The *YNT1* gene encoding the nitrate transporter in the yeast *Hansenula polymorpha* is clustered with genes *YNI1* and *YNR1* encoding nitrite reductase and nitrate reductase, and its disruption causes inability to grow in nitrate

M. Dolores PÉREZ, Celedonio GONZÁLEZ, Julio ÁVILA, Nélida BRITO and José M. SIVERIO*

Departamento de Bioquímica y Biología Molecular, Universidad de La Laguna, E-38206 La Laguna, Tenerife, Canarias, Spain

DNA sequencing in the phage *λJA13* isolated from a λ EMBL3 *Hansenula polymorpha* genomic DNA library containing the nitrate reductase- (*YNR1*) and nitrite reductase- (*YNI1*) encoding genes revealed an open reading frame (*YNT1*) of 1524 nucleotides encoding a putative protein of 508 amino acids with great similarity to the nitrate transporters from *Aspergillus nidulans* and *Chlamydomonas reinhardtii*. Disruption of the chromosomal *YNT1* copy resulted in incapacity to grow in nitrate and a significant reduction in rate of nitrate uptake. The disrupted strain is still sensitive to chlorate, and, in the presence of 0.1 mM nitrate, the expression of *YNR1* and *YNI1* and the activity of nitrate reductase and nitrite reductase are significantly reduced compared with the wild-type. Northern-blot analysis showed that *YNT1* is expressed when the yeast is grown in nitrate and nitrite but not in ammonium solution.

INTRODUCTION

The methylotrophic yeast *Hansenula polymorpha* is able to use nitrate as the sole nitrogen source [1]. The nitrate-assimilation pathway in this yeast follows that described for plants and filamentous fungi [2,3]. Once nitrate enters the cells, it is reduced to ammonia by the consecutive action of nitrate reductase (NR) and nitrite reductase (NiR). Since nitrate transport is the first step in the nitrate-assimilation pathway and induces expression of the genes related to its assimilation, the activity of nitrate transporters is important in the regulation of this pathway. Nitrate transport is difficult to assay because of the absence of suitable radiolabelled tracers, but despite this, the kinetic approach has been a useful way of studying it [4,5]. However, to achieve a better understanding of the structure, types and mechanisms of regulation of nitrate uptake it is necessary to isolate the genes encoding the nitrate transporters and characterize the products they encode. As part of our work on the regulation of nitrate metabolism in yeast, we have isolated a gene encoding a nitrate transporter in *H. polymorpha*. Over the last few years, the eukaryote nitrate-transporter-encoding genes *crnA*, *nar-3* and *CHL1* have been isolated from *Aspergillus nidulans* [6,7], *Chlamydomonas reinhardtii* [8] and *Arabidopsis thaliana* [9] respectively. However, these systems are less amenable to molecular genetic techniques than *H. polymorpha*. Therefore the isolation of the *YNT1* gene and the construction of the Δ*ynt1::URA3* null allele by gene disruption offer an attractive tool for the study of the nitrate-transport system in yeast as well as in other organisms such as plants. Here we describe the *YNT1* gene, which encodes the nitrate transporter from the yeast *H. polymorpha*, its clustered localization with *YNR1* and *YNI1* [10,11] and a partial characterization of a Δ*ynt1::URA3* strain.

MATERIALS AND METHODS

Yeast strains and growth conditions

H. polymorpha strain NCYC 495 was used to construct a genomic DNA library in λ EMBL3. A NCYC495 derivative strain

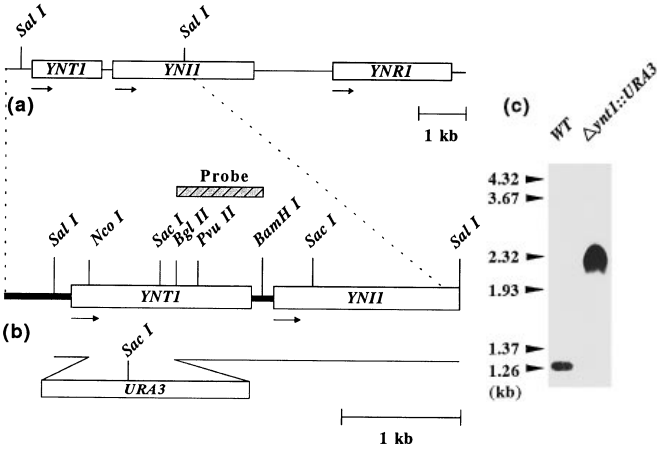

Figure 1 Gene cluster containing genes *YNT1*, *YNI1* and *YNR1* (a), the strategy followed to disrupt the *YNT1* gene (b) and Southern-blot analysis, showing its disruption (c)

(**a**) Genomic DNA region containing *YNT1* clustered with *YNI1* and *YNR1*. (**b**) To disrupt the *YNT1* chromosomal copy, an internal *YNT1 Nco*I–*Bgl*II in the plasmid *pNJ2* was replaced by the *H. polymorpha URA3* gene. The resulting plasmid *pTNB21* was digested with *Sal*I and the fragment containing Δ*ynt::URA3* was used to transform an *H. polymorpha ura3,leu1* strain. (**c**) Southern blot of genomic DNA from the wild-type (WT) and Δ*ynt1::URA3* strains digested with *Sac*I and probed with a *Bgl*II–*Bam*HI fragment from *YNT1*.

Abbreviations used: *YNT1*, *YNI1* and *YNR1* genes encoding nitrate transporter, nitrite reductase and nitrate reductase respectively; NR, nitrate reductase; NiR, nitrite reductase.

* To whom correspondence should be addressed.

J. Avila and N. Brito have contributed equally to this work.

The nucleotide sequence of the *YNT1* gene reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under accession number Z69783.

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                                                                                     AGCTTCGC  -653
CTTGGAAGCTGGGACCAATTCCCAAATATTGCCAAGTGGCAGAAGAAGGTCCTTGCACGCCCGGCTGTTGCTAAGGCATACGAGAAC  -565
TAGTAGGCGTGTTCGAGGTAAAGGTGATCCTCCAAAGGTGCCCAAGAATCAACTGTAGCCCGATGATAGTACGGATGAGGTGGTTTC  -479
AAGTGGAAATTCCTCCTAAACCGTTTACGCCTACTTCATTGAAGACTTTATACACGATATGGACCAGCTTGATGTTCATTGTTTTTG  -392
ATAGTAAGCTATGCAAAGATCTTCAAGAGAGAAATCCGAAGTAGACGTTCATAACGATTAGAAATCACTATCGCATAATTTTTCATA  -305
GCATACCTCACGTTGGAGCGGTACAAAAATGGGCGCACTTACATGTGATAGTGTTTGGTAAATAGACTTTTTGGCTAGCATCCCGCT  -218
CATCGGAGAATATTCTGCTCAAAATTAAGGATGCCCATTACGGAAATAGGTATTTTATCTTCAAATATTGTCGACAGGAGATTTTTT  -131
TGCATACTTTTGCTACCCTATTAATATGATAACGACGAAAAAAGTTGATTTTTTTTGGTACAATAGTGTACATATATAAGAGTGTTA   -44
CTGCAACCTAAAAAATGCTCCCATACAAACAATACCCTCCACA ATG CAA CTG TCT ACC TTA TGG GAA CCG CCA ATA   33
                                             M   Q   L   S   T   L   W   E   P   P   I     11
GTG AAT CCA AGA AAC CTG AAA GCG ACC TCA ATA CCA ATT TTT AAC CTA TGG AAC GTC TAT GGA AGA   99
 V   N   P   R   N   L   K   A   T   S   I   P   I   F   N   L   W   N   V   Y   G   R    33
AAC TTC TTT TTC GGG TGG TTT GGT TTT TTT GTT TGT TTT CTT TCT TGG TTC GCG TTT CCG CCT CTT  165
 N   F   F   F   G   W   F   G   F   F   V   C   F   L   S   W   F   A   F   P   P   L    55
CTC CAT GGC ATG TTG AAG AAG GAC CTA AGA CTC ACT GCA GTG GAT ATA TCC AAT AAT AAC ATA TGT  231
 L   H   G   M   L   K   K   D   L   R   L   T   A   V   D   I   S   N   N   N   I   C    77
GGA CTG ACA GGA ACT TTA CTA GGC AGA TTC ATT TTG GGG CCC CTT AAC GAC AAG TAT GGC CCA CGG  297
 G   L   T   G   T   L   L   G   R   F   I   L   G   P   L   N   D   K   Y   G   P   R    99
ATT ACT TTG ACA GGC GTG CTG GTT GCA GGA GCA ATT CCG ACT GCA TTT GTT CCT TTG GTT ACA AAT  363
 I   T   L   T   G   V   L   V   A   G   A   I   P   T   A   F   V   P   L   V   T   N   121
GTT GCA GGT CTA CAT GCC ATT CGC TTT TTT ATC AGC TTT CTA GGC TCC TCG TTT ATT TGT TGC TCC  429
 V   A   G   L   H   A   I   R   F   F   I   S   F   L   G   S   S   F   I   C   C   S   143
CAA TTC TGC GCT GTA TTT TTT GAT AAC AAC ATT ATA GGG ACA GCA AAT GCC ATC TCT GCC GGT TGG  495
 Q   F   C   A   V   F   F   D   N   N   I   I   G   T   A   N   A   I   S   A   G   W   165
GGA AAT GCT GGA GGA GGT GTG GCA TTC TTT GTC ATG CCT GCC ATT TCA AAT GCG TTG GAA AAT AGA  561
 G   N   A   G   G   G   V   A   F   F   V   M   P   A   I   S   N   A   L   E   N   R   187
GGT TAC TCT TTG CAC CAT TCT TGG AGC TAC TCT TTT GTG ATT GGG CCG TTC TTA ATT TTG ATG ATC  627
 G   Y   S   L   H   H   S   W   S   Y   S   F   V   I   G   P   F   L   I   L   M   I   209
ACG GCA ATA CTG ATC TTT GTA TTT GGA AGC GAC TGC CCG AGA GGC AGA TGG TCC CTT CGT GGA GAT  693
 T   A   I   L   I   F   V   F   G   S   D   C   P   R   G   R   W   S   L   R   G   D   231
ATC CTT GGT ATC AAC ATG GAT AAT ATG CTC GTG AAG TCT GTC TCT GTC ACA AGG CAC TTC TCT AAG  759
 I   L   G   I   N   M   D   N   M   L   V   K   S   V   S   V   T   R   H   F   S   K   253
GAA GGA GAG CTC ACT TCT GTA TTG TTG AGC CTG TTA ACG CAA TTG ATA AGG TCG GTG GTT GAG CCC  825
 E   G   E   L   T   S   V   F   V   E   L   V   N   A   I   D   K   V   V   V   E   P   275
AAT CAA GAC CAG GAA ATT CTT GAA GTC GCA GAT ATC ATA AAT GGT GAC GAA ATC ATT GAA GAC CCA  891
 N   Q   D   Q   E   I   L   E   V   A   D   I   I   N   G   D   E   I   I   E   D   P   297
TCG CTC AAT GAC GTG GTC AAG ATC TGT TTA TCC CCG CGG ACA ATG CTG GTC GGA CTT TGC TAC ATG  957
 S   L   N   D   V   V   K   I   C   L   S   P   R   T   M   L   V   G   L   C   Y   M   319
TGC TCG TTT GGT ACT GAG CTT GCA GTA GAG TCT ATT ATT TCC AAC CTG TTC GGG CAA AAG ATG ACA 1023
 C   S   F   G   T   E   L   A   V   E   S   I   I   S   N   L   F   G   Q   K   M   T   341
AAC TGG AGC ACC TCT AAA GCT GGA GCA TGG GGC TCA ATG CTT GGA CTC CTG AAC GTG GTG GCA AGA 1089
 N   W   S   T   S   K   A   G   A   W   G   S   M   L   G   L   L   N   V   V   A   R   363
CCA GCT GGA GGG ATC ATC TCC GAT TTT TTA TAC CAA AGA TTC AAA ACC ACA AAG GCT AAA AAG TTC 1155
 P   A   G   G   I   I   S   D   F   L   Y   Q   R   F   K   T   T   K   A   K   K   F   385
TGG ATG ATC TTC ACT GGC CTG ATG CAG GGC ATT TTT TTG ATT TGG ATT GGA CTA GTT CCG GAA TTA 1221
 W   M   I   F   T   G   L   M   Q   G   I   F   L   I   W   I   G   L   V   P   E   L   407
TCC ATC GCG GGA CTC ATA GTG TCC GTT TCG TTT TTG TGT CTT TGG TTT GAG ATG GGT AAT GGT GCA 1287
 S   I   A   G   L   I   V   S   V   S   F   L   C   L   W   F   E   M   G   N   G   A   429
AAT TAT GCA TGT GTT CCC GTT GTG AAT AGA CAT CAC AGT GGT ATT GTG AGT GGA GTT ACG GGA GCA 1353
 N   Y   A   C   V   P   V   V   N   R   H   H   S   G   I   V   S   G   V   T   G   A   451
ATG GGT AAC TTA GGA GGC ATT TTG TTT AGT TTA GTG TTC AGG TAC ACT ATA TCA AAT GGA GTG AAC 1419
 M   G   N   L   G   G   I   L   F   S   L   V   F   R   Y   T   I   S   N   G   V   N   473
AAC TAC TTC AAG GCG TTT TGG ATT ATA GGA ATT GTT TGC ACT GCT GTA AAT CTG GTC TGT GTG CTT 1485
 N   Y   F   K   A   F   W   I   I   G   I   V   C   T   A   V   N   L   V   C   V   L   495
ATT CCA ATT AGA GAG GAG AGG CCA AGG AAA GCG GAA AAT TGATTGAAATCTCGGTTACATTTTTTTATTTATGC 1559
 I   P   I   R   E   E   R   P   R   K   A   E   N   -                                   508
AATGCAAGTTCAGTTTTGTTATTGATAAGATTATTTTTTTTTTTGTAGTGGATCCGATCCTGTGATAAAGTAATCTGCTAAGCGGAGA 1646
TTAATTTTAGGGACGGAAATTATGTAGTATTCTAAGGGGATGAACAGCCTGACAAATAAAAGCAGTTGCAAAAGTCATATTCCATTT 1733
TGTAAAACTTGATGACTTGCTCTGTTCCACCCTTGCCAGAGGACATCAGTCCTCGGGCAGCTAAGAAG                    1820
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Figure 2 Nucleotide sequence of the *H. polymorpha YNT1* gene and deduced amino acid sequence

In the 5′-coding region, a TATATAA element and the putative regulatory GATA sequences are underlined.

(*leu1*,*ura3*) was used as the recipient for the gene disruption and it is referred to during this work as wild-type.

Cells were grown at 37 °C with shaking in liquid medium containing 0.17 % yeast nitrogen base without amino acids and $(NH_4)_2SO_4$ (Difco), 2 % (w/v) glucose and either 5 mM sodium nitrate or 5 mM NH_4Cl as nitrogen source, unless otherwise stated. When required, 0.23 mM leucine and 0.19 mM uracil were added to the medium. The medium used to test chlorate sensitivity contained 0.17 % yeast nitrogen base, 2 % (w/v) glucose, 4 mM sodium nitrate, 0.4 mM NH_4Cl and 100 mM potassium chlorate. Solid media contained in addition 2 % (w/v) agar (Difco).

Gene disruption

The strategy followed to disrupt the *YNT1* gene is shown in Figure 1(b). The plasmid *pNJ2* harbouring a *Sal*I–*Sal*I fragment of 3.5 kb containing *YNT1* was used. An internal 726 bp *Nco*I–*Bgl*II fragment from *YNT1* was replaced by a 1796 bp *Bam*HI–*Bgl*II fragment containing *H. polymorpha URA3* gene

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Hp   M---------QLSTLW-EPPIVNPRNL----KATSIPIFNLWNVYGRNFFFGWFGFFVC   45
An   M---------DFAKLLVASPEVNPNNR----KALTIPVLNPFNTYGRVFFFSWFGFMLA
Cr   MAEKPATVNAELVKEMDAAPKKYPYSLDSEGKANYCPVWRFTQPHMMAFHLSWICFFMS
     *         ..      .*   * .     **   *.    .     * ..*. * .

Hp   FLSWFAFPPLLHGMLKKDLRLTAVDISNNNICGLTGTLLGRFILGPLNDKYGPRITLTG  104
An   FLSWYAFPPLLTVTIRDDLDMSQTQIANSNIIALLATLLVRLICGPLCDRFGPRLVFIG
Cr   FVATFA-PASLAPIIRDDLFLTKSQLGNAGVAAVCGAIAARIFMGIVVDSIGPRYGAAA
     *.. .* *. *   .. ** ..  ...*  . .. ...  *.. * . *  ***    .

Hp   VLVAGAIPTAFVPLVTNVAGLHAIRFFISFLGSSFICCSQFCAVFFDNNIIGTANAISA  163
An   LLLVGSIPTAMAGLVTSPQGLIALRFFIGILGGTFVPCQVWCTGFFDKSIVGTANSLAA
Cr   TMLMTAPAVFCMALVTDFSTFACVRFFIGLSLCMFVCCQFWCGTMFNVKIVGTANAIAA
      ..  . .     ***    .  .****..    *. *   *   *.  *.****...*

Hp   GWGNAGGGVAFFVMPAISNAL-ENRGYSLHHSWSYSFVIGPFLILMITAILIFVFGSD-  220
An   GLGNAGGGITYFVMPAIFDSLIRDQGLPAHKAWRVAYIV-PF-ILIVAAALGMLFTCDD
Cr   GWGNMGGGACHFIMPLIYQG-IKDGGVPGYQAWRWAFFV-PGGIYILTATLTLLLGIDH
     * ** ***   *.** * ..   . * .   .*  .. . *  * ...* *  ..  *

Hp   CPRGRWS-----LRGDILGINMDNMLVKSVSVTRHFSKEGELTSVFVELVNAIDKVVVE  274
An   TPTGKWSERHIWMKEDTQTASKGNIV--DLSSGAQSSRPSGPPSIIAYAIPDVEKKGTE
Cr   -PSGK----------DYRDLKKEGTL----------KAKGAMWPVVKC-----------
      * *.          *         .            .   ..

Hp   PNQDQEILEVADIIN-GDEIIEDPSLNDVVKICLSPRTMLVGLCYMCSFGTELAVESII  332
An   TPLEPQSQAIGQFDAFRANAVASPSRKEAFNVIFSLATMAVAVPYACSFGSELAINSIL
Cr   --------GLGNYRSW----------------------ILALTYGYSFGVELTVDNVI
              ...                            ... *  *** **......

Hp   SNLFGQKMTNWSTSKAGAWGSMLGLLNVVARPAGGIISDFLYQRFKTTKAKKFWMIFTG  391
An   GDYYDKNFPYMGQTQTGKWAAMFGFLNIVCRPAGGFLADFLYRKTNTPWAKKLLLSFLG
Cr   VEYLFDQFG-LNLAVAGALGAIFGLMNLFTRATGGMISDLVAKPF-GMRGRIWALWIIQ
      .    .      . .*  ....*..*.  *..** ..*.. .      ..   . .

Hp   LMQGIFLIWIGLVPELSIAGLIVSVSFLCLWFEMGNGANYACVPVVNRHHSGIVSGVTG  450
An   VVMGAFMIAMGFSDPKSEATMFGLTAGLAFFLESCNGAIFSLVPHVHPYANGIVSGMVG
Cr   TLGGIFCIVLGKVSN-SLSSTIVIMIVFSIFCQQACGLHFGITPFVSRRAYGVVSGLVG
      . * * * .*     * .  .     . .  .   *  ..  * *     *.***. *

Hp   AMGNLG---------------------------------GILFSLVF-RYTISNGV---  472
An   GFGNLG---------------------------------GIIFAIIF-RYSH-------
Cr   AGGNTGAAITQAIWFAGTAPWQLTLSKADGFVYMGIMTIGLTLPLFFIWFPMWGSMLTG
     . ** *                                  *. ... * ..

Hp   -------NNYFKAFWIIGIVCTAVNLVCV--LIPIREERPRKAE---------------  507
An   -------HDYARGIWILGVISMAV-FISVSWVRPVPKSQMR-----------------
Cr   PREGAEEEDYYMREWSAEEVASGLHQGSMRFAMESKSQRGTRDKRAAGPARVPQQLRLG
            ..*    *    .  ..    .         .

Hp   -------------------------N                                  508
An   -------------------------E
Cr   ARCCQARGGLIRCVLAQSLGATGCSD
```

Figure 3 Alignment of the nitrate transporter encoded by *YNT1* (Hp) with those encoded by *crnA* from *A. nidulans* (An) and *nar-3* from *C. reinhardtii* (Cr)

Identities are indicated by asterisks and conservative changes by dots.

[12]. The resulting plasmid, *pTNB21*, digested with *Sal*I produced a fragment of about 4.5 kb, containing the *URA3* marker flanked by *YNT1* regions. This fragment was separated by electrophoresis, isolated from the agar and used to electrotransform the *H. polymorpha* (*ura3*,*leu1*) strain [13]. Afterwards, cells were plated on synthetic medium without uracil and ammonium as nitrogen source. Prototroph cells for uracil were further screened in a medium with 1 mM nitrate as nitrogen source. Of 100 uracil prototrophs, seven were unable to grow in nitrate. To check the correct replacement of *YNT1* by Δ*ynt1::URA3*, genomic DNA from the recipient and the disrupted strain were digested with *Sac*I and analysed by Southern blot, using the 697 bp *Bgl*II–*Bam*HI DNA fragment as probe. To restrict the possible growth sustained by leucine, the Δ*ynt1::URA3*,*leu* strain was transformed to leucine prototrophy by a plasmid containing the *LEU2* gene from *Saccharomyces cerevisiae* [13].

Nucleic acid isolation and λ EMBL3 *H. polymorpha* genomic DNA library

Yeast DNA was isolated [14], harvesting the cells in the early exponential phase of growth (2 mg wet weight/ml). Total yeast RNA was isolated [15] and fractionated by electrophoresis on a formaldehyde–agarose gel. DNA λ phage and plasmids were isolated as described previously [16]. The library used was described in [10]; genomic DNA was partially digested with *Sau*3AI and DNA fragments of about 15 kb were cloned into the *Bam*HI site of EMBL3 DNA phage.

Southern- and Northern-blot analyses

Southern- and Northern-blot analyses were carried out as described in [16,17]. Nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) and positively charged nylon membranes (Boehringer-Mannheim, Mannheim, Germany) were used in Southern- and Northern-blot analysis respectively. Nucleic acids were fixed to the filter by UV radiation. The probes were labelled with the digoxigenin system from Boehringer-Mannheim. The detection method used in Southern-blot analysis was the ECL chemiluminescent system (Amersham, Madrid, Spain), using an anti-digoxigenin antibody conjugated to peroxidase. In Northern-blot analysis the detection was carried out with the CDP-Star system (Boehringer-Mannheim), using an anti-digoxigenin antibody conjugated to alkaline phosphatase. RNA molecular size was determined using the RNA digoxigenin-labelled standard marker (Boehringer-Mannheim). The 743 bp *Nco*I–*Bgl*II, 931 bp *Sac*I–*Hin*dIII and 454 *Xba*I DNA fragments from *YNT1*, *YNI1* and *YNR1* respectively were used as probes in Northern-blot analysis.

DNA sequencing

Bluescript phagemid plasmids from Stratagene (Heidelberg, Germany) were used. Exonuclease III unidirectional deletions and single-stranded DNA were prepared by following the manufacturer's instructions (Promega, Madison, WI, U.S.A.). DNA was sequenced on single strands by the dideoxy chain-termination method [18], using Sequenase (USB, Cleveland, OH, U.S.A.). Sequencing was performed on both strands. Multiple protein sequence alignments were carried out with the CLUSTAL V program [19].

Hydropathy profile

Analysis was carried by the Kyte and Doolittle method [20] with a window of 11 residues by using the *Seqanal* package of programs for analysis of protein sequences for information on secondary and tertiary structures, developed by A. R. Crofts (1987), University of Illinois. The hydropathy profile obtained for the *YNT1* product was compared with that obtained by the same method from the mean index of hydropathy of the residues at each position in the sequence resulting from the alignment of nitrate transporters encoded by *YNT1*, *cnrA* and *nar-3* [6–8].

Nitrate uptake

Cells grown in ammonium solution were incubated for 2 h in nitrogen-free medium at 10 mg (wet weight)/ml, resuspended at 50 mg (wet weight)/ml, and 0.25 mM or 1 mM nitrate was added. At the indicated time, 1 ml of cell suspension was taken, placed in 9 ml of ice-cold water and centrifuged for 3 min. Nitrate was determined in the supernatant: it was chemically reduced to nitrite and measured colorimetrically [21] on an Autoanalyser Technicon AII.

Other methods

Extraction and determination of NR activity were performed as in [1]. NiR was assayed as in [22]; the reaction mixture contained in a final volume of 0.5 ml 80 mM potassium phosphate, 0.5 mM nitrite, 0.75 mM Methyl Viologen, reduced with 14 mM dithionite, and crude extract (about 0.2 mg of protein). The time course of the reaction was followed by determining nitrite disappearance.

Nucleotide sequence accession number

The GenBank/EMBL accession number for the *YNT1* gene sequence reported in this paper is Z69783.

RESULTS

Molecular cloning and sequence analysis of the *YNT1* gene

DNA sequencing in phage *JA13* containing the NR- (*YNR1*) and NiR- (*YNI1*) encoding genes [10,11] revealed an open reading frame of 1524 bp (named *YNT1*, yeast nitrate transporter gene), encoding a putative protein of 508 residues with great similarity to nitrate transporters encoded by genes *nar-3* and *crnA* from *C. reinhardtii* [8] and *A. nidulans* [6,7] respectively. The genomic region containing the genes *YNT1*, *YNI1* and *YNR1* and the transcriptional directions are shown in Figure 1(a).

In the 5′-non-coding region of *YNT1* the canonical TATATAA sequence [23] is found at −58 from the translation-initiation codon. Moreover, five regions containing the core sequence GATA, involved in the binding of GATA proteins, are present (Figure 2). GATA proteins related to the regulation of genes involved in the utilization of different nitrogen sources have been described in yeast and fungi [24,25].

The identity between nitrate transporters from *H. polymorpha* and *A. nidulans* was 39.1 % in a 496-amino acid overlap region, and the identity between the protein encoded by *YNT1* and *nar-3* from *C. reinhardtii* was 34.2 % in a 408-residue overlap region. Of the less similar proteins involved in nitrate or nitrite transport found via the similarity searches BLAST program [26] were a nitrite-extrusion protein from *Bacillus subtilis* [27] and a nitrate-transport from *B. subtilis* [28]. No significant similarity to the nitrate transporters encoded by *CHL1* and *nrtA* from *Arabidopsis* and *Synechococcus* [9,29] respectively was found.

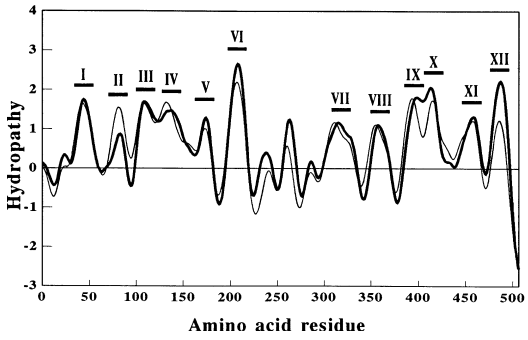

Figure 4 Hydropathy profile of the putative nitrate transporter encoded by the *YNT1* gene

Analysis was performed by the Kyte and Doolittle method [20] with a window of 11 residues; hydrophobic regions were given as a positive hydropathy index. The thick line corresponds to the profile obtained for the *YNT1* product and the thin line to the profile obtained from the mean index of hydropathy of the nitrate transporters encoded by YNT1, *cnrA* and *nar-3*. Tentative membrane-spanning helices are indicated by roman numerals.

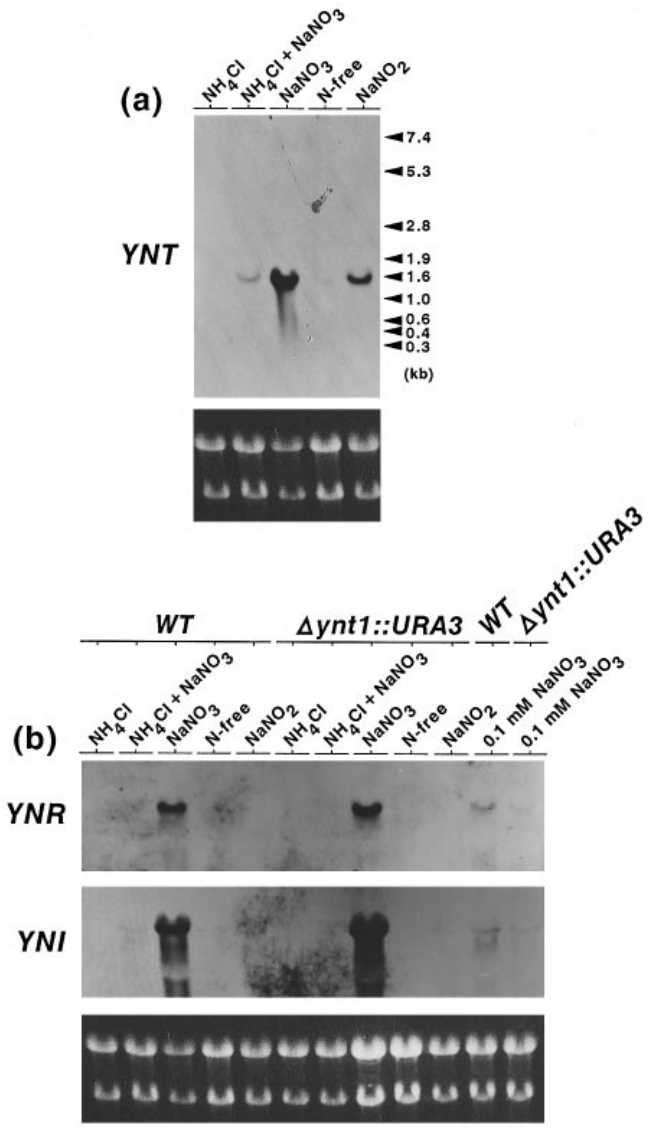

Figure 5 Expression of *YNT1*, *YNR1* and *YNI1*

Total RNA (10 μg) from cells grown in ammonium solution, washed and incubated at 10 mg of cells (wet weight)/ml for 2 h in the nitrogen sources indicated. All the nitrogen sources were used at 5 mM except when otherwise indicated. *YNT*, *YNR* and *YNI* indicated the probe used. At the bottom of each panel the RNA samples stained with ethidium bromide are shown. (**a**) Levels of *YNT1* mRNA. The relative molecular size calculated for the *YNT1* transcript is about 1.6 kb. (**b**) Levels of *YNR1* and *YNI1* mRNA in the wild-type (WT) and the Δ*ynt1::URA3* strains.

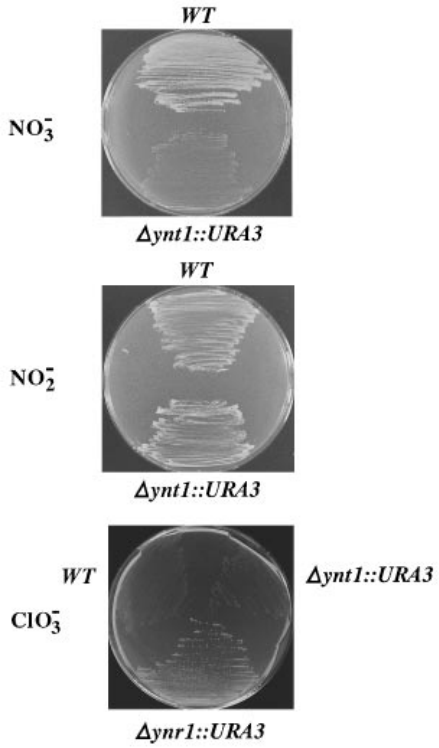

Figure 6 Growth test on nitrate, nitrite and chlorate

The growth of wild-type (WT) and Δ*ynt1::URA3* strains on nitrate and nitrite was tested, and also wild-type (WT), Δ*ynt1::URA3* and Δ*ynr1:URA3* strains in the presence of 100 mM chlorate.

The alignment of the deduced amino acid sequence of 508, 507 and 547 residues encoded by *YNT1*, *crnA* [6,7] and *nar-3* [8] respectively is shown in Figure 3. The greatest similarity between the three proteins is found in the N-terminal region. In contrast, at the C-terminus, regions from *C. reinhardtii* are not present in either the *H. polymorpha* or *A. nidulans* nitrate transporters.

Analysis of the α-helical transmembrane domains by the Kyte and Doolittle method [20] showed up some ambiguities with regard to the membrane-spanning domains, and the Eisenberg analysis [30] demonstrated 11 transmembrane domains (results not shown). These results could mean that regions of *YNT1*-encoded protein with a high degree of identity with those encoded by *crnA* and *nar-3* may differ in membrane orientation. Moreover, the *YNT1*-encoded protein C-terminus may be facing the outer side of the membrane, which disagrees with what has previously been proposed for this type of protein [20,31,32]. To attempt to understand these discrepancies on the secondary structure of the *YNT1* product, a hydropathy analysis was carried out by the Kyte and Doolittle method [20] and compared with that obtained by the same method from the mean index of hydropathy of the residues at each position in the sequence resulting from the alignment of nitrate transporters encoded by *YNT1*, *nar-3* [8] and *cnrA* [6,7]. The tentative secondary structure shown in Figure 4 shows 12 transmembrane domains. The regions with high similarity between the proteins compared have the same orientation with respect to the membrane, and the putative glycosylation site at position 342, between the VII and VIII membrane-spanning domains, faces the outer side of the membrane.

YNT1 expression

The levels of *YNT1* expression determined by Northern blot in cells grown in ammonium solution and transferred to different nitrogen sources for 2 h are shown in Figure 5(a). The *YNT1* gene is expressed in cells transferred to 5 mM nitrate or 5 mM nitrite, but scarcely at all in nitrate plus ammonium ions, and it is not expressed in ammonium or nitrogen-free medium. In all the nitrogen sources in which *YNT1* was expressed, a single transcript of about 1.6 kb was observed.

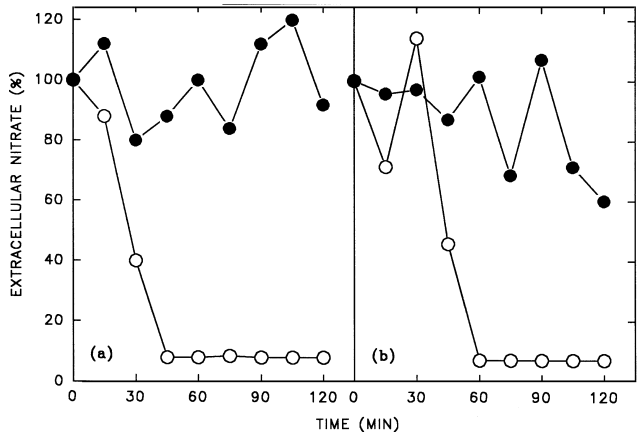

Figure 7 Nitrate uptake in the wild-type and Δ*ynt1::URA3* strains

Cells grown in ammonium solution were resuspended in nitrogen-free medium at 10 mg of cells (wet weight)/ml for 2 h, resuspended in the same medium at 50 mg of cells/ml, and 0.25 mM (**a**) or 1 mM (**b**) nitrate was added. Nitrate uptake was determined in the wild-type (○) and Δ*ynt1::URA3* (●) strains by measuring disappearance of extracellular nitrate. Experiments were repeated five times without significant differences; the results shown are from a single experiment.

Table 1 Levels of NR and NiR activity in the wild-type (WT) and in the Δ*ynt1::URA3* mutant

NR and NiR activities were determined in cells grown in 5 mM ammonium solution, resuspended in nitrogen-free medium at 10 mg/ml for 2 h, divided into four aliquots, and incubated for 2 h at different nitrate concentrations. Values are means ± S.D. for three independent experiments.

	Enzyme activity (nmol/min per mg of protein)			
	NR		NiR	
Nitrate (mM)	WT	Δ*ynt1::URA3*	WT	Δ*ynt1::URA3*
0.1	7 ± 1	1 ± 1	6 ± 2	4 ± 0.2
0.2	9 ± 2	5 ± 1	7 ± 1	4 ± 0.5
1	19 ± 4	6 ± 2	15 ± 2	5 ± 3
5	19 ± 6	8 ± 2	15 ± 6	15 ± 0.2

Construction and characterization of a Δ*ynt1::URA3* disrupted strain

An *H. polymorpha* strain in which about 50 % of the *YNT1* gene was deleted and replaced by the *H. polymorpha URA3* gene was created by the gene-replacement method [33] (Figure 1b). Southern-blot analysis of the disrupted strain confirmed the correct replacement of the chromosomal *YNT1* copy by the disrupted one (Figure 1c). The disrupted strain does not grow in nitrate but does in nitrite (Figure 6) and displays a significant reduction in the rate of nitrate uptake (Figure 7). In 0.1 mM nitrate the Δ*ynt1::URA3* strain showed reduced *YNR1* and *YNI1* expression (Figure 5b) as well as reduced NR and NiR activity (Table 1) with respect to the wild-type. However, it is noteworthy that, in 5 mM nitrate, *YNR1* and *YNI1* expression (Figure 5b) and NiR activity (Table 1) were similar in the wild-type and the disrupted strain, whereas NR activity was about 50 % less in the disrupted strain. The Δ*ynt1::URA3* strain was slightly less sensitive to chlorate than the wild-type. However, both were much more sensitive to chlorate than a strain deficient in NR (Δ*ynr1::URA3*) (Figure 6).

DISCUSSION

The great similarity of the product of the *YNT1* gene to those encoded by *crnA* [6,7] and *nar-3* [8] and the phenotype of the Δ*ynt1::URA3* strain reveals that *YNT1* encodes a nitrate transporter in the methylotrophic yeast *H. polymorpha*. The relative positions of *YNT1*, *YNI1* and *YNR1* in the cluster are very similar to those described for *A. nidulans*. However, *YNT1*, *YNI1* and *YNR1* are transcribed in the same direction, whereas in *A. nidulans* the genes encoding NR and NiR are divergently transcribed [34]. The genes involved in nitrate assimilation have been reported to be clustered in the fungi *A. nidulans* [34], *Aspergillus niger* [35] and *Ustilago maydis* [36] and in the alga *C. reinhardtii* [8]. However, in *Neurospora crassa* and in plants, no association of these genes has been found [24].

The significance of the clustering of *YNT1*, *YNI1* and *YNR1* could be in some way related to the co-ordinated regulation of these genes, which are induced by nitrate and repressed in the presence of depleted nitrogen sources. It is worth noting that only one transcript corresponding to *YNT1* was detected, in contrast with the situation in *A. nidulans* where a regulated and a constitutive transcript for *cnrA* have been reported [6].

In contrast with mutants affected in *cnrA* [6] or *CHL1* [31] in *A. nidulans* and *Ar. thaliana* respectively, those affected in *YNT1* are unable to grow in nitrate. The fact that the Δ*ynt1::URA3* strain fails to grow in nitrate whereas nitrate induces *YNR1* and *YNI1* appears to be contradictory and means that, in spite of the deletion of *YNT1*, sufficient nitrate to induce the genes involved in the nitrate assimilation must be entering the cell. The lack of growth of the Δ*ynt1::URA3* mutant could be explained by assuming that the internal concentration of nitrate is well below that necessary to allow substantial assimilation of nitrate. No information on the internal concentration of nitrate in yeast is available. Nitrate could also enter the cell by means of unspecific transport systems, especially when high extracellular concentrations are present in the medium. The slightly decreased sensitivity of the Δ*ynt1::URA3* to chlorate in comparison with the wild-type is in good agreement with the levels of NR activity in both strains. In the Δ*ynt1::URA3* strain chlorate probably enters the cell by the same unspecific way as nitrate. The lack of growth in nitrate of the Δ*ynt1::URA3* strain as well as the results of the Southern- and Northern-blot experiments make the existence of other specific nitrate transporters in addition to the *YNT1* product in *H. polymorpha* unlikely. The fact that, in the nitrate-transporter mutant, growth in nitrite occurs indicates that alternative nitrite-uptake systems exist in *H. polymorpha*, but this does not rule out transport of nitrite by the nitrate transporter.

Since the nitrate transporters (Figure 3) encoded by *cnrA* [6,7], *nar-3* [8] and *YNT1* showed significant similarity, and all of them including *CHL1* [31] have very similar secondary structure, it is conceivable that the nitrate-transport mutant reported here will be useful in the isolation of, by functional complementation, genes involved in nitrate transport from plants. The discovery of the mutant opens up the possibility of studying the structure–function relationship of the nitrate transporter protein by site mutagenesis of nitrate-transport-encoding genes.

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