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Aerobic and anaerobic nitrate and nitrite reduction in free-living cells of *Bradyrhizobium* sp. (*Lupinus*)

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Abstract

Induction, energy gain, effect on growth, and interaction of nitrate and nitrite reduction of *Bradyrhizobium* sp. (*Lupinus*) USDA 3045 were characterized. Both nitrate and nitrite were reduced in air, although nitrite reduction was insensitive to ammonium inhibition. Anaerobic reduction of both ions was shown to be linked with energy conservation. A dissimilatory ammonification process was detected, which has not been reported in rhizobia so far. Nevertheless, anaerobic conversion of nitrate to ammonium was lower than 40%, which suggests the presence of an additional, nitrite reductase of denitrifying type. Nitrite toxicity caused a non-linear relationship between biomass produced and >2 mM concentrations of each N oxyanion consumed. At ≥ 5 mM initial concentrations of nitrate, a stoichiometric nitrite accumulation occurred and nitrite remained in the medium. This suggests an inhibition of nitrite reductase activity by nitrate, presumably due to competition with nitrate reductase for electron donors. Lowering of growth temperature almost completely diminished nitrite accumulation and enabled consumption as high as 10 mM nitrate, which confirms such a conclusion.
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Keywords: Dissimilatory ammonification; Nitrate reduction; Nitrite reduction; Regulation; *Bradyrhizobium* sp. (*Lupinus*)

1. Introduction

Aside from well-known adaptations to symbiotic cooperation, rhizobia have retained the ability to function efficiently *ex planta*. Rhizobia reveal a very good capacity for expansion and have been found in soils where their macrosymbionts have never been grown [1]. Substantial parts of bacteroid populations from both the determinate and the indeterminate type of nodules are able to recover the saprophytic way of life [2].

Under sufficiently low oxygen concentrations (microaerobic), some of free-living as well as symbiotic rhizobia induce synthesis of the enzymes of the denitrifying pathway [1]. Dissimilatory nitrate reduction, linked with ATP synthesis, is the advantage of denitrifiers over other bacteria and enables them to colonize soils under fluctuating oxygen conditions [3,4]. In our previous work, we described nitrate reductase present in symbiotic *Bradyrhizobium* sp. (*Lupinus*) cells [5]. The enzyme occurs in association with membranes and has some functional features in common with bacterial nitrate dissimilation.

It is currently supposed that symbiotic rhizobia have evolved from non-symbiotic ancestors that acquired nodulation and nitrogen-fixation genes by lateral transfer, which resulted in transformation of many distantly related soil bacteria into nodulating rhizobia [6,7]. Rhizobial nitrate reduction reflects this heterogeneity since it was shown to be so diverse that, depending on strain or physiological state of cells, it could be only of assimilatory type or could be restricted to the first stage of denitrification–nitrate respiration [8]. Considering this diversity, it is necessary to elaborate the strain-specific regulatory model to properly predict how in the given soil and symbiotic conditions nitrate and nitrite reduction would proceed.

In the present paper we analyze factors affecting aerobic and anaerobic growth on nitrate or nitrite of free-living *Bradyrhizobium* sp. (*Lupinus*) of the USDA 3045 strain. Induction, energy gain, and interaction of nitrate and nitrite reduction were characterized.

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2. Materials and methods

2.1. Culture conditions

Bradyrhizobium sp. (*Lupinus*) USDA 3045 strain was obtained from Beltsville Rhizobium Culture Collection, Beltsville, MD, USA. Cultures were grown at 32°C in liquid yeast extract/mannitol medium [9], pH 7.5, containing (in g l^{-1}): NaCl (0.2), K_2HPO_4 (0.5), $MgSO_4$ (0.2), $CaSO_4$ (0.1), $CaCO_3$ (0.1), mannitol (10) and yeast extract (0.5), with omission of sodium glutamate. A non-fermentable minimal medium was obtained by replacing mannitol and yeast extract with glycerol (10 g l^{-1}). A 24-h culture grown aerobically was transferred to the fresh medium for anaerobic (or aerobic in some cases) overnight preincubation. Then cells were centrifuged, resuspended in 70 ml of fresh yeast extract/mannitol medium (initial OD_{680} = 0.3–0.4), supplemented with nitrate or nitrite, and cultures were used for measurements. In some experiments inhibitory factors: NH_4Cl (10 mM) or L-methionine-D,L-sulfoximine (1 mM), were added at this step. Chloramphenicol (0.1 mg ml^{-1}) inhibition was initiated at the begining of the overnight preincubation. Anaerobic conditions were initiated by flushing N_2 through the flask for 15 min. Aerobic conditions were maintained by flushing the medium continuously with air.

Experiments measuring N ion production and consumption at 32°C were carried out up to 8–10 h (depending on initial nitrate or nitrite concentrations), when the culture entered early-stationary phase. Cultures incubated at 20°C were grown anaerobically without preceding preincubations and reached stationary growth phase after 60 h.

2.2. Analytical methods

Nitrite was determined according to Senn et al. [10]. Nitrate and ammonium ions were measured using 'Spectroquant' test kits from Merck KGaA, Darmstadt, Germany. Protein concentration was estimated by the procedure of Heckman and Drevon [11]. The values obtained for each culture condition were independently determined at least three times, and less than 10% variation was found.

2.3. Enzyme assays

Cells for enzyme assays were harvested by centrifugation at the mid-exponential phase. It was after 5 or 6 h of growth on 2 mM nitrite or 2 mM nitrate, respectively. Cells were washed and resuspended in 1 ml of N_2-saturated Tris–HCl buffer (100 mM, pH 7.5). The buffer was N_2-saturated and contained 10 mM dithiotreitol, which was important for succesful nitrite reductase (NiR) activity measurements. The assay mixture (total volume, 0.3 ml) was N_2-saturated and composed of 90 mM Tris–HCl, 10 mM KNO_3 or 0.1 mM $NaNO_2$, 1 mM benzyl viologen, 30 mM dithionite and 50 µl of bacterial cells (5–6 mg of protein per ml). The reaction (at 30°C) was carried out for 5 min for NR or 10 min for NiR. Then cells were removed by centrifugation and nitrite produced (NR activity) or nitrite consumed (NiR activity) was measured in the remaining supernatant. For NiR activity, initial nitrite concentration was also measured immediately after dithionite addition.

3. Results and discussion

3.1. Induction of aerobic nitrate reduction

Cells of *B.* sp. (*Lupinus*), grown on an aerated liquid medium, showed a weak nitrate reductase activity (NRA) (Table 1). Nitrate addition (2 mM) caused a four-fold stimulation of NRA through de novo enzyme synthesis, since chloramphenicol addition prevented such stimulation. Induction of NRA was strongly inhibited by ammonium ions, which indicates that the enzyme is of assimilatory type [12,13]. As a consequence, ammonium

Table 1
Effect of nitrate, nitrite and ammonium ions on induction of specific NR and NiR activities under aerobic and anaerobic conditions[a]

Growth conditions	Specific activity[b] (nmol NO_2^- (mg protein)$^{-1}$ min^{-1})			
	Nitrate reductase		Nitrite reductase	
	$+O_2$	$-O_2$	$+O_2$	$-O_2$
No N ions	34 (±2)	206(±14)	21 (±3)	43(±5)
NO_3^-+chloramphenicol	37 (±2)	39(±2)	20 (±2)	20(±4)
NO_3^-	137 (±10)	504(±45)	109 (±11)	99(±10)
$NO_3^-+NH_4^+$	48 (±5)	570(±57)	147 (±14)	127(±15)
NO_2^-	37 (±2)	492(±38)	127 (±11)	220(±18)
$NO_2^-+NH_4^+$	39 (±2)	600(±52)	78 (±5)	172(±8)
$NO_3^-+NO_2^-$	nd	962(±68)	nd	319(±21)

[a]Cells of USDA 3045 strain were grown in the medium supplemented with 2 mM KNO_3, 2 mM $NaNO_2$, 10 mM NH_4Cl and chloramphenicol (0.1 mg ml^{-1}) as indicated in the table. NR and NiR activities were measured at the mid-exponential growth phase.

[b]The specific activity data are means and standard deviations of results from three replicate vials and three independent experiments. nd = not determined.

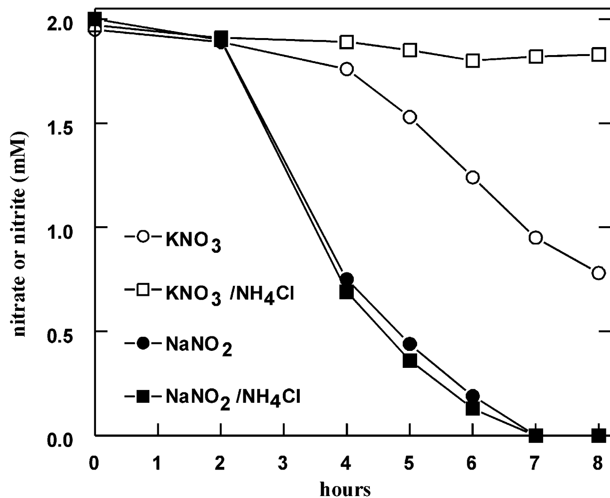

Fig. 1. Effect of ammonium ions on aerobic reduction of nitrate and nitrite in cells of *Bradyrhizobium* sp. (*Lupinus*). After 24 h aerobic preincubation, cells were transferred to fresh, aerated medium supplemented with 2 mM KNO_3 or 2 mM $NaNO_2$ and 10 mM NH_4Cl. The data are representative of three replicate experiments.

addition to aerobic culture entirely restrained nitrate utilization (Fig. 1). The rhizobial assimilatory nitrate reduction pathway is under genetic control of the central nitrogen regulation system (Ntr). Under a relatively high ammonium concentration, dephosphorylation of NtrC occurs, which makes it impossible to activate transcription of nitrate assimilation genes [14].

3.2. Induction of aerobic nitrite reduction

Aerobically grown rhizobia revealed a weak nitrite reductase activity (NiRA) (Table 1). The activity increased five-fold after nitrate (2 mM) addition through de novo enzyme synthesis. 2 mM nitrite induced aerobic NiRA to a similar extent as 2 mM nitrate. Surprisingly, addition of 10 mM NH_4Cl did not inhibit nitrate induction and only slightly lowered nitrite induction (Table 1). Induction in the presence of nitrogen excess indicates that the enzyme is regulated differently from aerobic nitrate reduction. In accordance with that conclusion, ammonium had no influence on aerobic nitrite consumption during growth (Fig. 1).

3.3. Dissimilatory growth on N oxyanions and energy conservation efficiency

Under anaerobic conditions, the USDA 3045 strain was capable of fermentative growth, using mannitol as an energy source (Fig. 3 and data not shown). Nevertheless, an addition of each N oxyanion (2 mM) resulted in a significant growth enhancement. Nitrate increased net protein production by up to 180%, while nitrite did so by up to 100% (Fig. 3). These results imply that *B.* sp. (*Lupinus*) is capable of dissimilatory reduction of N oxyanions. In fact, when mannitol was substituted with non-fermentable glycerol, cells were still able to grow efficiently if nitrate or nitrite were used as the sole external electron acceptor (Fig. 2). These data lead to the conclusion that anaerobic reduction of both ions is linked to energy conservation. However, Fig. 3 demonstrates that growth yield on 2 mM nitrite was 40% lower than on 2 mM nitrate. The growth yield on nitrate under anaerobiosis was 62% of that under aerobic conditions (data not shown). This is in good agreement with the findings of Bhandari et al. [15], who estimated the efficiency of ATP production during nitrate respiration in *Bradyrhizobium japonicum* at 40–50% of oxidative phosphorylation.

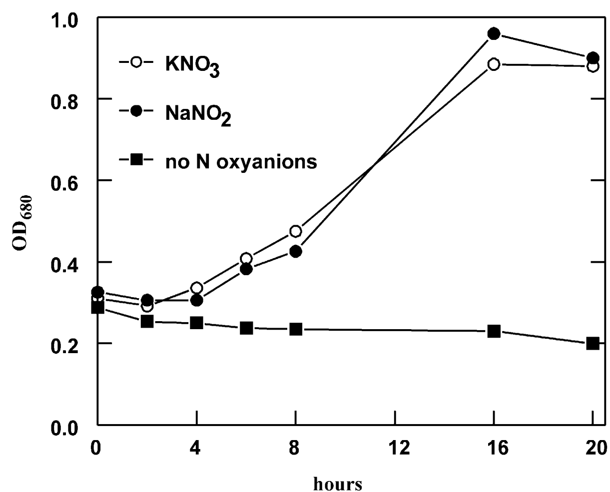

Fig. 2. Anaerobic growth of *Bradyrhizobium* sp. (*Lupinus*) in non-fermentable minimal medium. In each variant, yeast extract and mannitol were substituted with glycerol as non-fermentable carbon source. 2 mM nitrate or 2 mM nitrite was used as the sole nitrogen source and the sole external electron acceptor. The control variant contained no N oxyanions. The data are representative of three replicate experiments.

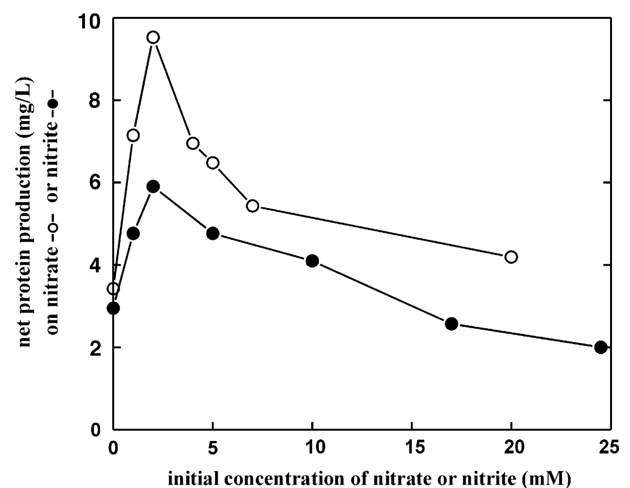

Fig. 3. Growth yield of *Bradyrhizobium* sp. (*Lupinus*) on 2 mM nitrate and 2 mM nitrite under anaerobiosis. Protein content was measured when the culture entered the stationary growth phase. Biomass production at 0 mM nitrate or nitrite resulted from fermentative metabolism. Each value was calculated by substraction of initial protein content from total protein produced per liter. The data are representative of three replicate experiments.

3.4. Induction of anaerobic NRA

Anaerobiosis induced NRA to a level six times higher than the activity in air (Table 1). Nitrate addition (2 mM) enhanced induction to a factor 2.5 more strongly through de novo enzyme synthesis. Nitrite (2 mM) was shown to be an equally effective inducer of the enzyme. NRA of anaerobically grown bacteria was not affected by the presence of 10 mM ammonium (Table 1). As a consequence, ammonium addition to anaerobic culture had no influence on nitrate utilization (data not shown). The data presented indicate that the enzyme is of respiratory type [4].

Simultaneously given 2 mM nitrate and 2 mM nitrite caused a twice as high induction of the enzyme than when given separately (Table 1). This implies that nitrate and nitrite cause induction or depression of dissimilatory NR through supplementary regulatory pathways (see discussion below).

3.5. Nitrate reduction during anaerobic growth

Anaerobic reduction of ≤2 mM nitrate led to transient external nitrite accumulation (Fig. 4A and data not shown). However, at ≥5 mM initial concentration of nitrate, stoichiometric nitrite accumulation occurred and nitrite remained at a constant level in the growth medium (Fig. 5). Moreover, the cells could not utilize more than 5 (±1) mM nitrate, even if the cultivation time was prolonged to 25 h (Fig. 5). The lack of reduction of higher nitrate levels was apparently due to intracellular accumulated nitrite, which could cause inhibition of nitrate uptake [16]. As a result, nitrate transport came to a halt before completion of its conversion.

Due to nitrite accumulation, the optimal initial concentration of nitrate was 2 mM. Fig. 3 demonstrates that at higher nitrate concentrations, a non-linear relationship occurred between biomass produced and nitrate consumed. Nevertheless, the growth yield on 2 mM nitrate was 180% higher than under fermentative conditions (Fig. 3).

3.6. Induction of anaerobic nitrite reductase activity

Anaerobiosis induced NiRA to a level two times higher than the activity in air (Table 1). Nitrate addition (2 mM) enhanced anaerobic induction two-fold through de novo enzyme synthesis. Nitrite (2 mM) was shown to be a much better inducer, enhancing anaerobic enzyme induction five-fold. Addition of 10 mM NH_4Cl did not inhibit nitrate induction and only slightly lowered nitrite induction (Table 1). As a result, ammonium had no influence on in vivo anaerobic nitrite reduction (Fig. 4B). The data presented indicate that the enzyme is of dissimilatory type [4].

Simultaneously given 2 mM nitrate and 2 mM nitrite caused a much higher induction of the enzyme than when given separately, which is similar to the effect of these ions on NR induction (Table 1). This demonstrates that in cells

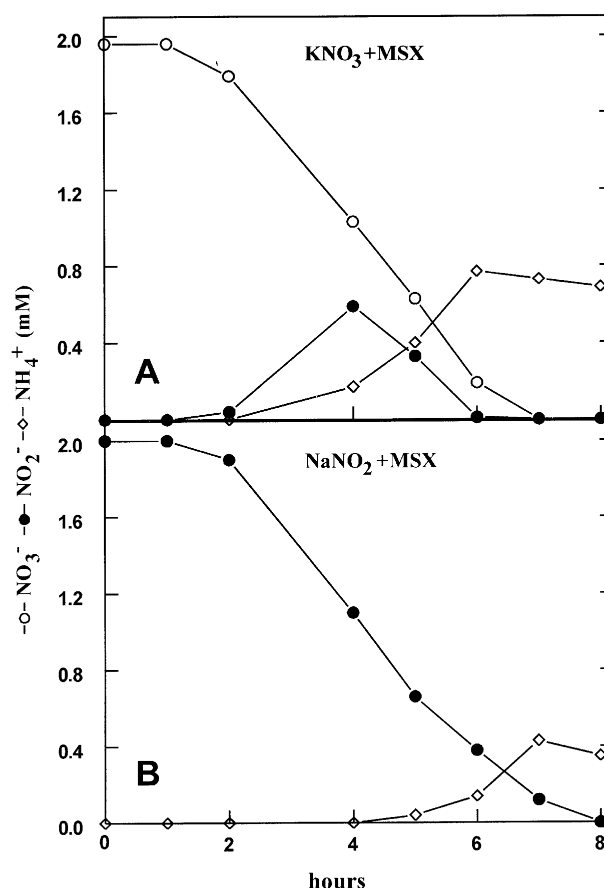

Fig. 4. Anaerobic reduction of 2 mM nitrate (panel A) or 2 mM nitrite (panel B) to ammonium in cells of *Bradyrhizobium* sp. (*Lupinus*) in the presence of MSX – an inhibitor of glutamine synthetase. In the absence of MSX, nitrite excretion and N oxyanion reduction proceeded in the same way (not shown). The data are representative of five replicate experiments. MSX, L-methionine-D,L-sulfoximine.

of *B.* sp. (*Lupinus*) such a synergy of nitrate and nitrite is a common phenomenon for anaerobic induction of both NR and NiR activities. In *Escherichia coli* the induction of dissimilatory NR and NiR expression in response to N oxyanions is mediated by two NarX and NarQ homologous sensors and corresponding NarL and NarP regulators [13]. These regulators are not supplementary since NarL and NarP bind competitively at the activation sites of the promoter region of the *narG* gene of NR [17] as well as of the *nirB* gene of NiR [18]. Considering that model, it is not clear how in the cells of *B.* sp. (*Lupinus*) the induction of the enzymes in response to one N oxyanion can be stimulated by the presence of another.

We propose that such a synergistic effect of N oxyanions on NR and NiR activities is a consequence of stimulation of nitrate transport by nitrite. Nitrate uptake in the absence of nitrite is possible mainly through H^+/NO_3^- symport. Since nitrite can cross biological membranes relatively freely (even by passive diffusion), addition of these ions to extracellular medium could induce the NO_3^-/NO_2^- antiport mechanism, which facilitates nitrate transport [16]. Accumulation of intracellular nitrate could in turn

enhance induction of both membrane-bound NR and cytoplasmic NiR activities. Nevertheless, it should be noted that a substantial part of anaerobic nitrite reduction in *B.* sp. (*Lupinus*) USDA 3045 could be associated also with the periplasmic form of the enzyme (see below).

3.7. Nitrite reduction during anaerobic growth

At an optimal initial concentration of nitrite (2 mM), the growth yield was 100% higher than under fermentative conditions (Fig. 3). However, nitrite added at ≥5 mM appeared to be toxic and stimulation of growth was significantly retarded. When the initial concentration of nitrite exceeded 15 mM, cell yield was below 70% of the level of fermentative growth (Fig. 3). The USDA 3045 strain was incapable of utilizing more than 6 (±1) mM of nitrite. As a result, nitrite still remained in the medium after growth ceased (data not shown).

3.8. Inhibition of anaerobic nitrite reduction in the presence of nitrate

Anaerobic nitrate reduction led to external nitrite accumulation. At an initial nitrate concentration of ≤2 mM, the accumulation was transient but nitrite reduction was blocked until the nitrate level decreased to 0.8 mM (±0.2, $n=10$) (Fig. 4A and data not shown). However, when nitrate was added at ≥5 mM, reduction of excreted nitrite did not occur at all, even if cultivation time was prolonged to 25 h (Fig. 5). These results indicate that the presence of nitrate at a concentration higher than 0.8 mM inhibits nitrite reduction. Several mechanisms of such an inhibition are conceivable [19].

(i) Suppression of NiR synthesis by nitrate is unlikely since 2 mM nitrate was shown to be a good inducer of de novo NiR synthesis (Table 1). Higher nitrate concentrations also had no inhibitory effect on NiRA induction (manuscript in preparation). Moreover, addition of 2 mM nitrate did not prevent NiR induction by nitrite but stimulated it by 45% (Table 1).

(ii) Delayed induction time of NiR, as compared with NR, did not occur in the presence of nitrate since specific activities of both enzymes increased simultaneously during 2 mM nitrate consumption and peaked just at the highest nitrite accumulation (manuscript in preparation). Moreover, in vivo nitrite reduction could start after a lag phase no longer than for nitrate reduction (compare Fig. 4A,B).

(iii) Competition for electron donors between NR and NiR. According to this explanation, nitrite accumulates due to a lack of balance between nitrate and nitrite reduction rate. Since ATP is produced from the nitrate-to-nitrite step but not from nitrite reduction, it is more advantageous for the organism to divert its limited electron flow to the energy-producing step. This could result in nitrite accumulation. However, after nitrate depletion the need for a high-capacity electron sink became important, so nitrite reduction is expected from that moment [19]. Transient nitrite accumulation, depicted in Fig. 4A, seems to be in agreement with the idea of competition for electron donors between nitrate and nitrite reductase.

To verify the possibility (iii) of nitrate and nitrite reduction, it was analyzed at a temperature lowered to 20°C. Under such conditions NiR should be more efficient in competition for electron donors with NR due to a decreased nitrate transport rate. As expected, nitrite accumulation did not occur even at an initial concentration of nitrate as high as 10 mM (Fig. 6A). Moreover, addition of 10 mM nitrate doubled net protein production (data not shown). This contrasts with growth parameters at the commonly used temperature of 32°C, since under such conditions accumulation of the toxic nitrite could prevent biomass production from an increase at nitrate concentrations >5 mM (Fig. 3). These results indicate that nitrite reduction in the presence of higher nitrate concentrations is possible but at temperatures more typical of the natural habitat of rhizobia. Higher temperatures of growth led to a lowered rate of nitrite reduction, most probably due to the unbalanced kinetic parameters of reduction of nitrate, which oxidizes the bulk of NADH [19].

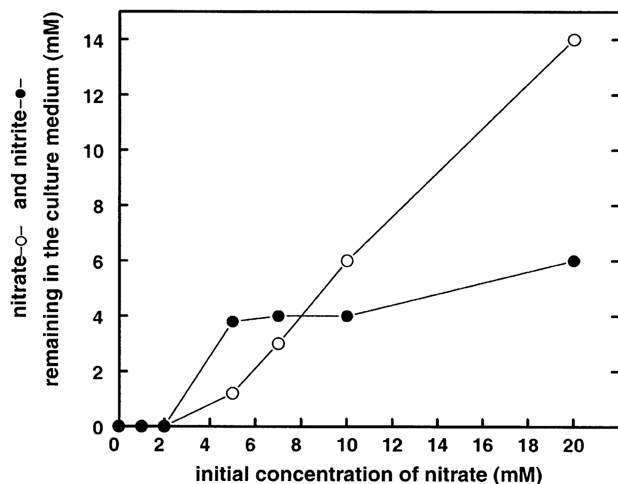

Fig. 5. Levels of nitrate remaining and nitrite accumulated during anaerobic growth of *Bradyrhizobium* sp. (*Lupinus*) with different concentrations of nitrate added. The concentrations of ions were measured after a cultivation time prolonged up to 25 h. The data are representative of three replicate experiments.

3.9. End product of anaerobic nitrite reduction

Fig. 2 demonstrates the efficiency of anaerobic growth on a minimal glycerol medium with nitrate or nitrite as the sole nitrogen source. This indicates that the investigated strain has the capacity not only for respiration but also for efficient anaerobic assimilation of N oxyanions. An essential stage of such a process is ammonia production. These findings prompted the intriguing question if the investigated strain could activate ammonia-producing NiR under anaerobiosis. Indeed, anaerobic, NADH-dependent NiRA

in cells of *B.* sp. (*Lupinus*) were found to be located within the cytoplasm (manuscript in preparation), which is in general agreement with the location of ammonia-producing NiR of *E. coli* encoded by the *nirB* gene [20]. Nevertheless, a search for this product in anaerobic culture of *B.* sp. (*Lupinus*) showed only a trace level of ammonium accumulation. Ammonium was eventually found as the end product of anaerobic reduction of both nitrate and nitrite when MSX (L-methionine-D,L-sulfoximine), a potent inhibitor of glutamine synthetase, was used (Fig. 4A,B). Nevertheless, only 40% of nitrogen from reduced nitrate (2 mM) was observed as external ammonium accumulation (Fig. 4A). At a temperature lowered to 20°C only 20% of nitrate (10 mM) was converted into ammonium in the presence of MSX (Fig. 6B).

Anaerobic ammonium excretion during nitrate reduction attests to a dissimilatory ammonification process [4,19]. There was no evidence of the presence of such a pathway in rhizobia so far. Nitrate respiration is the first reaction in this pathway and provides ATP through electron transport phosphorylation. The capacity for accepting six electrons per one reduced nitrite ion allows a very efficient reoxidation of NADH. Therefore, an electron-sink function is the most postulated role for the dissimilatory nitrite ammonification process [4].

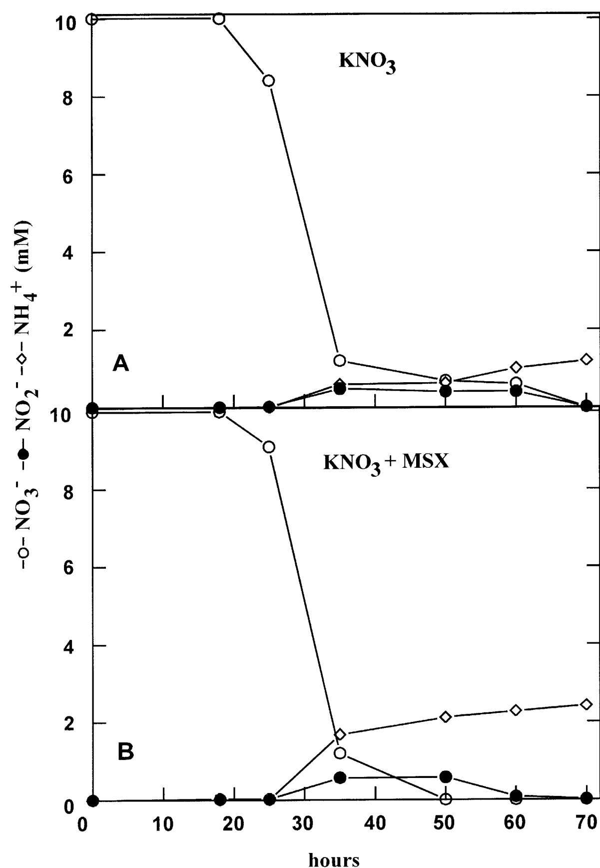

Fig. 6. Anaerobic reduction of 10 mM nitrate in cells of *Bradyrhizobium* sp. (*Lupinus*) at a temperature lowered to 20°C. A: In the absence of MSX. B: In the presence of MSX. The data are representative of three replicate experiments.

Two explanations of the deficit in nitrate conversion to ammonium are conceivable. One of them is partial ammonium incorporation into amino acids (even in the presence of MSX). The investigated strain was capable of efficient ammonium assimilation under anaerobiosis (data not shown and Fig. 2). Obviously, the conclusion about the fate of nitrite reduced under anaerobiosis would be better grounded if $^{15}NO_3^-$ conversion into $^{15}NH_4^+$ and the stoichiometry of N oxyanion transformation into glutamine would be estimated, so further work is needed. The second possibility is that nitrate is converted into gaseous N due to the activity of an additional – denitrifying – form of NiR. Detection of denitrification products has not been carried out, but in the periplasmic fraction of USDA 3045 cells we detected methylviologen-dependent NiRA, which confirms such a supposition (manuscript in preparation). O'Hara and coworkers [21] reported that N_2O is the end product of nitrate reduction in free-living cells and in bacteroids of *B.* sp. (*Lupinus*), which indicates a complete denitrification system. The recently completed identification of the nucleotide sequence of the *Sinorhizobium meliloti* genome uncovered genes coding for two nitrite reductases [22]. One of them is strictly denitrifying, encoded by the *nirK* gene, and the second is soluble, NADH-dependent and ammonia-producing NiR, encoded by the *nirB* gene. Recently, in *B. japonicum* USDA110, the *nirK* gene [23,24] and genes encoding nitric oxide reductase (*norCBQD*) [25] have been identified.

3.10. Is nitrite reduction in USDA 3045 cells driven by a single enzyme form regardless of oxygen conditions?

In cells of *B.* sp. (*Lupinus*), the effect of ammonium on aerobic NiRA reflected the effect on anaerobic nitrite reduction. Under both aerobic and anaerobic conditions, addition of 10 mM NH_4Cl did not inhibit induction of NiRA by nitrate and only slightly lowered nitrite induction (Table 1). This indicates that NiR induction was not repressed by conditions of nitrogen excess regardless of the oxygen state of cells. Such results prompt the question if both activities are driven by a single enzyme form of dissimilatory type. The work of Ka et al. [26] showed that constitutive production of Cu and heme-type denitrifying nitrite reductases in aerobic environments is a common phenomenon rather than an exception among denitrifiers. Considering the fact that NiRA was detected in the periplasmic fraction of anaerobically grown USDA 3045 cells (manuscript in preparation), it is conceivable that such a constitutive dissimilatory NiR is also present in this strain.

Additionally, since under aerobic conditions low levels of ammonium extraction were detected (data not shown), a second, ammonia-producing form of aerobic NiR seems to occur apart from the periplasmic one. Since nitrite ammonification was proved in USDA 3045 cells also under

anaerobiosis, the question remains to be elucidated if this process could be driven by a single enzyme form regardless of oxygen conditions. In *Bacillus subtilis* the soluble, ammonia-producing nitrite reductase, encoded by homologs of *E. coli nirB* and *nirD* genes, functions both in aerobic nitrogen assimilation and nitrite respiration [27]. Nevertheless, aerobic nitrite reduction in *B. subtilis* is repressed in excess of nitrogen. This is significantly different from the regulation observed in *B.* sp. (*Lupinus*) cells, where periplasmic NiRA could mask the response of the cytoplasmic enzyme.

References

[1] O'Hara, G.W. and Daniel, R.M. (1985) Rhizobial denitrification, a review. Soil Biol. Biochem. 17, 1–9.
[2] Sprent, J.I. and Raven, J.A. (1992) Evolution of nitrogen-fixing symbioses. In: Biological Nitrogen Fixation (Stacey, G., Burris, R.H. and Evans, H.J., Eds.), pp. 461–496. Chapman and Hall, New York.
[3] Philipott, L. and Højberg, O. (1999) Dissimilatory nitrate reductases in bacteria. Biochim. Biophys. Acta 1446, 1–23.
[4] Zumft, W.G. (1997) Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. 61, 533–616.
[5] Polcyn, W. and Luciński, R. (2001) Functional similarities of nitrate reductase from yelow lupine bacteroids to bacterial denitrification systems. J. Plant Physiol. 158, 829–834.
[6] Broughton, W.J. and Perret, X. (1999) Genealogy of legume-*Rhizobium* symbioses. Curr. Opin. Plant Biol. 2, 305–311.
[7] van Berkum, P. and Eardly, B.D. (1998) Molecular evolutionary systematics of the *Rhizobiaceae*. In: The *Rhizobiaceae*: Molecular Biology of Model Plant-Associated Bacteria (Spaink, H.P., Kondorosi, A. and Hooykaas, P.J.J., Eds.), pp. 1–24. Kluwer Academic, Dordrecht.
[8] Ishizawa, S. (1980) Note on nitrate reduction in *Rhizobium*. Soil Sci. Plant Nutr. 26, 447–450.
[9] Kennedy, I.R., Rigaud, J. and Trinchant, J.C. (1975) Nitrate reductase from bacteroids of *Rhizobium japonicum*: enzyme characteristics and possible interaction with nitrogen fixation. Biochim. Biophys. Acta 397, 24–35.
[10] Senn, D.R., Carr, P.W. and Klatt, L.N. (1976) Minimization of a sodium dithionite-derived interference in nitrate reductase-methyl viologen reactions. Anal. Biochem. 75, 464–471.
[11] Heckman, M.O. and Drevon, J.J. (1987) Nitrate metabolism in soybean root nodules. Physiol. Plant 69, 721–725.
[12] Moreno-Vivián, C., Cabello, P., Martinez-Luque, M., Blasco, R. and Castillo, F. (1999) Prokaryotic nitrate reduction: molecular properties and functional distinction among bacterial nitrate reductases. J. Bacteriol. 181, 6573–6584.
[13] Richardson, D.J., Berks, B.C., Russell, D.A., Spiro, S. and Taylor, C.J. (2001) Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. Cell. Mol. Life Sci. 58, 165–178.
[14] Kahn, M.L., McDermott, T.R. and Udvardi, M.K. (1998) Carbon and nitrogen metabolism in rhizobia. In: The *Rhizobiaceae*: Molecular Biology of Model Plant-Associated Bacteria (Spaink, H.P., Kondorosi, A. and Hooykaas, P.J.J., Eds.), pp. 461–485. Kluwer Academic, Dordrecht.
[15] Bhandari, B., Naik, M.S. and Nicholas, D.J.D. (1984) ATP production coupled to the denitrification of nitrate in *Rhizobium japonicum*, grown in cultures and in bacteroids from *Glycine max*. FEBS Lett. 168, 321–326.
[16] Moir, J.W. and Wood, N.J. (2001) Nitrate and nitrite transport in bacteria. Cell. Mol. Life Sci. 58, 215–224.
[17] Wang, H., Tseng, C.-P. and Gunsalus, R.P. (1999) The *napF* and *narG* nitrate reductase operons in *Escherichia coli* are differentially expressed in response to submicromolar concentration of nitrate but not nitrite. J. Bacteriol. 181, 5303–5308.
[18] Wang, H. and Gunsalus, R.P. (2000) The *nfrA* and *nirB* nitrite reductase operons in *Escherichia coli* are expressed differently in response to nitrate than to nitrite. J. Bacteriol. 182, 5313–5822.
[19] Tiedje, J. M. (1988) Ecology of denitrification and dissimilatory nitrate reduction to ammonium. In: Biology of Anaerobic Microorganisms (Zehnder, A.J.B., Ed.), pp. 179–244. Wiley, New York.
[20] Berks, B.C., Ferguson, S.J., Moir, J.W.B. and Richardson, D.J. (1995) Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. Biochim. Biophys. Acta 1232, 97–173.
[21] O'Hara, G.W., Daniel, R.M. and Steele, K.W. (1983) Effect of oxygen on the synthesis, activity and breakdown of the *Rhizobium* denitrification system. J. Gen. Microbiol. 129, 2405–2412.
[22] Galibert, F., Finan, T.M., Long, S.R., Puhler, A., Abola, P., Ampe, F., Barloy-Hubler, F., Barnett, M.J., Becker, A., Boistard, P., Bothe, G., Boutry, M., Bowser, L., Buhrmester, J., Cadieu, E., Capela, D., Chain, P., Cowie, A., Davis, R.W., Dreano, S., Federspiel, N.A., Fisher, R.F., Gloux, S., Godrie, T., Goffeau, A., Golding, B., Gouzy, J., Gurjal, M., Hernandez-Lucas, I., Hong, A., Huizar, L., Hyman, R.W., Jones, T., Kahn, D., Kahn, M.L., Kalman, S., Keating, D.H., Kiss, E., Komp, C., Lelaure, V., Masuy, D., Palm, C., Peck, M.C., Pohl, T.M., Portetelle, D., Purnelle, B., Ramsperger, U., Surzycki, R., Thebault, P., Vandenbol, M., Vorholter, F.J., Weidner, S., Wells, D.H., Wong, K., Yeh, K.C. and Batut, J. (2001) The composite genome of the legume symbiont *Sinorhizobium meliloti*. Science 293, 668–672.
[23] Nellen-Anthamatten, D., Rossi, P., Preisig, O., Kullik, I., Babst, M., Fischer, H.M. and Hennecke, H. (1998) *Bradyrhizobium japonicum* $FixK_2$, a crucial distributor in the FixLJ-dependent regulatory cascade for control of genes inducible by low oxygen levels. J. Bacteriol. 180, 5251–5255.
[24] Velasco, L., Mesa, S., Delgado, M.J. and Bedmar, E.J. (2001) Characterization of the *nirK* gene encoding the respiratory, Cu-containing nitrite reductase of *Bradyrhizobium japonicum*. Biochim. Biophys. Acta 1521, 130–134.
[25] Mesa, S., Velasco, L., Manzanera, M.E., Delgado, M.J. and Bedmar, E.J. (2002) Characterization of the *norCBQD* genes, encoding nitric oxide reductase, in the nitrogen fixing bacterium *Bradyrhizobium japonicum*. Microbiology 148, 3553–3560.
[26] Ka, J.-O., Urbance, J., Ye, R.W., Ahn, T.-Y. and Tiedje, J.M. (1997) Diversity of oxygen and N-oxide regulation of nitrite reductases in denitrifying bacteria. FEMS Microbiol. Lett. 156, 55–60.
[27] Nakano, M.N. and Zuber, P. (1998) Anaerobic growth of a 'strict aerobe' (*Bacillus subtilis*). Annu. Rev. Microbiol. 52, 165–190.