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MICROBIAL NITRATE REDUCTASE: CLASSIFICATION AND DETERMINATION

ALENA DVOŘÁKOVÁ[a], KATEŘINA DEMNEROVÁ[a], MARTINA MACKOVÁ[a], JARMILA PAZLAROVÁ[a], PAVEL RAUCH[a], ELIDA FERRI[b], and STEFANO GIROTTI[b]

[a]*Department of Biochemistry and Microbiology, Institute of Chemical Technology, Prague,* [b]*University of Bologna, Bologna, Italy*

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1. Introduction

Microbial nitrate reductase activity has been widely studied for several reasons. The theoretical interest in nitrate reduction has focused on nitrate reductases as models of trans-membranal proteins, on their synthesis and function and on the role of these enzymes in alternative pathways of the cellular metabolism of nitrogen compounds. This approach led to the discovery of three related nitrate reductases, participating in two alternative nitrate-metabolising pathways[1].

The first of the enzymes is the cytoplasmic assimilatory nitrate reductase catalysing the first step in the energy-requiring assimilatory nitrate reduction which occurs in all plants and in most fungi as well as in many bacteria. Nitrate reduced to ammonium ions by the assimilatory nitrate reductase is used by cells as a major nitrogen source for biosynthetic purposes.

The other two enzymes are the trans-membranal and periplasmic dissimilatory nitrate reductases respectively, which are a part of the dissimilatory nitrate reduction or denitrification of nitrate to nitrite, N-oxides, or to gaseous nitrogen. Here, the nitrate nitrogen serves, prevailingly under anoxic conditions, as a terminal electron acceptor in the membranal respiratory chain for the production of ATP.

From the point of view of agriculture and ecology, the greatest interest lies especially in the dissimilative nitrate reductase activity of bacteria which plays a significant role in soil and water systems. The microbial reduction of nitrate to nitrogen gas by denitrifying bacteria represents a major cause of nitrogen loss from agricultural soil - high amount of the nitrogen applied in the form of fertilisers is lost to the atmosphere. Denitrifying bacteria are also important in removing nitrate from waste- and underground water.

In the field of food industry, the nitrate-reducing bacteria represent one of the most undesirable forms of food contamination, as the enzymatic reduction of nitrate to nitrite and N-oxides facilitates further formation of the carcinogenic N-nitrosamines, known under the abbreviation of ATNC (Apparent Total N-Nitrosocompounds). This problem is especially pressing in some of the fermentation technologies, e.g. in the breweries, and calls for a development of a facile and fast method for determination of the total nitrate reductase activity resulting from bacterial food contamination.

2. Nitrate Reductase Classification

There are several related enzymes, all of them called nitrate reductases, involved in the two different pathways of nitrate reduction in microorganisms, as shown in Fig. 1.

Three major types of microbial nitrate reductases can be distinguished, according to the type of nitrate utilisation they are part of, to their localisation in the cell and their enzymatic function and properties: they are the assimilatory nitrate reductase (NAS), the dissimilatory (respiratory) ni-

trate reductase (NAR) and the periplasmic nitrate reductase (NAP). The summary comparison of the three types of nitrate reductases is shown in Table I.

2.1. Assimilatory Nitrate Reductase (NAS)

The assimilatory nitrate reductases occur in all plants, in most fungi, and in many bacteria (for the most studied prokaryotic nitrate reductases see Table II).

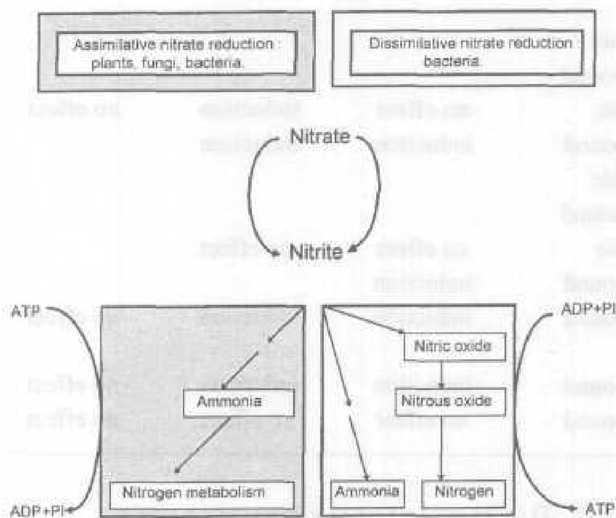


Fig. 1. **Nitrate** reducing **metabolic pathways** (Bonnefoy & **DeMoss** 1994)

In prokaryotes, the NAS belong to the less-known nitrate reduction catalysing enzymes. First described by Pichinoty[2], they catalyse the reduction of nitrate to nitrite - usually with a pyridine nucleotide as an electron donor - as the first step in the assimilatory nitrate reduction pathway.

There are three types of assimilatory nitrate reductases, according to the electron donor they use for the nitrate reduction:

- NADH:nitrate oxidoreductase (EC 1.6.6.1),
- NAD(P)H:nitrate oxidoreductase (EC 1.6.6.2) and
- NADPH:nitrate oxidoreductase (EC 1.6.6.3).

However, the known assimilatory nitrate reductases can use more than one electron donor, though sometimes with significantly decreased efficiency[3]. The NAS can also use synthetic electron donors, as e.g. the so-called viologen dyes. In the assimilatory nitrate reduction pathway, nitrite is further reduced to ammonia as the final reduction product which is then incorporated into the biomass[4]. In this way, nitrate functions as a source of nitrogen for biosynthetic purposes in the assimilatory pathway. The process may occur under aerobic or anaerobic conditions and requires energy[5].

The assimilatory nitrate reductases are constitutive or inducible with low constitutive activity[6,7]. The induction of the assimilatory nitrate reductase depends on the intracellular level of nitrate and sometimes nitrite or nitro-aromatic compounds, as 2,4-dinitrophenol, as well as on the carbon/nitrogen balance of the cell independently of the

Table I
Main characteristic properties of different nitrate reductases[7]

Property	Assmilatory nitrate reductase		Dissimilatory nitrate reductase	
Host	Eucaryotes	Procaryotes	Procaryotes	
Energy (ATP)	Consumption		Production	
Localisation		cytoplasmic	periplasmic	membrane-bounc
Azide		intensitive	intensitive	
Reduction of chlorate	yes	no	yes	
Cofactors	molybdopterin cyt *b* NAD(P)H FAD	molybdopterin cyt *c* Fe-S cluster	molybdopterin cyt *c*	molybdopterin cyt *c* Fe-S cluster
Anaerobiosis		no effect	constitutive	induction
Nitrate	induction	inductions	imulation	induction
Ammonium	repression	repression	no effect	no effect
General nitrogen		repression		no effect

Table II
The most studied prokaryotyc nitrate reductases and their properties[7]

Strains	NR Number	Pathway	Localisation	Regulation		
				Anaerobiosis	Nitrate	Ammonium
K. pneumoniae	2	assimilative	cytoplasmic	indirect repression	induction	repression
		dissimilative	membrane bound	induction	induction	no effect
P. aeruginosa	2	assimilative	cytoplasmic			repression
		dissimilative	membrane bound	induction	induction	
A. eutrophus	3	assimilative				
		dissimilative	periplasmic			
		dissimilative	membrane bound			
R. capsulatus	2	dissimilative	periplasmic	no effect	induction	no effect
R. sphaeroides		dissimilative	membrane bound	induction	induction	
P. denitrificans	2	dissimilative	periplasmic			
		dissimilative	membrane bound			
T. pantotropha	2	dissimilative	periplasmic	no effect	no effect	
		dissimilative	membrane bound	induction		
S. typhimurium	2	dissimilative	membrane bound	induction	induction	no effect
E. coli	2	dissimilative	membrane bound	induction	induction	no effect
		dissimilative	membrane bound	no effect	no effect	no effect

presence of oxygen[6-8]. The NAS are by rule soluble enzymes localised in the cell cytoplasm. However, there have been reports on membrane-bound assimilatory nitrate reductases in some bacteria, for example, in *Rhodobacter capsulatus*[8,9].

The enzyme is usually a homodimer or a homotetramer of subunits with molecular weight approximately 95 000 to 100 000, but a monomeric NAS has also been discovered in some micro-organisms[10]. Each of the subunits contains the prosthetic group of FAD, a b-type cytochrome with haem and molybdenum-pterin groups (Mo-pterin), in a 1:1:1 stoichiometry[3]. The flavin prosthetic group has been identified as the site of NAD(P)H oxidation, while nitrate is reduced at the Mo-pterin centre[3]. The NAS are inhibited by nitrite, slightly repressed[11] or fully inhibited[4,6] by reduced nitrogen sources, e.g. ammonia, inhibited by cyanides[4] and some trace metals as Cu^{2+}, probably due to the oxidation of sulfhydryl groups to disulfides[4]. Both NAD^+, $NADP^+$ and some of their analogues (e.g. the nicotinic acid) have been discovered to be competitive inhibitors of the NAS activity[3]. The assimilatory nitrate reductase is activated by EDTA and thiols, but the mechanism of the activation remains unknown[4].

2.2. Dissimilatory (Respiratory) Nitrate Reductase (NAR)

Respiratory nitrate reductases [1.7.99.4][12] function in the anaerobic respiratory process in which nitrate and nitrite serve as terminal electron acceptors instead of molecular oxygen and are reduced to nitric and nitrous oxides, or further up to gaseous molecular nitrogen[13]. The dissimilatory nitrate reduction is coupled to the generation of the electrochemical proton gradient across the membrane[14] and to generation of ATP (ref. [15]). Only some bacteria and yeasts possess the dissimilatory nitrate reductase activity[7] (for the most studied NAR see Table II).

The respiratory nitrate reductases are inducible enzymes, they are expressed in cells only under anoxic conditions in the presence of nitrate. The induction of NAR does not depend on the degree of reduction of the carbon source available to the cell[14]. The NAR are trans-membrane, highly hydrophobic enzymes with high degree of similarity in different bacterial strains[15-17]. Other substrates of the NAR, beside nitrate, are chlorate and bromate[18]. The enzyme is inhibited competitively by azides, probably by chelating the molybdenum or iron groups at

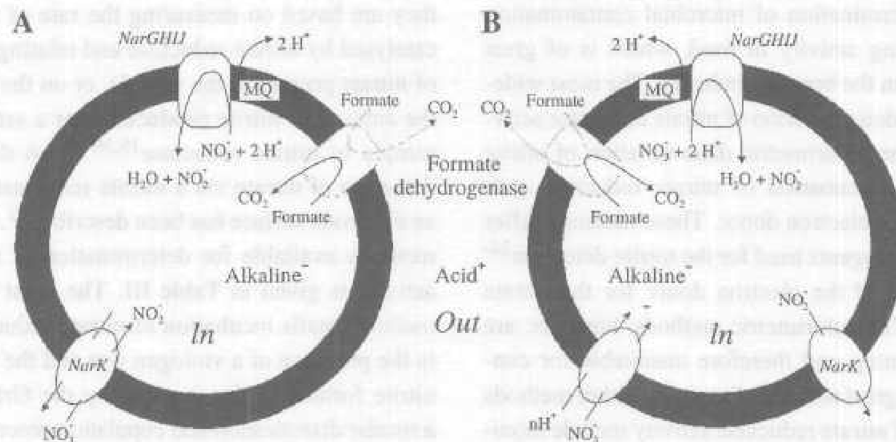


Fig. **2. Schemes of the trans-membrane fluxes of nitrate and nitrite according to the antiport model (A) and the uniport model (B).** MQ = menaquinone, Nar = nitrate reductases

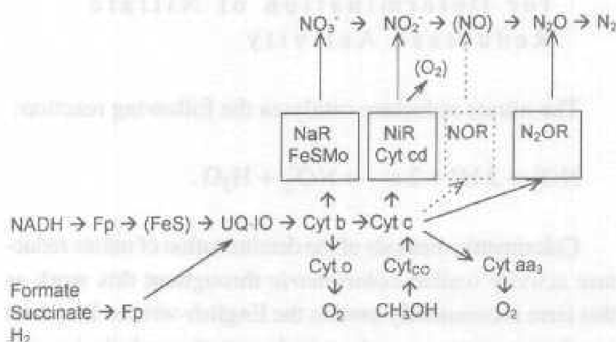


Fig. 3. **Model of probable pathway of electron transport in Paracoccus denitrificans (Knowles 1982).** Nar = nitrate reductase, NiR = nitrite reductase, NOR, N_2OR = N-oxides, Fp = flavoprotein, UQ = ubiquinone, Cyt = cytochrome

the active site of the enzyme[15]. The NAR are also inhibited by cystein[19].

The typical NAR is composed of three subunits: the α-subunit (MW = 140,000) which contains a molybdenum atom and four Fe-S clusters where the reduction of nitrate is supposed to take place at the cytoplasmic site of the membrane[18]; the β-subunit (MW = 60,000) and, finally, the y-subunit (MW = 19,500) which is a b-type cytochrome involved in the electron transport from NADH and in membrane association of the enzyme[15].

The innate electron donors of the NAR are quinols (mainly ubiquinol) and formate[20]. However, the NAR can use synthetic donors as well, especially the so-called viologen dyes[18]. As the nitrate reduction site seems to be oriented to the cytoplasm, the transport of nitrate and nitrite across the membrane remains a question. Nitrate uptake seems to depend on the rate of nitrate reduction[21]. Two hypotheses have been proposed for the nitrate transport:

1) The NAR functions as a nitrate/nitrite antiporter, ridding the cell of the toxic nitrite, or
2) as a nitrate/proton symporter, creating a proton gradient across the membrane[21] (see Fig. 2).

The mechanism of nitrate reduction and the electron flow remains unknown, and only general schemes have been proposed for it (Fig. 3). The reaction mechanisms for ubiquinol and for viologen dyes seem to be distinct[18].

2.3. Periplasmic Nitrate Reductase (NAP)

Very little is known about the periplasmic nitrate reductases and their function in the nitrate reduction processes is not clear[7]. So far they have been found only in some bacteria[7] (see Table I).

Unlike the NAR, the periplasmic nitrate reductase is expressed constitutively, both under oxic and anoxic conditions[7]. It is located in the periplasmic compartment[22]. The periplasmic nitrate reductases examined so far are α-β heterodimeric enzymes. The α-subunit binds a molybdenum cofactor-guanidine mononucleotide moiety and a 4Fe-4S cluster. The β-subunit is a di-haem c-type cytochrome[23]. The physiological role of the periplasmic nitrate reductase might be keeping the redox balance during the transfer from aerobic to anaerobic metabolism[7].

3. Determination of Nitrate Reductase Activity

The significance of developing a fast and easy methods for nitrate reductase activity determination is twofold:
7) Determination of nitrate reductase activity could be a

means for the determination of microbial contamination with nitrate-reducing activity in food, which is of great interest especially in the brewing industry. The most widespread methods of determination of nitrate reductase activity are based on the colorimetric determination of nitrite formed during the incubation of nitrate reductase with nitrate and a suitable electron donor. These methods differ in the colorimetric reagents used for the nitrite detection[24-26] or in the choice of the electron donor for the nitrate reductase[24,26,27]. The colorimetric methods, however, are mostly timeconsuming and therefore unsuitable for continuous analysis of great number of samples. Other methods of determination of nitrate reductase activity include monitoring the nitrate/nitrite concentration changes by nitrate specific electrode[28] or by HPLC[29,30]. A possible method for determination of the NAD(P)H-dependent nitrate reductase activity could be a bioluminescent continuous-flow system, described for other analytes[31].

2) Nitrate reductase activity assays could serve for the determination of nitrate in fertilisers, food and water. Though there exists a whole range of methods for the determination of nitrate, none of them is problem free in terms of specificity, sensitivity and time necessary for the analysis[32]. Sorne such methods have been already described:

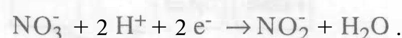
Table III
Overview of methods for determination of nitrate reductase activity

Method	Characteristics	Electron donor
Colorimetric: Measures the production of nitrate		
- m-Phenylendiamine reaction[25]	semi-quantitative	native[25]
- Griess reaction + similar[24,26]	quantitative	viologen dyes +NAD(P)H
HPLC: Measures the production of nitrate[30]	quantitative	viologen dyes +NAD(P)H
Spectrophotometric: Measures the consumption of NAD(P)H[24]	quantitative	NAD(P)H
Bioluminescent: Measures the consumption ofNAD(P)H[24]	quantitative	NAD(P)H

they are based on measuring the rate of nitrate reduction catalysed by nitrate reductase and relating it to the amount of nitrate present in the sample, or on the measurement of the amount of nitrite produced after a set exposure of the sample to nitrate reductase[16,26]. Also the amperometric detection of nitrate via a nitrate reductase immobilised at an electrode surface has been described[33]. The overview of methods available for determination of nitrate reductase activity is given in Table III. The most commonly used method entails incubation of nitrate reductase with nitrate in the presence of a viologen dye and the determination of nitrite formed in the reaction by the Griess reaction[34] or a similar diazotization and copulation procedure.

3.1. Colorimetric Methods for Determination of Nitrate Reductase Activity

The nitrate reductase catalyses the following reaction:



$$NO_3^- + 2\,H^+ + 2\,e^- \rightarrow NO_2^- + H_2O\,.$$

Colorimetric methods of the determination of nitrate reductase activity (called *colorimetric* throughout this work as this term is commonly used in the English-written literature in reference to them, and not in the strictly analytical sense) are based on a reaction of nitrite produced in the reaction with a suitable reagent forming a coloured complex whose absorbance is then measured at its absorbance maximum. The colorimetric methods can be divided into two groups according to whether the nitrate reductase activity is tested in a living biomass or in disrupted cells already containing a suitable electron donor necessary for the reaction or whether a partially isolated or commercially available enzyme is used for which the electron donor must be selected and added to the reaction mixture. In the second case, the choice of a suitable electron donor that does not interfere with the colorimetric reaction of nitrite is crucial for the determination.

4. Isolation and Purification of Nitrate Reductases

4.1. Assimilatory Nitrate Reductase

Isolation and purification procedures for the assimilatory nitrate reductase have been described. The NAS is a

soluble, light-sensitive enzyme though otherwise relatively stable, usually located in the cytoplasm. It has a rather broad pH optimum between 7 and 8. The optimal temperature for the assimilatory nitrate reductase activity depends on the bacterial strains from which the NAS has been isolated[10].

To obtain assimilatory nitrate reductase activity, cells have to be grown to the late exponential phase under heterotrophic conditions with aeration and with nitrate as the only or prevailing nitrogen source. Only little or none of assimilatory nitrate reductase activity occurs if nitrate is either omitted from the medium or some ammonium salt is supplied as an additional source of nitrogen[1].

4.2. Dissimilatory Nitrate Reductase

Isolation and purification procedure for the dissimilatory nitrate reductase has been described[24]. The NAR are trans-membrane, highly hydrophobic enzymes. When purified, the enzyme exhibits a characteristic tendency to aggregate to inactive oligomers (MW = 600,00—800,000) or to form a reversibly associating-dissociating system which can dissociate to monomers of molecular weight of 200,000 or associate to polymers as large as a tetramer of 800,000. This aggregation depends on the method of release of the enzyme from the membrane[35].

The NAR is a highly unstable, light-sensitive enzyme. The γ-subunit of the enzyme very often gets lost in the process of purification in consequence of a limited proteolysis or simply by dissociation[35]. Another characteristic property of the enzyme is its heat-activation which is an irreversible, time and temperature dependent process[16]. Both the pH and temperature optimum depend on the bacterial strain from which the NAR is isolated[1,24].

To obtain the dissimilatory nitrate reductase activity, cells have to be grown anaerobically in the presence of nitrate (to induce the NAR) and in the presence of ammonium ions in order to repress the NAS expression[1].

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REFERENCES

1. Warnecke-Eberz U., Friedrich B.: Arch. Microbiol. 759,405(1993).
2. Pichinoty F.: Bull. Soc. Chim. Biol. *47,* 1526 (1965).
3. Trimboli A. J.,Barber M. J.: Arch. Biochem. Biophys. *315(1),* 48(1994).
4. Smarrelli Jr. J., Campbell W.H.: Phytochem. *19,* 1601 (1980).
5. Rowe J. 1, Ubbink-Kok T., Molenaar D., Konings W. N., Driessen A. J. M.: Mol. Microbiol. *12(4),* 579 (1994).
6. Dobao M. M., Martinez-Luque M., Moreno-Vivian C, Castillo F.: Can. J. Microbiol. *40,* 645 (1994).
7. Bonnefoy V., DeMoss J. A.: Antonie van Leeuwenhoek *66,* 47(1994).
8. Martinez-Luque M., Dobao M. M., Castillo F.: FEMS Microbiol. Lett. *83,* 329 (1991).
9. Wesch R., Klemm J. H.: FEBS Lett. *8,* 37 (1980).
10. Gangeswaran R., Lowe R. H., Eady R. R.: Biochem. J. *289*, 335 (1993).
11. Gonzalez C., Gonzalez G., Avila J., Perez M. D., Brito N., Siverio J. M.: Microbiol. *140,* 2633 (1994).
12. Rosso J. P., Forget P., Pichinoti L: Biochim. Biophys. Acta *321,* 443 (1973).
13. Knowles R.: Microbiol. Rev. *46(1),* 43 (1982).
14. Boogerd F. C, Van Verseveld H. W., Stouthamer A. H.: Biochem. Biophys. Acta *723,* 415 (1983).
15. Carlson C. A., Ferguson L. P., Ingraham J. L.: J. Bacteriol. *151(1),* 162(1982).
16. Craske A., Ferguson S. J.: Eur. J. Biochem. *158,* 429 (1986).
17. Hochstein L. I., Lang.: Arch. Biochem. Biophys. *288(2),* 380 (1991).
18. Morpeth F. F., Boxer D. H.: Biochem. *24,* 40 (1985).
19. Yamaoka K., Kato M., Kamihara T.: Biosci. Biotech. Biochem. *58(6),* 995 (1994).
20. Smith N. A., Smith P.: Lett. Appl. Microbiol. *14,* 61 (1992).
21. Wu Q., Knowles R.: Can. J. Microbiol. *40,* 576(1994).
22. Richardson D. J., Ferguson S. J.: Arch. Microbiol. *157,* 535 (1992).
23. Sears H. J., Bennett B., Spiro S., Thompson A. J., Richardson D. J.: Biochem. J. *310,* 311 (1995).
24. MacGregor, in: *Methods in Enzymology LIII* (Fleischer, Packer, ed.) Academic Press, New York 1978.
25. Šavel J., Prokopova M., Zdvihalova D.: Kvasný Prum. *38(12),* 350(1992).
26. Verdon C. P., Burton B. A., Prior R. L.: Anal. Biochem. *224,* 502 (1995).
27. Schmidt H. H. W.: Biochem. Boehringer Mannheim 2,30(1995).

28. John P.: J. Gen. Microbiol. *98,* 2318 (1977).
29. Crowther D., Monaghan J. M., Cook K., Gara D.: Anal. Commun. *33,* 51 (1996).
30. Wais S., Sussmuth R.: J. Microbiol. Methods *19,* 215 (1993).
31. Roda A., Girotti S., Carrea G., in: *Methods in Enzymology 133.* Academic Press, New York 1986.
32. Sah R. N.: Commun. Soil Sci. Plant Anal. *25(17&18),* 2841 (1994).
33. Cosnier S., Innocent C., JouanneuY.: Anal. Chem. *66,* 3198 (1994).
34. Griess J. P.: Phil. Trans. R. S. *154,* 667 (1864).
35. Lund K., DeMoss J. A..: J. Biol. Chem. *251(8),* 2207 (1976).
36. Dvořáková A.: *Diploma thesis.* Institute of Chemical Technology, Prague 1996.

A. Dvořáková[a], K. Demnerová[a], M. Macková[a], J. Pazlarová[a], P. Rauch[a], E. Ferri[b], and S. Girotti[b] *([a]Department of Biochemistry and Microbiology, Institute of Chemical Technology, Prague, [b]University of Bologna, Bologna, Italy):* **Microbial Nitrate Reductase: Classification and Determination**

The review summarises the contemporary knowledge on occurrence, production, and estimation of different microbial nitrate reductases (NR). The main interest is focused on two basic NR types: dissimilatory and assimilatory NR, both playing an important role in nitrate metabolism of bacteria. NR are putatively responsible for the presence of nitrosamines in many food items. In the second part, quantitative and qualitative methods of NR estimation are compared.