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Regular paper

Nitrate-related down-regulation of respiratory nitrate reductase from *Bradyrhizobium* sp. (*Lupinus*)

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Previously, we showed that anaerobic induction of respiratory nitrate reductase (NR) activity in *Bradyrhizobium* sp. (*Lupinus*) USDA 3045 is strongly enhanced by nitrate or nitrite through *de novo* synthesis. Here, multiple NR-active soluble forms, ranging from 75 kDa to 190 kDa, were observed under anaerobic conditions. Electrophoretic activity band patterns differed depending on the level and the type of the N oxyanion added. The intensity of the membrane-bound NR activity band of 230 kDa changed with time along with consumption of 2 mM nitrate. It was associated with a parallel 5-fold increase and then 2-fold reduction in the amount of membrane-bound NR protein. In contrast, on 4 mM nitrate, the level of NR protein was much more stable, apparently due to slower nitrate depletion. Moreover, in cells anaerobically grown without nitrate addition, a 42-kDa derivative of NR degradation was immunodetected, which was not observed if nitrate was present in the medium. These findings suggest that the amount of the respiratory NR protein could be negatively regulated by endogenous proteases in relation to the level of nitrate available. It seems, therefore, that multiple native forms might be not different isoenzymes but immature complexes or derivatives of the enzyme protein turnover. This report adds to a modest list of bacterial enzymes apparently regulated by proteolysis, such as GS, MurAA, EnvA, GdhA, and MetA.

Keywords: *Bradyrhizobium* sp. (*Lupinus*), down-regulation, membrane-bound nitrate reductase [EC 1.7.99.4], multiple enzyme forms, nitrate and nitrite induction, stability of subunits

INTRODUCTION

Bradyrhizobium sp. USDA 3045, a symbiont of *Lupinus luteus*, exhibits respiratory nitrate reductase (NR) activity strongly induced by anaerobiosis, which can be further enhanced through *de novo* synthesis after addition of N oxyanions (Polcyn & Luciński, 2003). In a recent work the α- and β-subunits of NR were isolated from this rhizobial strain, and the enzyme was identified by tandem mass spectrometry as a homolog of membrane-bound NarGH-type NRs (Polcyn, 2008).

Previously we purified from soluble and membrane fractions of nitrate-respiring *Bradyrhizobium* sp. (*Lupinus*) USDA 3045 two NR-active electrophoretic forms: NRI of 140 kDa and NRII of 190 kDa. The obtained specific antibodies showed strong immuno-cross-reaction between these native species, indicating that NRI and NRII are two forms of the same enzyme (Polcyn & Luciński, 2006). Several authors have reported multiple, particulate or soluble, native forms of bradyrhizobial NRs. Formation of several NR isoenzymes by polymerization of 90-kDa subunit (from 90 to 720 kDa) was suggested in free-living *Bradyrhizobium* sp. (*Lupinus*) and their bacteroids from *Lupinus albus* (Chamber-Pérez *et al.*, 2002). In *Glycine max* bacteroids, microaerobically incubated in the absence or presence of nitrate, two soluble NR forms were reported (76 kDa and 160 kDa), whereas the membrane-bound NR form was uniformly of 230 kDa (Delgado *et al.*, 1998). Fernández-López *et al.* (1994) suggested that two activity-bands of 160 kDa and 200 kDa, observed in electrophoretically resolved membrane proteins of

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Abbreviations: NR, nitrate reductase, PIC, protease inhibitor cocktail; PMSF, phenylmethylsulfonyl fluoride.

free-living *Bradyrhizobium japonicum*, represent differentially regulated NR isoenzymes. They concluded that the enzyme is of a membrane-bound type and that the faster-migrating form was induced by anaerobiosis alone, whereas induction of the second form occurred when nitrate was present. Although the periplasmic nature of the respiratory NR has been recently established for USDA 110 strain of *B. japonicum* (Delgado *et al.*, 2003), the presence of a membrane-bound type of NR in the strain investigated by Fernández-López and coworkers could not be excluded.

A model of the maturation of *Escherichia coli* membrane-bound NR has been proposed recently (Blasco *et al.*, 1998; Vergnes *et al.*, 2004). In that pathway, first the soluble NarGH and next the mature membrane-attached NarGHI complexes are formed. Interaction of NarGH with a specific chaperone (NarJ) is required for incorporation of the molybdopterin cofactor into NarG. When these processes are disturbed, some NarGH complexes remain in the cytoplasm or irreversibly separate from the membrane. One of the purposes of destructive proteolysis in bacterial cells is the clean-up of non-functional proteins (Gottesman, 1996). The kinetics of partitioning between chaperones and proteases determines whether a protein will be destroyed before it folds properly (Jenal & Hengge-Aronis, 2003). Eventually, degradation of premature complexes could occur with time.

The aim of this study was to provide arguments for the suggestion that nitrate depletion makes respiratory NR from *Bradyrhizobium* sp. (*Lupinus*) sensitive to endogenous proteolysis. Multiple forms of bradyrhizobial NR might be, therefore, not different isoenzymes but immature complexes or derivatives of the enzyme protein turnover related to the level of nitrate available.

MATERIALS AND METHODS

Culture conditions. *Bradyrhizobium* sp. (*Lupinus*) USDA 3045 was obtained from the Beltsville Rhizobium Culture Collection (Beltsville, MD, USA). An aerobic culture, grown at 30°C on yeast-extract-mannitol broth (Somasegaran & Hoben, 1994), was spun, divided into aliquots, frozen in liquid nitrogen with 10% glycerol and stored at –20°C for future use. The pellets were then transferred to fresh medium to achieve initial $OD_{580} \approx 0.350$ and divided into 70 ml batches, supplemented or not with various concentrations of nitrate or nitrite, then grown for 6 h to the late-exponential growth phase. Anaerobic conditions were achieved in sealed flasks after a short time through consumption of residual oxygen by the inoculated bacteria. For the time-course experiments, cells were grown in 1-liter batch cultures containing 2 or 4 mM nitrate for 6 h or to the early-stationary phase (10 h) and 70-ml samples were taken at indicated intervals.

Analysis of NR complexes and subunits. Pelleted cells were supplemented with 3 mM EDTA and 10 μl per ml of PIC (protease inhibitor cocktail; Sigma-Aldrich P8849), converted to spheroplast (Polcyn & Luciński, 2006) and then disrupted by short ultrasonication or 30-min treatment with 1% Triton X-100, followed by separation of soluble and membrane fractions at 240 000 × *g*.

For SDS/PAGE, samples were solubilized by 5-min boiling in Roti Load 1 (Roth) sample buffer + PIC (10 μl per ml) and resolved on 10% polyacrelamide gels (Laemmli, 1970). For non-denaturing PAGE, the membranes (10 mg of protein per ml^{-1}) were solubilized for 30 min, room temp., with 2% Triton X-100 in 0.1 M Tris, pH 8, containing 0.2 M NaCl, 11% (v/v) glycerol, 3 mM EDTA and 20 μl per ml of PIC. The cytosolic proteins were concentrated under protection of PIC with ammonium sulfate at 60% saturation. Samples were mixed with 4× sample buffer + PIC (10 μl per ml) and analyzed by 4–15% non-denaturing PAGE according to Polcyn and Luciński (2006).

In-gel NR activity was detected by immersing the gel in 10 mM methyl viologen in 50 mM Tris buffer (pH 7.5) with 2 mg per ml of sodium dithionite (all reagents from Sigma-Aldrich) until the gel was evenly dyed dark blue. Then the gel was transferred to 1 M KNO_3 and NR activity was rapidly visualized as a clear band of oxidized methyl viologen.

For immunoblotting cell extracts resolved by PAGE were transferred onto Immobilon-P membrane (0.45 μm, Millipore) by wet transfer (500 mAmp, 3 h, with stirring and ice cooling) using 25 mM Tris, 192 mM glycine buffer with 0.01% SDS but without methanol. After transfer, blots were treated according to the rapid immunodetection protocol from Millipore (2000). The IgG fraction of anti-NR_{II} serum (Polcyn & Luciński, 2006) was used at 0.75 μg/ml concentration. Rabbit ExtrAvidin Peroxidase Staining Kit (Sigma-Aldrich) and 3,3-diaminobenzidine Enhanced Liquid Substrate System (Sigma-Aldrich, D6815) were used to visualize the IgG-antigen complex. Both primary and secondary antibodies were diluted with 10% Roti-Block (Roth) in water.

Native HMW Calibration Kit (Amersham Biosciences) and denatured PageRuler Protein Ladder (Fermentas) were used as protein standards. Shimadzu CS-9000 scanner was used for densitometric measurements. Nitrite and nitrate estimation were done as described previously (Polcyn & Luciński, 2003).

RESULTS

Amount of respiratory NR forms varies in relation to the level of nitrate available

Multiple NR-active electrophoretic forms were found in the soluble cell fraction of *Bradyrhizobium* sp. (*Lupinus*) USDA 3045 incubated anaerobically with various concentrations of nitrate or nitrite. In the nitrate-fed cells, the 190-kDa form was the most abundant and its activity increased with increasing nitrate concentration (Fig. 1A). A different activity band pattern, comprising mainly 140-kDa and 125-kDa bands (Fig. 1B), was the attribute of soluble NR induced with nitrite — another, less effective, inducer of the enzyme (Wang *et al*., 1999; Polcyn & Luciński, 2003). The activity of the 125-kDa complex increased strikingly from 0.1 mM to 1 mM nitrite added. However, within the higher range of available nitrite, the intensity of this form decreased dramatically, whereas the activity of the 140-kDa band started to grow in strength.

Interestingly, leaving the sonicated extracts from nitrate-deprived *Bradyrhizobium* sp. (*Lupinus*) USDA 3045 cells for a prolonged period of time at 4°C without the protection from protease inhibitors led to partial or even complete conversion of the 140-kDa NR-active form into a 125-kDa band with a remarkably increased activity (not shown). These findings suggested that truncated but active enzyme forms of NR could be formed, presumably due to the action of endogenous proteases.

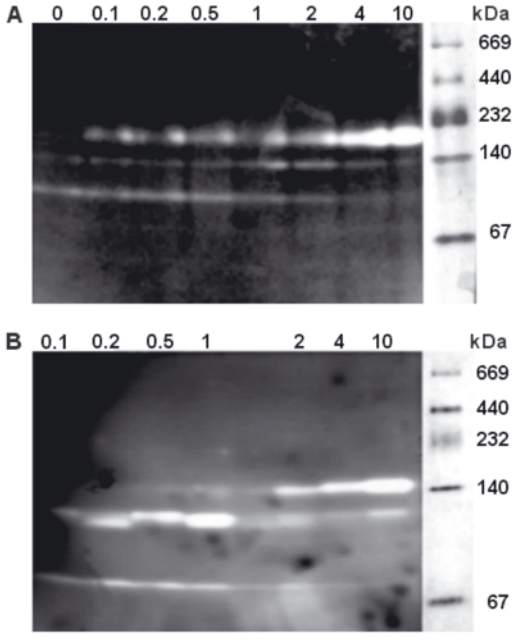

Figure 1. Non-denaturing-PAGE analysis of soluble NR forms.
Rhizobia were induced anaerobically for 6 h with indicated concentrations (mM) of (A) nitrate or (B) nitrite. Cells grown with 0–1 mM concentrations were harvested after 3-th hour to avoid nitrate depletion. Samples of soluble proteins of 300 μg were resolved on 4–15% gradient gel and NR-active complexes were visualized.

The soluble NR forms differ from the 230-kDa enzyme form predominating in the membrane fraction (Fig. 2). Previously, 190-kDa and 140-kDa NR-active forms were isolated not only from the soluble fraction but also from membranes of nitrate-respiring *Bradyrhizobium* sp. (*Lupinus*) USDA 3045 (Polcyn & Luciński, 2006). However, in the present study, a gentler method of cell disruption was used and, more importantly, both spheroplast sonication and solubilization of membrane proteins were done under protection of a protease inhibitor cocktail of a much broader specificity to bacterial proteases than PMSF and benzamidine used in the earlier procedure. This apparently limited the proteolytic cleavage *in vitro* and resulted in a slower migration in the gel of the main membrane-bound NR complex. Nevertheless, the 125-kDa form of low, constant NR activity (Fig. 2) and two faint intermediate forms of 190 kDa and 140 kDa, could be sporadically observed in overloaded lanes (not shown).

The intensity of the 230-kDa activity-band increased several-fold, up to hour 4 of growth on 2 mM nitrate. However, during the next 2 h, the NR activity of this complex decreased remarkably. Similarly, SDS/PAGE immunoblot analysis with anti-NR_{II} antibodies showed a very dynamic NR synthesis in the membrane fraction. The amount of the α-subunit of the enzyme increased 5-fold during the first 3 h after 2 mM nitrate addition (Fig. 3A), but decreased 2-fold during the next 4 hours as a result of nitrate depletion (Fig. 3B), although the culture was still in the log phase of growth. After 10 h of growth, the amount of the α-subunit was negligible. In contrast, in membranes of cells grown on 4 mM nitrate the amount of both α- and β-subunits did not decrease up to hour 7 (Fig. 3C), apparently due to uncon-

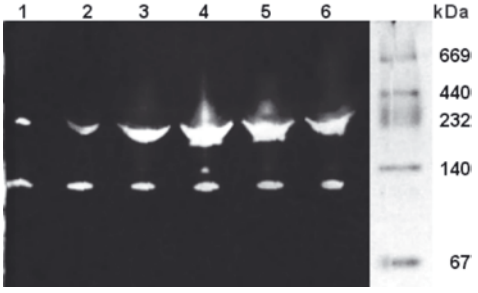

Figure 2. Changes in the level of membrane-bound NR activity in rhizobia induced anaerobically with 2 mM nitrate.
At indicated intervals (hours), 50-μg samples of membrane proteins were solubilized and resolved on 4–15% gradient polyacrylamide gel, containing 0.2% Triton X-100 and NR-active complexes were visualized.

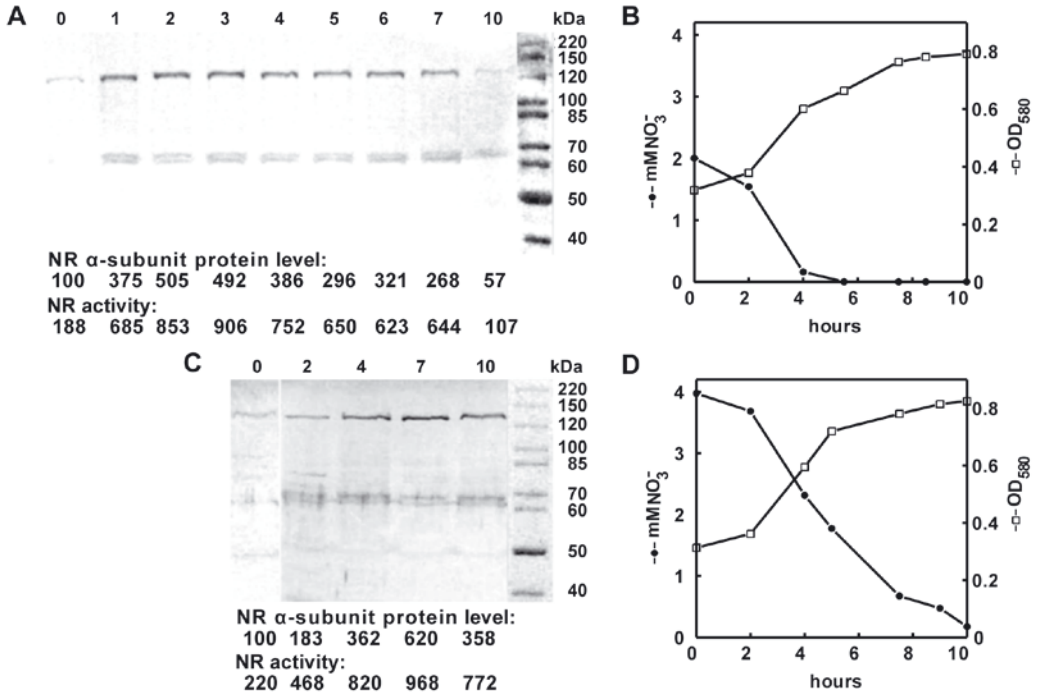

Figure 3. Changes in the level of respiratory NR protein and activity.
Rhizobia were induced anaerobically with (A) 2 mM nitrate or (C) 4 mM nitrate. B and D: Changes in nitrate level and OD_{580}. At indicated intervals (hours), cells were harvested and 15-μg samples of membrane proteins were analyzed by SDS/PAGE and immunoblotting. The protein level of NR α-subunit, estimated by densitometry scanning, is indicated in arbitrary units, relative to the control variant grown for 3 h without nitrate addition. The NR activity was expressed as nmol $NO_2^- \times min^{-1} \times (mg\ protein)^{-1}$. The data are means of densitometric and NR activity measurements from at least two independent experiments. Standard deviations are not shown, however, less than 15% variation was found.

sumed nitrate remaining in the culture medium (Fig. 3D). Furthermore, the level of the increase and decrease of the NR protein was strongly correlated with changes in NR activity (Fig. 3A). These data indicate that nitrate exhaustion from culture medium was associated with a parallel reduction in the amount of membrane-bound NR protein and loss of NR activity.

Nitrate depletion makes rhizobial respiratory NR sensitive to proteolytic degradation

In the soluble fraction of nitrate-respiring rhizobia native-PAGE Western blots showed both 140-kDa and 190-kDa forms and, in addition, an immunoreactive 230-kDa band which presumably was an inactive enzyme complex (Fig. 4). However, when rhizobia were grown anaerobically for 6 h without nitrate addition, an additional, presumably degradative band of 96 kDa was also immunodetected (Fig. 4, "$-O_2$"). A similar proteolytic band appeared when previously nitrate-induced cells were transferred to aerobic conditions (Fig. 4, "$+O_2$").

The observed variation in the protein level (Fig. 3), various activity of the detected NR forms (Fig. 1 and Fig. 2) and degradation shown in Fig. 4 prompted me to investigate whether NR subunits are susceptible to proteolysis during anaerobic growth. To determine this, the degradation products of NR subunits were searched for by immunoblotting in subcellular fractions of rhizobia induced anaerobically for 6 h in the absence of nitrate. One-half of each sample was processed under protection of the protease inhibitor cocktail (see Materials and Methods). Cells were converted to spheroplasts and then disrupted by short sonication (S) or 30-min treatment with 1%

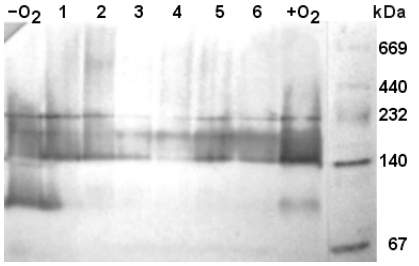

Figure 4. Immunoreactivity of native NR complexes found in the soluble fraction.
Rhizobia, grown anaerobically on 2 mM nitrate, were harvested at indicated intervals (hours). The culture remaining after 6 h was vigorously aerated for 2 h (variant "$+O_2$"). Sample of variant "$-O_2$" was taken from a parallel anaerobic culture grown for 6 h without nitrate addition. Samples of soluble proteins of 150-μg were resolved on non-denaturing 4–15% gradient polyacrylamide gel and analyzed by immunoblotting.

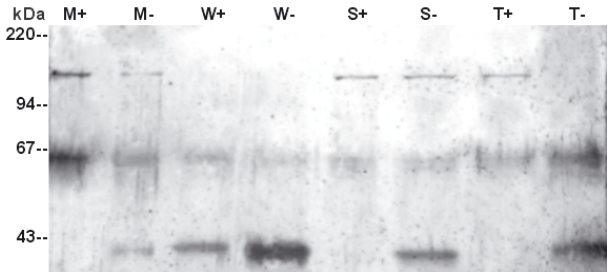

Figure 5. Effect of nitrate availability on the stability of subunits of respiratory NR.
Two parallel cultures were grown anaerobically for 6 h with (+) or without (–) 4 mM nitrate. Then cells were converted to spheroplasts, supplemented with protease inhibitors, disrupted by short sonication, and fractionated by centrifugation. Samples of 20 μg of protein were then subjected to SDS/PAGE immunoblot analysis. (lanes M+, M–) membrane fractions; (lanes S+, S–) supernatants after sonication; (lanes T+, T–) supernatants after 30-min permeabilization of spheroplasts with 1% Triton X-100; (lanes W+, W–) ultrasonic total cell extracts prepared without protease inhibitors. Numbers on the left indicate molecular masses of protein standards in kDa.

Triton X-100 (T) and fractionated by centrifugation into cytosolic (S and T) and membrane (M) fractions. For comparison, in the other half of each sample (W), ultrasonic total cell extract was obtained without protease inhibitors to allow *in vitro* proteolysis (Fig. 5).

Irrespective of nitrate availability, the anti-NRII serum reacted with α- and β-subunits of NR (Fig. 5). However, for cells deprived of nitrate (–), an additional, 42-kDa peptide was immunodetected in cytosolic (S– and T–) proteins. In the membrane fraction (M–), a small amount of the 42-kDa peptide was also present, indicating that it might be a membrane-associated protein. The 42-kDa peptide was the most abundant in total cell extract (W–) obtained without the protection of protease inhibitors. The presented data suggested that nitrate reductase, induced exclusively by anaerobiosis, could undergo some proteolytic degradation when no nitrate was available.

In contrast, extracts from nitrate-fed cells (+) did not contain the immunoreactive 42-kDa peptide in spite of applying the same solubilization methods (Fig. 5). Neither the ultrasonic (S+) or 1% Triton X-100 (T+) cell disruption, nor solubilization of membranes with 4% Triton X-100 (not shown), did liberate it. Moreover, the 42-kDa peptide could not be released from the membrane fraction by standard 5-min boiling with 2% SDS (M+). Nevertheless, the 42-kDa peptide could be detected in total extract from cells processed without the protection of protease inhibitors (W+).

DISCUSSION

According to the model of maturation of *E. coli* membrane-bound NR (see Introduction), the 230-kDa NR complex from nitrate-fed bradyrhizobial strain (Fig. 2) is the mature form of the enzyme. Its molecular mass, confirmed by gel filtration (Polcyn, 2008), is in agreement with the mass of the mature NarGHI complex of bacterial respiratory NR. However, the variable NR-active forms ranging from 75 kDa to 190 kDa found in the soluble cell fraction (Figs. 1 and 4) could be rationalized in terms of enzyme protein turnover related to the level of available nitrate. When cells had not enough nitrate available for respiration then some enzyme complexes might be improperly folded or not attached to the membrane. As a result, immature or degraded NR complexes could be formed as soluble, faster-migrating bands.

The present observations are similar to the result of trypsin digestion of *Pseudomonas stutzeri* membrane-bound NR producing from a 140-kDa complex a degraded but active form of 95 kDa (Blümle & Zumft, 1991). The α subunit of NR from this bacterium has a molecular mass of 112 kDa or even 130 kDa (Hettmann *et al*., 2003), so it is apparent that in spite of extended proteolytic degradation of this subunit, the methyl-viologen-dependent activity of the enzyme could have been largely unaffected. Similar dissociation of truncated NR complexes, with retained catalytic activity, might therefore occur also in *Bradyrhizobium* sp. (*Lupinus*) cells.

The intensity of the 230-kDa activity-band varied with time (Fig. 2), suggesting that the amount of rhizobial respiratory NR could be altered by the level of available nitrate. This supposition was supported when a strong synthesis of membrane-bound NR subunits, followed by their partial disappearance, was immunodetected. The degradation occurred during a short time (Fig. 3A), along with 2 mM nitrate depletion (Fig. 3B). It contrasted with the longer stability of NR from a culture grown in 4 mM nitrate (Fig. 3C). Immunodetection of a degraded complex of native soluble NR (Fig. 4) and the 42-kDa proteolytic derivative of the NR subunits (Fig. 5) indicated that in cells grown without nitrate, respiratory NR became apparently unstable and vulnerable to the action of endogenous protease(s), which was not the case when nitrate was present.

It seems that the above-mentioned observations are not due to *in vitro* proteolytic degradation after cell lysis. Since the extraction and PAGE conditions were uniform for cells induced with or without nitrate, similar degradation should occur in both cases were it caused by *in vitro* proteolysis, which was not observed. Moreover, rhizobia deprived of nitrate were actually not stressed since they were grown on yeast-mannitol broth and, as we showed previously, it enabled efficient fermentative growth of *Bradyrhizobium* sp. (*Lupinus*) USDA 3045 (Polcyn & Luciński, 2003). Fig. 3A illustrate that depletion of

2 mM nitrate did not prevent cell growth and the culture entered stationary phase much later than a two-fold reduction in NR protein level occurred. It can be, therefore, suggested that down-regulation of membrane-bound NR may help to switch respiratory metabolism to fermentative pathways when nitrate respiration is limited. Indeed, such a nitrate/nitrite regulation of fermentative pathways could be observed in *Bradyrhizobium* sp. (*Lupinus*) USDA 3045 and in several other rhizobia (Polcyn & Podeszwa, unpublished).

Although only a minor number of cases of proteolysis-related regulation in bacteria apply to metabolic enzymes, there are several reports describing degradation of nonfunctional soluble NR from *E. coli* mutants (Hackett & MacGregor, 1981), glutamine synthetase oxidized due to a lack of glutamate (Roseman & Levine, 1987; Lee *et al*., 1988), and of MetA (Biran *et al*., 2000), MurAA (Kock *et al*., 2004), EnvA (Ogura *et al*., 1999) and GdhA (Maurizi & Rasulova, 2002).

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