Review

Assimilation of nitrate by yeasts

José M. Siverio *

Departamento de Bioquímica y Biología Molecular, Grupo del Metabolismo del Nitrógeno, Universidad de La Laguna, E-38206 La Laguna, Tenerife, Spain

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Abstract

Nitrate assimilation has received much attention in filamentous fungi and plants but not so much in yeasts. Recently the availability of classical genetic and molecular biology tools for the yeast Hansenula polymorpha has allowed the advance of the study of this metabolic pathway in yeasts. The genes YNT1, YNR1 and YNI1, encoding respectively nitrate transport, nitrate reductase and nitrite reductase, have been cloned, as well as two other genes encoding transcriptional regulatory factors. All these genes lie closely together in a cluster. Transcriptional regulation is the main regulatory mechanism that controls the levels of the enzymes involved in nitrate metabolism although other mechanisms may also be operative. The process involved in the sensing and signalling of the presence of nitrate in the medium is not well understood. In this article the current state of the studies of nitrate assimilation in yeasts as well as possible venues for future research are reviewed. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Nitrate and nitrite assimilation; Yeast; Nitrate transport; Hansenula polymorpha; Pichia angusta

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1. Introduction

Yeasts are able to use a great variety of compounds as nitrogen sources [1]. However, the use of nitrate and nitrite is restricted to relatively few species of different genera (Table 1); curiously, some yeasts are able to use nitrite but not nitrate (Table 1) but the biochemical basis of this property has not been investigated. The fact that yeasts of the genera Saccharomyces and Schizosaccharomyces are unable to use nitrate or nitrite as sole nitrogen source [1] may partly explain why this area of metabolism has received scarce attention in yeasts in comparison to the broad and intensive studies carried out in filamentous fungi [2]. Another reason for this neglect has been the lack of adequate tools for genetic analysis and molecular biology studies in those yeasts able to assimilate nitrate. However, the situation changed when these types of tools were developed in the last decade for the yeast Hansenula poly-
Nitrate and nitrite transport

The chemical characteristics of nitrate and nitrite make necessary the existence of transport systems to carry these molecules to the interior of the cells in which they are utilised. Hipkin [7] provided the first evidence on the connection between nitrate induced protein synthesis and nitrate uptake in yeast. Shortly before, Eddy and Hopkins [8] showed that in Candida utilis nitrate uptake is concomitant with the entrance of two equivalents of protons and the exit of one equivalent of K⁺. The lack of easy assays for labelled nitrogen containing compounds has seriously hindered the study of nitrate or nitrite transport systems in comparison with other transport systems, for example those of sugars. Several strategies have been used to bypass this limitation and to measure the activity of nitrate transport. Since in H. polymorpha nitrate reductase is not a rate-limiting step in the nitrate assimilation pathway [9], nitrate uptake rates measured at low nitrate concentration (0.1 mM) for short periods of time have been assumed to be a measure of nitrate transport [9]. Alternative strategies have been used for the same purposes in different organisms. In algae the measurement of intracellular content of nitrate has been used to determine nitrate transport in mutants lacking this enzyme [10]. More recently, in Aspergillus nidulans nitrate influx was measured using ¹³N, a short-lived isotope [11]. An indirect strategy has been to inject the nitrate transporter mRNA in oocytes of Xenopus where nitrate transport can be studied by electrophysiological methods [12–14]. Using all these strategies a corpus of knowledge on nitrate transport in different organisms has been acquired [15–17]. Kinetically two groups of nitrate transporters have been characterised: one with high affinity, Kᵣ in the μM nitrate range, found in yeasts, filamentous fungi, algae and plants [15–17] and one low affinity group, Kᵣ in the mM nitrate range, found mainly in plants, although there is indirect evidence of its presence in yeast and algae [18,19].

The cloning of a gene named YN1 encoding a nitrate transporter from H. polymorpha helped to dissect nitrate and nitrite transport in yeast [20]. YN1 encodes a high affinity nitrate transporter (Kᵣ 2–3 μM) which constitutes quantitatively the main nitrate transporter activity in H. polymorpha [18,20]. Ynt1 also transports nitrite with high affinity and belongs to the proposed NNP (nitrate nitrite porter) family involved in nitrate and nitrite transport [17]. This family in turn belongs to the major facilitator superfamily (MFS), constituted by transmembrane proteins in which 12 membrane spanning helices connect cytosolic N-terminal and C-terminal domains [21]. Although Ynt1 presents similarity in sequence, putative secondary structure and membrane topology with the Aspergillus nitrate transporter CRNA [22] and the plant and algae high affinity nitrate transporters [15–17], the shorter C-terminal region and the longer length of the loop between the sixth and the seventh transmembrane

Table 1

Yeast genera among which nitrate and nitrite utiliser species are found [1]

<table>
<thead>
<tr>
<th>Yarrowia</th>
<th>Bullera*</th>
<th>Candida</th>
<th>Citeromyces</th>
</tr>
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<tbody>
<tr>
<td>Cryptococcus</td>
<td>Cystoflocibasidium</td>
<td>Chionosphera</td>
<td>Debaryomyces*</td>
</tr>
<tr>
<td>Dekkeria</td>
<td>Fellomyces</td>
<td>Fibulobasidium*</td>
<td>Filobasidium</td>
</tr>
<tr>
<td>Hansenula</td>
<td>Geotrichum</td>
<td>Hormoascus</td>
<td>Leucosporidium</td>
</tr>
<tr>
<td>Mrakia</td>
<td>Pachysolen</td>
<td>Pichia</td>
<td>Rhodosporidium</td>
</tr>
<tr>
<td>Rhodotorula</td>
<td>Saitoella</td>
<td>Sporidiobolus</td>
<td>Sporobolomyces</td>
</tr>
<tr>
<td>Trichosporon*</td>
<td>Wickerhamiella</td>
<td>Wilgopsis</td>
<td></td>
</tr>
</tbody>
</table>

*Some species in these genera utilise nitrite but not nitrate.
domains are characteristics distinctive of Ynt1 and CRNA with respect to the plant nitrate transporters.

In several organisms where nitrate uptake has been more deeply studied, multiple nitrate uptake systems have been identified [15,17]; for example in A. nidulans, in addition to CRNA [22], a second high affinity nitrate transporter designated NRTB has been isolated [11]. In Chlamydomonas reinhardtii where nitrate/nitrite transport is the best characterised, so far, four systems involved in nitrate and nitrite transport have been reported [19,23,24].

In H. polymorpha the existence of a second nitrate transporter has been inferred from different experimental evidence but the gene encoding it has not yet been identified [18].

Nitrite also enters H. polymorpha through a transport system different to Ynt1; however, several questions remain unsolved concerning this process. It is not clear whether nitrite enters through a specific transport system or if it shares a nitrate transport [18]. The study of its entrance in those yeast species able to use nitrite but not nitrate (see Table 1) may help to understand this process and the characteristics of the implicated proteins.

2.2. Nitrate reductase

The ultimate fate of intracellular nitrate is its reduction to ammonium. This reduction occurs in two successive steps (see Fig. 1). The first step is catalysed by nitrate reductase, a protein which requires different cofactors like molybdopterin, haem-iron and FAD [25,26]. The enzyme from the yeast H. polymorpha as most yeast nitrate reductases studied is able to use NADH and NADPH as electron donors [7], but its nitrate reductase activity in vitro is about 3-fold higher with NADPH [27].

Genes encoding nitrate reductase have been isolated from filamentous fungi [28,29], plants [26], algae [30] and the yeasts H. polymorpha [6] and Hansenula anomala [31]. The putative proteins encoded by the genes of these yeasts, named YNRI, share a high similarity with the nitrate reductases from Neurospora crassa [29], Aspergillus [28,32] and plants [26]. The regions that bind molybdopterin, haem and FAD respectively have been identified by their similarity with regions of different proteins like mammalian sulfite oxidase [33], cytochrome b5 [34] and NADPH cytochrome b5 reductase [35], proteins that present domains involved in molybdopterin, haem and FAD binding respectively. Similar to the situation in other nitrate reductases the cofactor binding regions in the yeast protein are located consecutively with the molybdopterin binding region near the N-terminus, followed by the haem, and the FAD–NADPH binding regions at the C-terminus.

Different experimental evidences indicate that in H. polymorpha the YNRI gene is the only one that encodes nitrate reductase [6].

2.3. Nitrite reductase

The reduction of nitrite to ammonium is catalysed by nitrite reductase, a protein that in fungi and bacteria uses NAD(P)H as electron donor. This differentiates it from the nitrate reductases from plants and algae, that use ferredoxin [36]. Nitrite reductases possess two prosthetic groups, an iron–sulfur centre and a sirohaem group [37–39]. In addition the bacterial and fungal proteins possess FAD [37,40].

Genes encoding nitrite reductase named YNII have been isolated from the yeasts H. anomala and H. polymorpha [31,41]. The gene YNII from H. polymorpha encodes a putative protein of a calculated molecular mass of 116.6 kDa and with about 50% identity with nitrite reductases from other fungi [25,42]. Significant identity was also found with nitrite reductases from Klebsiella pneumoniae [43] and Escherichia coli [44]. Comparison of the sequence of the H. polymorpha protein with the fungal ones [45] and with a region of the sulfite reductase from E. coli [46] allowed the localisation of the putative iron–sulfur centre and sirohaem domain. In this region the consensus sequence CXXXXXChnGCXXX [37] is found and the cysteine residues have been proposed to be involved in the binding of the tetranuclear iron–sulfur centre and sirohaem [36]. Analysis of the sequence also revealed the existence of a β-sheet–α-helix–β-sheet structure with a motif sequence GXGXGG, that is found in some proteins that interact with FAD, NAD or NADP [47]. This motif is found twice near the N-terminus of the reductase from H. polymorpha as well as in those of A. nidulans and N. crassa [28,29,41] and is likely implicated in the interaction with the nucleotide.
The gene YNI1 is present in a single copy and there is no evidence for other genes encoding additional nitrite reductase in \textit{H. polymorpha} [41].

3. Genomic organisation of the genes involved in nitrate assimilation in \textit{H. polymorpha}

During the isolation of the genes involved in nitrate assimilation in \textit{H. polymorpha} it was observed that certain clones which carried DNA corresponding to YNI1 hybridised with a probe designed for YNRI and vice versa. Further analysis revealed that the genes encoding the enzymes for the nitrate assimilation pathway were clustered in \textit{H. polymorpha}. It was also found that the genes encoding the transcriptional factors Yna1 and Yna2 [48,49] (Fig. 2) were located in the same cluster. Clustering of genes encoding enzymes from nitrate metabolism has also been observed in other organisms such as \textit{A. nidulans} and \textit{Aspergillus niger} [32] or the alga \textit{C. reinhardtii} [50] but not in \textit{N. crassa} [51]. Also in \textit{H. anomala} the genes YNR1 and YNI1 are closely linked [31]. What appears to be a peculiarity of the \textit{H. polymorpha} gene cluster is the presence in it of the genes encoding the two factors involved in the transcriptional regulation of the other cluster members. Another difference of the \textit{H. polymorpha} gene cluster is that the YNR1 and YNI1 genes are convergently transcribed, a situation not found in filamentous fungi [49,52].

In total, the fragment of DNA devoted to nitrate metabolism in \textit{H. polymorpha} represents 11,040 bp, of which roughly 92% is coding DNA. This coding density is higher than the average in \textit{Saccharomyces cerevisiae}, where coding DNA accounts for about 72% of the genome [53].

4. Regulation of the expression levels of YNT1, YNR1 and YNI1

Measurements of steady-state levels of specific mRNAs are assumed to reflect the degree of transcription of the corresponding genes. Although this is not absolutely true, we will also follow this assumption in the analysis of YNT1, YNR1 and YNI1 expression from \textit{H. polymorpha}.

Levels of the respective mRNAs determined by Northern blot in cells grown with ammonium as nitrogen source and transferred for 2 h to nitrate or nitrite were very high, while they were undetectable in the original culture. Since low but detectable levels of the mRNAs specific for the three genes were demonstrated after transfer to media with nitrate plus ammonium, it was concluded that the expression of these genes is induced by nitrate and subjected to nitrogen catabolite repression [48,49]. Curiously, in \textit{H. anomala} the level of mRNA corresponding to YNR1 and YNI1 in ammonium plus nitrate medium was similar to that found in nitrate [31], indicating that in this yeast nitrogen catabolite repression for these genes is less stringent than in \textit{H. polymorpha} [49].

The strength of the promoters of YNT1, YNR1 and YNI1 has been estimated using fusions with the \textit{E. coli lacZ} gene [45]. The highest \(\beta\)-galactosidase activity was found with the fusion expressed from the YNRI promoter, and the lowest in the fusion with the YNT1 promoter [45]. Similar results were obtained when the measurements were carried out in cells transferred to nitrite [45]. No \(\beta\)-galactosidase activity was found in cells grown in ammonium. These results suggest that the strongest promoter is that corresponding to YNR1 followed by YNT1 and YNI1, and contrast with the assumption that nitrite reductase levels are the highest in the nitrate assimilation pathway followed by those of nitrate reductase [45]. The low level of nitrite reductase (about 40 mU mg\(^{-1}\) protein) measured in \textit{H. polymorpha} is consistent with the low level of expression observed in the fusion with the YNI1 promoter [41].

Studies of the mechanisms by which the transcriptional regulation of the genes encoding enzymes is involved in nitrate assimilation in yeasts have been performed only in \textit{H. polymorpha}, but what has been found is reminiscent of the situation in filamentous fungi [2]. In these organisms two transcriptional factors specifically involved in the induction by nitrate and their counterparts involved in nitrogen catabolite repression have been studied in detail. The activators are NIT4 from \textit{N. crassa} and NirA from \textit{A. nidulans}, and they belong to the Zn(II)\(\_\)2Cys6 family of transcriptional factors [54,55]. However, although usually the fungal Zn(II)\(\_\)2Cys6-type transcriptional factors recognise in the target gene promoter the sequence CCG \(X_c\) CCG [56], the sequences of NIT4 and NirA binding sites are far from that consensus. NIT4 binds to sites containing the symmetrical octameric sequence TCCGCGGA, while in \textit{A. nidulans} NirA binds four sites with the consensus sequence CTCCCGHGCG in the intergenic region of the divergently transcribed genes \textit{niiD} and \textit{niiA} encoding nitrate and nitrite reductases [57].

NIT2 and AreA are positive regulatory factors from \textit{N. crassa} and \textit{A. nidulans} respectively, involved in nitrogen catabolite derepression from ‘good’ nitrogen sources such as ammonium or glutamine. These proteins belong to the family of GATA transcriptional factors, present in different organisms from fungi to humans [58,59]. The mecha-
nism proposed for the NIT2 in \( N.\ crassa \) repressive function is that NMR1, a negatively acting protein, interacts with NIT2 in the presence of nitrogen catabolic repressors preventing the binding of NIT2 to DNA and as a consequence no transcription of the target gene is carried out [59]. \( N.\ crassa \) NMR1, the orthologue of Ure2 in \( S.\ cerevisiae \), a central protein in the control of nitrogen metabolism in this organism [58], interacts with GLN3 and GAT1 products. \( H.\ polymorpha \) mutants defective in NMR1 (formally \( Hp\text{-Ure}2 \)) have been isolated [60].

In \( H.\ polymorpha \) two Zn(II)_2Cys_6 transcriptional factors encoded by the genes \( YNA1 \) and \( YNA2 \) have been found clustered with the structural genes [48,49]. The corresponding proteins show a high similarity with the factors NirA [54] and NIT4 [55] previously mentioned. They show a typical Cys-X_2-Cys-X_6-Cys-X_5-Cys-X_2-Cys-X_4-Cys sequence where the sixth cysteine region is followed by a basic region, which could be involved in the DNA binding specificity of the transcriptional factor linked to a leucine zipper motif likely implicated in a possible dimerisation of these transcriptional factors [61,62].

The similarity of Yna1p and Yna2p with the proteins NirA and NIT4 [48,49] is highly suggestive of a role of \( YNA1 \) and \( YNA2 \) genes in the transcriptional activation of nitrate inducible genes in \( H.\ polymorpha \). This role is shown in fact by the behaviour of \( \Delta yna1::URA3,\ yna2::URA3 \) and the double mutant. These mutants were unable to grow in nitrate or nitrite while they grew in glutamate, proline or hypoxanthine [48,49]. Moreover they were unable to produce detectable mRNA corresponding to \( YNT1,\ YNR1 \) or \( YNI1 \) after transfer to nitrate containing medium.

\( YNA1 \) appears to control expression of \( YNA2 \), while \( YNA2 \) has no effect on the transcription of \( YNA1 \) [49]. Thus, a regulatory pathway \( YNA1 \rightarrow YNA2 \rightarrow (YNT1,\ YNR1,\ YNI1) \) might be suggested. An alternative could be the formation of a heterodimeric complex Yna1p–Yna2p that would be involved in the transcriptional activation of the nitrate assimilatory genes. Such heterodimers have been found in \( S.\ cerevisiae \) for the GATA factors Dal80p and Deh1p [63] and Zn(II)_2Cys_6 Oaf1p and Pip2p factors [64,65].

5. Non-transcriptional regulation of the proteins involved in nitrate utilisation

Although control of the transcription of \( YNT1,\ YNI1 \) and \( YNR1 \) in response to the nitrogen sources of the medium is the main mechanism that controls the levels of proteins involved in the nitrate assimilation pathway in yeasts, there are other mechanisms, still poorly understood, that also contribute to the rapid adjustment of the levels of those proteins to changes in the availability of the nitrogen source.

A rapid loss of Ynt1 activity has been observed in \( H.\ polymorpha \) after addition of glutamine to cells grown in nitrate [9]. In \( H.\ anamala \) the absence of nitrogen sources or the presence of ammonium or glutamine produces a loss of nitrate reductase activity and protein [7,66]. In addition, the nitrate reductase undergoes inactivation as a response to nitrite, uncouplers and heat shock [67,68]. This inactivation is reversible and does not affect a non-physiological activity of the protein in which only the molybdopterin moiety is involved. Curiously, the inactive enzyme was found to be associated with membranes presumably from mitochondria [67,68]. A satisfactory explanation to this localisation has not yet been found; however, a similar situation has been found in plants [69,70]. In \( C.\ utilis \) nitrite reductase undergoes a phosphorylation–dephosphorylation reaction that may be of regulatory significance. This enzyme is a heterodimer composed of two monomers of 58 and 66 kDa, which in vivo are differentially phosphorylated in response to nitrate and ammonium. During growth in nitrate the enzyme presents a high activity and the 66-kDa subunit is highly phosphorylated while the 58-kDa subunit is not. Dephosphorylation of this enzyme form in vitro by alkaline phosphate correlates with a decrease of nitrite reductase activity. In ammonium-grown cells nitrite reductase activity was 20% of that in cells grown in nitrate and the phosphorylation degree was decreased about 80%, but both subunits were phosphorylated [71,72]. Unfortunately no further information is available either on the phosphorylation sites or on the participants in the reaction.

<table>
<thead>
<tr>
<th>N-Source</th>
<th>Yeast nitrate assimilation mutant strains</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>( \Delta ynt1::URA3 )</td>
</tr>
<tr>
<td>Nitrate</td>
<td>±</td>
</tr>
<tr>
<td>Nitrite</td>
<td>+</td>
</tr>
<tr>
<td>Ammonium</td>
<td>+</td>
</tr>
<tr>
<td>Proline</td>
<td>+</td>
</tr>
<tr>
<td>Glutamate</td>
<td>+</td>
</tr>
</tbody>
</table>

*, growth; ±, weak growth; –, no growth. From [20,41,48,69,60].
6. Conclusions and perspectives

The availability of a set of mutants (Table 2) of *H. polymorpha* affected in either genes encoding metabolic or regulatory proteins involved in nitrate metabolism situates this yeast in an excellent position to be used as a model organism to study nitrate assimilation. In addition these mutants open the possibility to study plant genes involved in nitrate assimilation in this organism. In this respect work towards the cloning of plant genes involved in certain areas of nitrate metabolism and the study of their function, or structure-function studies of known proteins could be considered. Studies of this type could be done in a yeast able to assimilate nitrate far more easily than in the classical model yeasts *S. cerevisiae* and *Schizosaccharomyces pombe*, since these yeasts are unable to synthesise the molybdopterin cofactor. An integrative expression vector for *H. polymorpha* is available that could be used for these tasks [73].

Something that ought to be studied carefully is the relationship of the nitrate assimilatory pathway with the redox metabolism of the cell. It would be interesting to see how mutations in systems that provide NADPH, such as glucose-6-phosphate dehydrogenase, affect nitrate assimilation.

Among the still unsolved problems of nitrate metabolism in yeast is that of the signal that triggers induction of the proteins of the assimilatory pathway. Isolation of mutants defective in their response to nitrate would contribute to our understanding of the system.

Finally, to understand from an evolutionary point of view why only a limited number of yeast species is able to assimilate nitrate would contribute to draw a more accurate picture of the position of yeasts in Nature.

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