delta G_0'$) is only 162 KJ/mol for the reduction of nitrate to nitrite by NADH, but is 220 or 229 KJ/mol for the reduction of O_2 or NO_2^-, respectively, by NADH (11, see Table 1). The data indicate that the same amount of energy (ATP) is obtained when O_2 or NO_2^- is used as respiratory electron acceptor, but only 1/3 of that with nitrate. The same conclusion follows from other independent measurements which will be published elsewhere (Zimmer and Bothe, in preparation).

Table 1. Molar growth yields (Y_S^{max}) of a malate grown, chemostatic culture of Azospirillum brasilense Sp 7 using different respiratory electron acceptors

Respiratory electron acceptor:	O_2	NO_2^-	NO_3^-
Y_S^{max} (g/mol):	48	48	15.3
ratio Y_S^{max}: Y_S^{max} of the O_2-culture:	---	1:1	0.32:1
$G^{o'}$ (KJ/mol) *	220	229	162

* The $\Delta G^{o'}$- values are taken from Thauer et al. (11) and refer to the reactions:



a) for O_2: $1/2\ O_2 + NADH + H^+ \longrightarrow H_2O + NAD^+$
b) for NO_2^-: $NO_2^- + NADH + 2H^+ \longrightarrow 3/2\ H_2O + 1/2\ N_2O + NAD^+$
c) for NO_3^-: $NO_3^- + NADH + H^+ \longrightarrow H_2O + NO_2^- + NAD^+$

Two different types of dissimilatory nitrite reductases have been described for denitrifying bacteria (12,13). The more commonly distributed enzyme (occurring in Paracoccus denitrificans, Pseudomonas strains and Thiobacillus denitrificans, e. g.) contains cytochrome c,d in the prosthetic group and is blocked by NH_2OH. A non-heme dissimilatory nitrite reductase containing Cu is produced by Alcaligenes faecalis or Rhodopseudomonas sphaeroides f. denitrificans. The latter enzyme catalyzes the reduction of NH_2OH. Previous experiments with intact cells showed that NH_2OH blocks dissimilatory nitrite reduction by Azospirillum sug-

gesting that this bacterium possesses a cytochrome c, d type enzyme (6). This can directly by shown in a preparation from Azospirillum brasilense Sp 7. The preparation was obtained by centrifuging the crude extract at 100 000 g for 2 h, and the supernatant was then fractionated by $(NH_4)_2SO_4$ precipitation. The fraction precipitating between 55 and 90 % $(NH_4)_2SO_4$ could be reduced by phenazine methosulfate/NADH and reoxidized by $NaNO_2$.The difference spectra (reduced by phenazine methosulfate/NADH minus oxidized as obtained after the isolation =straight line) or reduced by phenazine methosulfate/NADH and reoxidized by $NaNO_2$ minus oxidized as obtained after the isolation = dashed line) revealed the presence of six different bands (Fig 1). The bands at 415, 519 and 548 nm might be associated with cytochrome c and those at 460 , 606 and 650 nm are indicative for the occurrence of cytochrome d (cytochrome c_2) (see 14, 15, 16). Thus Azospirillum possesses a cytochrome c,d dissimilatory nitrite reductase. The bacterium may also contain a Cu-enzyme which has not yet been investigated. However, this would be an unusual feature because no bacterium has been described up-till-now which contains both types of dissimilatory nitrite reductases.

Dissimilatory nitrite reductase should be membrane-bound, because it is functioning in respiratory energy generation (6). Table 2 compares the specific activities of dissimilatory nitrite reductase in crude extracts and in the particulate and soluble fraction obtained after centrifugation at high speed. The crude extract significantly reduced nitrite. However, the stoichiometry between N_2O-formation and NO_2^--disappearance was far from the expected relation (1:2). Thus other products besides N_2O were likely produced from nitrite. The crude extract did not reduce N_2O further to N_2. This could be shown by adding C_2H_2 which specifically blocks N_2O-reductase in denitrifying bacteria (see 7). The addition of C_2H_2 did not enhance the rate of N_2O-formation (see Table 3). Crude extracts, however, converted nitrite to nitric oxide to a large extent. When the extract was centrifuged, most of the NO_2^--reducing activity was recovered in the supernatant (Table 2). The supernatant containing the soluble dissimilatory nitrite

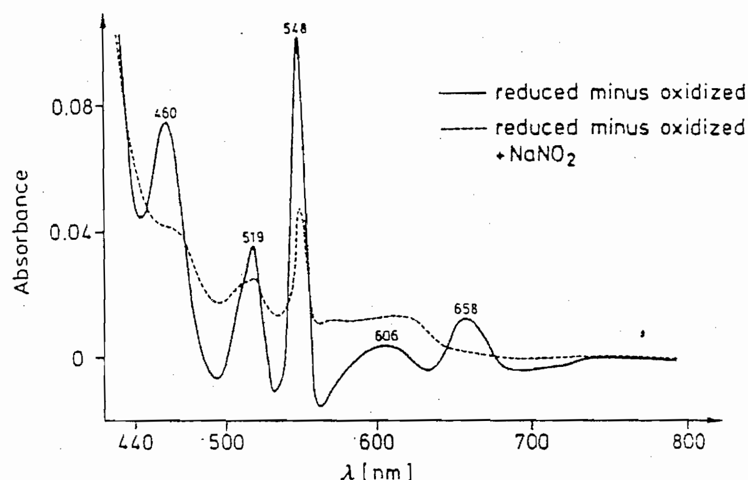


Fig. 1. Difference spectra of the solubilized dissimilatory nitrite reductase enriched by ultracentrifugation and $(NH_4)_2SO_4$-precipitation

straight line: difference spectrum: enzyme reduced by NADH and phenazine methosulfate minus oxidized enzyme (in the reference cuvette)

dashed line: difference spectrum: enzyme reduced by NADH and phenazine methosulfate and reoxidized by $NaNO_2$ minus oxidized enzyme (in the reference cuvette)

For this assay, the crude extract obtained by French Press treatment was centrifuged (2 h, 100 000 g), and the supernatant was fractionated by $(NH_4)_2SO_4$. The fraction precipitating between 55 and 90 % $(NH_4)_2SO_4$ was dissolved in 10 mM K-phosphate buffer pH 7.5 and dialyzed overnight against the same buffer. The spectra from this preparation were recorded in a Perkin-Elmer 555 spectrophotometer. The cuvette contained for the reduction of the nitrite reductase: NADH 0.5 mM, phenazine methosulfate 0.025 mM, K-phosphate buffer pH 7.5 50 mM and protein 0.7 mg. The gas phase was argon. $NaNO_2$ (final concentration 12.5 mM) was added to the cuvette for the reoxidation of the sample.

Table 2. The distribution of dissimilatory nitrite reductase activity after ultracentrifugation

I. Specific activity:
(in nmol/ min x mg protein)

	NO_2^- consump-tion	N_2O forma-tion	NO forma-tion	ratio $NO_2^-:N_2O$	ratio $NO_2^-:NO$
a) crude extract	354	42.2	118	8.4:1	3.0:1
b) supernatant after ultra-centrifuga-tion	415	22.2	97	18.7:1	4.3:1
c) pellet after ultra-centrifuga-tion	131	38.9	28	3.4:1	4.7:1

II. Total activity:
(in nmol/ min)

	protein content	NO_2^--consump-tion		N_2O-forma-tion		NO-forma-tion	
	mg	total	%	total	%	total	%
a) crude extract	69.8	24556	100	2947	100	7403	100
b) supernatant after ultra-centrifugation	54.6	22667	92	1210	41	5288	71
c) pellet after ultra-centrifugation	13.6	1778	7	528	18	382	5

The crude extract (15 ml, obtained by French Press treatment at 140 000 KPa, 2x and centrifugation at 12 000 g, 30 min) was centrifuged (2 h, 100 000 g). The supernatant (11 ml) was directly assayed and the pellet was suspended in 10 mM K-phosphate buffer pH 7.5 (4ml). The preparations were assayed for NO_2^--reduction using phenazine methosulfate and Na-ascorbate as electron donors

Table 3. Dissimilatory formation of nitrous oxide by a membrane fraction from Azospirillum brasilense Sp 7

Assay condition	nmol N_2O formed/ min x mg protein
1. complete	68.4
2. complete + 2 % C_2H_2	69.6
3. complete, but gas phase air	17.1
4. - $NaNO_2$	3.4
5. - phenazine methosulfate	1.7
6. - Na-ascorbate	0
7. - extract	0
8. complete, but extract boiled	0

The membrane fraction was assayed for the phenazine methosulfate and Na - ascorbate dependent formation of nitrous oxide from nitrite. The pellet obtained after ultracentrifugation (2 h, 100 000 g) was assayed for activity. The exact assay conditions are described under Materials and Methods

reductase fraction produced much NO, but rather little N_2O. The sum of the NO and N_2O productions could not account for the nitrite disappearance, indicating that another, still unidentified product must have been formed during nitrite reduction. By contrast, the overall activity in the pellet was low. However, the stoichiometry between NO_2^--disappearance and N_2O-formation approached to 2:1, and much smaller amounts of NO were formed in the pellet than in the supernatant(Table 2).

Nitric oxide formation from nitrite is believed to be an artifact of cell-free preparations in Pseudomonas denitrificans (17), and this is presumably also the case in Azospirillum. Dissimilatory nitrite reductase appears to fall off the membranes easily, and the enzyme catalyzes the formation of free nitric oxide only in the solubilized form.

The dissimilatory nitrite reduction in the pellet was characterized further (Table 3). This preparation catalyzed the formation of N_2O which was strictly dependent on nitrite and on the reductants sodium ascorbate and phenazine methosulfate. The reaction required anaerobic conditions and proceeded linearly for at least 45 min and 1 mg protein/vessel. We are currently trying to characterize this preparation in more detail.

Dissimilatory nitrous oxide reductase was reported to be a copper enzyme (18,19) or not to contain this metal (20). Up-till now, this enzyme has only been investigated biochemically from the marine Pseudomonas perfectomarinus. Azospirillum grows anaerobical-

Table 4. Growth of Azospirillum brasilense Sp 7 under anaerobic conditions with N_2O as the respiratory electron acceptor: Dependence on the metal-ion in the medium

	EDTA (300 µM)	salt (250 µM)	after 16 h (O.D.$_{560nm}$)	association constant of the metal-EDTA complex (log K_S)
1.	---	---	0.266	---
2.	+	---	0.018	---
3.	+	$CuSO_4$	0.173	18
4.	+	$CuCl_2$	0.165	18
5.	+	$CaSO_4$	0.027	9
6.	+	$CaCl_2$	0.029	9
7.	+	$NiCl_2$	0.069	18
8.	+	$CoSO_4$	0.074	16
9.	+	$ZnSO_4$	0.114	16
10.	+	$MgSO_4$	0.019	9
11.	+	$MnCl_2$	0.067	13.5

The growth and assay conditions are described under Materials and Methods

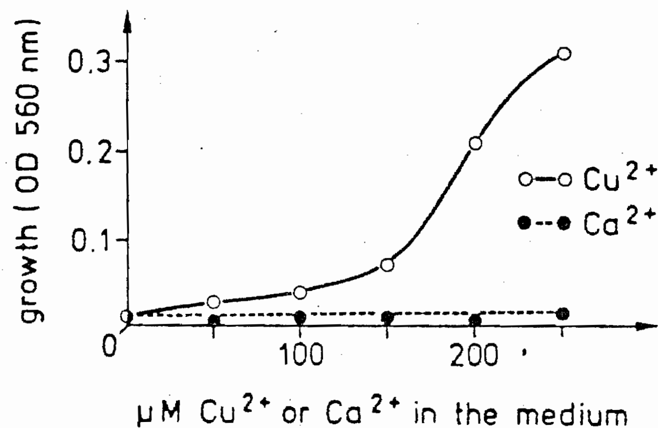


Fig. 2. Dependence of the growth of EDTA-treated Azospirillum on the presence of $CuSO_4$ or $CaSO_4$ in the medium

The experimental details are described under Materials and Methods (last section)

ly with N_2O as the sole respiratory electron acceptor. The bacterium must synthesize nitrogenase to meet the requirements for nitrogen unless the medium is supplemented with NH_4Cl(7). N_2O-dependent growth was further investigated in the present study. When the medium was supplemented with 300 μM EDTA, growth stopped (Table 4). EDTA did not unspecifically affect growth. This follows from the findings that Cu^{++} and to some extent Zn^{++}, but virtually not Mg^{++},Ca^{++}, Ni^{++} or Mn^{++} restored growth (measured by the increase in turbidity which had resulted 16 h after the inoculation) (Table 4). The stability constant for the Cu-EDTA-complex is similar or higher than those for the other metal-EDTA-complexes (21, see Table 3). This means that Cu is not less effective in binding EDTA and thus in removing the chelator out of the medium than other metals. The effects of the different salts are, therefore, not due to differences in the concentrations of free EDTA in the assays, but are caused by the metal-cations itself. It is, therefore, safe to conclude that Cu is involved in the expression of dissimilatory nitrous oxide reduction by Azospirillum. Fig. 2 indicates that 250 μM $CuCl_2$ was saturating for maximal growth under the conditions employed.

Concluding remarks

Our attempts to characterize denitrification by Azospirillum biochemically are still at an initial state. However, the present investigation showed that dissimilatory nitrite reductase is a cytochrome c,d containing enzyme. Further investigations have to elucidate the natural donor(s) for dissimilatory nitrate, nitrite and nitrous oxide reductases and the biochemical properties of the enzymes involved. Experiments are currently performed to measure directly the amount of ATP formed and the coupling to H^+-uptake in vesicle preparations. From the molecular growth yield measurements reported here it is likely that NO_2^- and O_2-respiration provide 3 ATP/2e whereas NO_3^- -respiration yields only 1 ATP/2e transferred. The present investigation also showed that Cu is involved in the expression of N_2O-reduction activity in Azospirillum. This means that Cu is a constituent of the dissimilatory N_2O-reductase itself or is required for the biosynthesis of a factor involved in dissimilatory N_2O-reduction (e. g. an electron donor). It is planned to purify nitrous oxide reductase under anaerobic conditions similar as performed with the enzyme from Pseudomonas perfectomarinus (19). This should allow to show the presence of Cu in the prosthetic group of the enzyme.

Acknowledgement. This work was kindly supported by grants from the Bundesministerium für Forschung und Technologie (PTB Jülich).

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