The genes *YNI1* and *YNR1*, encoding nitrite reductase and nitrate reductase respectively in the yeast *Hansenula polymorpha*, are clustered and co-ordinately regulated

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The nitrite reductase-encoding gene (*YNI1*) from the yeast *Hansenula polymorpha* was isolated from a lambda EMBL3 *H. polymorpha* genomic DNA library, using as a probe a 481 bp DNA fragment from the gene of *Aspergillus nidulans* encoding nitrite reductase (*niiA*). An open reading frame of 3132 bp, encoding a putative protein of 1044 amino acids with high similarity with nitrite reductases from fungi, was located by DNA sequencing in the phages λ NB5 and λ JA13. Genes *YNI1* and *YNR1* (encoding nitrate reductase) are clustered, separated by 1700 bp. Northern blot analysis showed that expression of

YNI1 and *YNR1* is co-ordinately regulated; induced by nitrate and nitrite and repressed by sources of reduced nitrogen, even in the presence of nitrate. A mutant lacking nitrite reductase activity was obtained by deletion of the chromosomal copy of *YNI1*. The mutant does not grow in nitrate or in nitrite; it exhibits a similar level of transcription of *YNR1* to the wild type, but the nitrate reductase enzymic activity is only about 50 % of the wild type. In the presence of nitrate the $\Delta yni1::URA3$ mutant extrudes approx. 24 nmol of nitrite/h per mg of yeast (wet weight), about five times more than the wild type.

INTRODUCTION

The methylotrophic yeast *Hansenula polymorpha* can use nitrate as its sole nitrogen source [1]. Nitrate is reduced to nitrite by nitrate reductase (NR), and nitrite is reduced to ammonia by nitrite reductase (NiR). Two types of assimilatory NiRs with different electron donors have been reported [2]: a ferredoxin-NiR, characteristic of plants and algae, and an NAD(P)H-NiR, found in fungi and bacteria. Assimilatory NiR possesses two prosthetic groups, an iron-sulphur centre and a haem group termed sirohaem [3–6]. In addition to sirohaem and iron-sulphur prosthetic groups, NAD(P)H-NiR from fungi and bacteria possesses FAD [7,8]. Independent of the electron donor, both enzymes catalyse the stoichiometric reduction of nitrite to ammonia by transferring six electrons to nitrite.

A NiR-encoding gene has been isolated from several organisms, but not so far from any yeast [9–12]. In fungi, with the exception of *Neurospora crassa*, the genes involved in nitrate assimilation have been reported to be clustered [9,13,14]. In contrast to the situation in filamentous fungi and plants [15], nitrate assimilation has been scarcely studied in yeast. The NR-encoding gene (*YNR1*) has been isolated, and preliminary results indicate its clustering with the NiR-encoding gene (*YNI1*) [16]. In the yeast *H. polymorpha*, molecular biology techniques have reached an advanced stage of development, and heterologous or homologous gene overexpression is being successfully performed [17]. Therefore this yeast is an excellent system in which to overexpress NiR for crystallization, as well as to analyse *in vivo* by complementation of a NiR null strain the mutations performed on NiR-encoding genes by site-directed mutagenesis. We have thus attempted the isolation of the NiR-encoding gene from *H.*

polymorpha to provide a simple, useful tool for the biochemical study of NiR protein, as well as to gain insight into nitrate assimilation gene organization and regulation in yeast. In this paper we report the isolation of the NiR-encoding gene from *H. polymorpha*, and the genetic and biochemical characterization of a NiR null mutant ($\Delta yni1::URA3$).

MATERIAL AND METHODS

Yeast strains and growth conditions

Hansenula polymorpha strain NCYC 495 was used to construct a genomic DNA library in lambda EMBL3. The NCYC 495 derivative strain (*leu1,ura3*) was used as recipient strain to perform gene disruption, and it is referred to in this work as wild type (WT). Cells were grown at 37 °C with shaking in liquid medium containing 0.17 % yeast nitrogen base without amino acids or ammonium sulphate (Difco), 2 % (w/v) glucose and either 5 mM sodium nitrate or 5 mM ammonium chloride as nitrogen source, unless otherwise stated.

Construction of a lambda EMBL3 *H. polymorpha* genomic DNA library

Genomic DNA from *H. polymorpha* was partially digested with *Sau3AI* and separated by agarose gel electrophoresis. DNA fragments of 15 kb average size were isolated from the agarose gel by electroelution and ligated into the *Bam*HI site of the EMBL3 DNA phage. The resulting recombinant DNA was

Abbreviations used: *YNI1*, nitrite reductase-encoding gene; NiR, nitrite reductase; *YNR1*, nitrate reductase-encoding gene; NR, nitrate reductase; ORF, open reading frame; *niiA*, 481 bp DNA fragment from *Aspergillus nidulans* nitrite reductase-encoding gene, used as probe; WT, wild type.

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packaged *in vitro* with a lambda DNA packaging kit (Promega Corp., Madison, WI, U.S.A.).

Gene disruption

The strategy used to disrupt *YNII* is shown in Figure 1(c). The plasmid pNiA2, harbouring a *SalI*–*Bam*HI fragment of about 3 kb containing the C-terminal moiety of NiR, was used. A *Bgl*II fragment of 726 bp was replaced by a 1796 bp *Bam*HI–*Bgl*II DNA fragment containing the *H. polymorpha* *URA3* gene [18]. The resulting plasmid, pNic39, was digested with *SalI*–*Bam*HI to produce a fragment of about 4 kb, containing the *URA3* marker flanked by *YNII* regions. This fragment was separated by electrophoresis, isolated from the agar and used to electrotransform [19] the *H. polymorpha* (*leu1,ura 3*) strain. Cells were then plated on synthetic medium consisting of 0.67% yeast nitrogen base without amino acids, supplemented with 0.23 mM leucine and 2% (w/v) glucose. Prototroph cells for uracil were further screened in a medium consisting of 0.17% yeast nitrogen base without amino acids or ammonium sulphate, supplemented with 0.23 mM leucine and 1 mM nitrate as nitrogen source. Of 100 prototroph cells (*URA* +), 20 were unable to grow in nitrate. The disruption was checked by Southern analysis with the 796 bp *Pst*I–*Eco*RV DNA fragment as probe.

Nucleic acid isolation

Yeast DNA was isolated as described in [20] from cells harvested in the early exponential phase of growth. Total yeast RNA was isolated as in [21] and fractionated by electrophoresis on a formaldehyde agarose gel. Lambda DNA was isolated as described in [22].

Library screening, and Southern and Northern blot analysis

Southern blot and Northern blot analyses were carried out as described in [22,23]. Nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) and positively charged nylon membranes (Boehringer Mannheim) 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 chemiluminescence system (Amersham, Madrid, Spain), using an anti-digoxigenin antibody conjugated to peroxidase. For Northern blot analysis 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 digoxigenin-labelled RNA standard markers (Boehringer Mannheim).

The heterologous probe, a *Hind*III–*Eco*RI fragment of 481 bp from the *Aspergillus nidulans* NiR-encoding gene (*niiAp*) [9], was labelled with [α -³²P]dCTP using random oligonucleotide primers [22]. Hybridization with the *niiAp* probe was carried out at 37 °C overnight in 6 × SSPE (1 × SSPE is 150 mM NaCl, 10 mM Na₂HPO₄ and 1 mM EDTA), 0.05 × BLOTTO [1 × BLOTTO is 5% (w/v) non-fat dried milk dissolved in water containing 0.02% sodium azide] and 40% (v/v) formamide. The filters were washed with 2 × SSC (1 × SSC is 150 mM NaCl and 15 mM sodium citrate) and 0.5% SDS for 30 min, first for 15 min at room temperature and then for 15 min at 55 °C. The same conditions were used for Southern blotting as in the library screening.

The 931 bp *Sac*I–*Hind*III fragment from *YNII* and the 454 bp *Xba*I fragment from *YNRI* [16] 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 using the manufacturer's recommendations (Promega Corp.). DNA was sequenced on single strands by the dideoxy chain termination method [24], using Sequenase (U.S.B, Cleveland, OH, U.S.A.). Sequencing was performed on both strands. Multiple protein sequence alignments were carried out with the CLUSTAL V programme [25].

Other methods

Crude extracts were prepared and NR activity was assayed as described in [1]. NiR activity was assayed as in [26] with slight modifications. 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 0.125 ml of crude extract of about 1 mg/ml protein. The reaction was followed by measuring nitrite disappearance.

Cell death as a result of nitrite treatment was monitored by fluorescence microscopy using propidium iodide as a marker of dead cells [27].

RESULTS AND DISCUSSION

Molecular cloning of the NiR-encoding gene (*YNII*)

A Southern blot of *H. polymorpha* DNA probed with a 481 bp *Hind*III–*Eco*RI DNA fragment (*niiAp*) from the *niiA* gene encoding NiR from *A. nidulans* [9] showed different hybridization bands (results not shown). Assuming that this hybridization corresponds to DNA from the NiR-encoding gene from *H. polymorpha*, the *niiAp* fragment was used as a probe to isolate *YNII* by screening an EMBL3 *H. polymorpha* genomic DNA library. From about 30000 phages screened, 12 were found to contain inserts that hybridized with the probe. The phages released an insert of average length 12 kb after *Sal*I digestion. A 4.5 kb *Sal*I fragment hybridized with the probe in four of these, while a 3.4 kb fragment was positive in another, λ NB5. The 3.4 kb fragment from phage λ NB5 was cloned in plasmid Bluescript (KS+) at the *Sal*I site. DNA sequencing of the ends of the 3.4 kb fragment and searching for protein similarity using BLASTA program analysis [28] showed that one end of the 3.4 kb *Sal*I DNA fragment encoded a protein sequence with high similarity to the central region of fungal NiR sequences, indicating that the 3.4 kb fragment contains only part of NiR-encoding region. Since in fungi it has been reported that the NR- and NiR-encoding genes are clustered [9,13,14], the phage λ JA13, harbouring the *H. polymorpha* NR-encoding gene (*YNRI*) [16], was digested with *Sal*I and subjected to Southern blotting, revealing a band of 3.4 kb that also hybridized with *niiAp*. Conversely, the phage λ NB5 hybridized with a probe from the *YNRI* gene of *H. polymorpha* [16]. Further Southern and restriction analysis indicated that the phages λ JA13 and λ NB5, with inserts of about 12 kb, contain overlapping regions. To localize the complete *YNII* open reading frame (ORF), the ends of the 7.4 kb *Sal*I fragment from the phage λ JA13, which contained *YNRI*, were sequenced [16]. Similarity searches using BLASTA program analysis [28] of the sequenced regions showed similarity with the NiRs from fungi. The strategy of DNA sequencing used to localize *YNII* in the fragments hybridizing with *niiAp* and *YNRp* contained in the phages λ NB5 and λ JA13 is shown in Figure 1(a). DNA sequencing revealed an ORF of 3132 nucleotides, encoding a putative protein of 1044 residues

Figure 1 Strategy used to locate the *YNI1* gene in the isolated phages, structure of the region containing *YNI1* and *YNR1*, and construction of a $\Delta yni1::URA3$ strain

(a) The *YNI1* gene was located in two DNA fragments of 3.4 kb and 7.4 kb from the phages λNB5 and λJA13 respectively. Phages λNB5 and λJA13 were isolated independently using probes *niiAp* and *YNRp*, and both phages contain inserts of about 12 kb with overlapping regions. Only the *SalI* fragments that hybridize with the probes are depicted. (b) Region containing *YNI1* and *YNR1*. The ORFs are enclosed in boxes; the thick lines outside the boxes indicate the additional regions sequenced. Arrows indicate the direction of transcription. The vertical line in the *YNI1* box indicates the *SalI* site between the fragments of 3.4 kb and 7.4 kb. (c) Restriction map of *YNI1* and strategy used to disrupt the chromosomal copy of *YNI1*, and Southern blot from genomic DNA from the recipient and $\Delta yni1::URA3$ strains. DNA fragments of 2555 bp and 2540 bp were obtained in the recipient strain genomic DNA digested with *EcoRV* and *HindIII* respectively. In the $\Delta yni1::URA3$ strain, *EcoRV* and *HindIII* gave DNA fragments of 1255 bp and 1973 bp respectively. The *H. polymorpha* *URA3* gene contains *EcoRV* and *HindIII* restriction sites.

(Figure 2) with a calculated molecular mass of 116.6 kDa and high sequence identity with NiRs from other fungi [9,10]. A *BamHI* DNA fragment of about 3 kb was isolated from phage λJA13, cloned in plasmid Bluescript (KS+) and the *SalI* region between the *SalI* fragments of 3.4 and 7.4 kb sequenced to assess the singularity of the *SalI* site.

The positions of *YNR1* and *YNI1* in the *H. polymorpha* genome and the direction of transcription are shown in Figure 1(b); the distance between the *YNR1* and *YNI1* ORFs is about 1700 bp. Therefore *YNI1* and *YNR1* in *H. polymorpha* are clustered, as in *A. nidulans*, *A. niger* and *Ustilago maydis* [9,13,14], but unlike *N. crassa* [10] and plants [15]. Genes encoding proteins

involved in nitrate assimilation are also clustered in the alga *Chlamydomonas reinhardtii* [29]. The relative positions of *YNR1* and *YNI1* in the cluster and the transcriptional direction are similar to the corresponding *A. nidulans* genes [9].

In the 5' non-coding region of *YNI1*, canonical TATATA or TATAAA sequences [30] are not found. There are only two TATA sequences, situated at -344 and -302 from the translation initiation codon, with TA-rich sequences situated at -194 and -97. Two stretches containing the core sequence GATA, implicated in binding of GATA family proteins, some of which are related to the regulation of expression of genes involved in utilization of nitrogen sources [31], are present in the same region (Figure 2). However, the participation of these GATA sequences in *YNI1* regulation remains to be ascertained.

Comparison of the putative NiR protein sequence encoded by *YNI1* from *H. polymorpha* with NiRs from other organisms

Similarity searches by FASTA program analysis [32] of the predicted NiR encoded by *YNI1* showed identities of 44.7% and 56.0% in the 1029- and 591-amino-acid regions of overlap with NiRs from *A. nidulans* [9] and *N. crassa* [10] respectively. Significant identity was also found with bacterial NiRs from *Klebsiella pneumoniae* [33] and *Escherichia coli* [34]. Of the 40 proteins with the best score from the FASTA analysis, no plant NiRs were included; this low similarity was also revealed by simple sequence alignment, using the CLUSTAL V program, of the NiR encoded by *YNI1* and NiRs from several plants (results not shown).

The alignment of the deduced amino acid sequences encoded by *YNI1* (*H. polymorpha*), *niiA* (*A. nidulans*) [9] and *nasB* (*K. pneumoniae*) [33] is shown in Figure 3. The putative iron-sulphur centre and sirohaem domain was localized in the predicted *H. polymorpha* NiR sequence by comparison with fungal NiRs and with the corresponding domain of the sulphite reductase from *E. coli* [35]. This region possesses the consensus sequence CXXXXXC_nGCXXXC [5], where the cysteine residues have been proposed to be involved in the binding of the tetranuclear iron-sulphur centre and sirohaem to the NiR [6]. The region around the cysteine consensus sequence is highly conserved among NiRs, but poorly conserved between NiRs and sulphite reductase. Likewise, comparison of the cysteine-containing sequences from plants with those from fungi reveals low identity (results not shown).

Concerning the FAD and NAD(P)H binding domains, analysis of the structures and amino acid sequences of six proteins interacting with FAD, NAD⁺ or NADP⁺ revealed a common fold $\beta\alpha\beta$ (where β is β -sheet and α is α -helix), with a motif sequence GXGXXG [36]. This motif has been found twice in the N-termini of NiRs from *A. nidulans* and *N. crassa* [10], as well as in *H. polymorpha*. These two sequences could be involved in the FAD and NAD(P)H binding domains of NiR.

Levels of *YNI1* and *YNR1* expression

The levels of *YNI1* expression determined by Northern blotting in cells grown in ammonium salts and transferred to different nitrogen sources for 2 h are shown in Figure 4(a). *YNI1* is expressed in nitrate but not in reduced nitrogen sources or nitrogen-free media. The capacity of nitrite to induce *YNR1* and *YNI1* expression is shown in Figure 4(b). The induction produced by nitrite at 1 mM was even greater than that in nitrate. *YNR1* expression determined in the NiR null mutant presents the same behaviour under the conditions studied as in the recipient strain

H.p.	M-----TCSPVPLPEDISPAAK-----KLVVTVGLQMVGLSFLEKLLSNDKLNYSIMVYGEFPYLAHYRVLTEYFQHFREFK	75
A.n.	MEPLDGRNGEVTASAHNGIPIIDGVDPSTLRGDIDQDFNRRQXIVVGLQMVAVAFIEKLVKLDSEERRKQDIVVIGEPEPHAYNRVGLSSYFEEHKKIE	
K.n.	MT-----KPVLLVGLQMVGLSFLEKLLSNDKLNYSIMVYGEFPYLAHYRVLTEYFQHFREFK	
H.p.	***** NILLSPEEFYQ--LRGEKWNVAIDEKVVDIDRRARIITDKGNRASYDVVLCTGSAAILPTDLLPLTRKSYREMGCFVYRTIDDLYSMDYCQGAUKTR	174
A.n.	DYLYNPKMYGSEFKDRSFDYILNTRVTDVFPQHKTKTSTGDIIVSYDILVLATGSADVLET-----STPGHDAKGIFFVYRTISDLRLMEFAANHKGQT	
K.n.	SLSLVEGDFTT---QHGIELRLSESVASIDREARVVRDAFGEHETHWOKLVLATGSYP-----FVFPV--PGHNLGCFVYRTIDDLQDIA--ARAATARR	
H.p.	..***** AIVVGGGLGLEAAKALYDMESFEDITIVHRSHWLLSQQMDQKGGSLT SKVKELGITCRTGTVSELLFDEDQNLKGVKYNQNGQIEECILLCTYIGIKP	274
A.n.	GVTVGGGLGLEAAKAMTDLEDGSKVLIDRNKGVFLARQLDGDAGSLVTKKIRDLGLEVLHEKRVAKIHTDDNNVTGILFDGQGLDCCICFAIGIRP	
K.n.	GVTVGGGLGLEAAKALQQLGL--ETHVVEFAPNLMAVQLDNGGAAMLREKISELGVGVHTSKATTEIVRNEQG--LQLNFRDGSLLATIMVTSAGIRP	
H.p.	..***** RDELTS--GLNAGSAGGFKVNMLOTSDENIYAIGECASWNNMTFGLIAPGYEMADILAFNLQCKLHQKFESEPSMGTSLKLMGVDAVSGDFFADRN	373
A.n.	RDELGGTGTQCAKRGFVIDESLRTSVNDIYAIGECASWNNMTFGLIAPGYEMADILAFNLQCKLHQKFESEPSMGTSLKLMGVDAVSGDFFADRN	
K.n.	QDALRSGGLSVGERGGICIDNQCTSDPDVLAIGECALWENKIYGLVAPGYQAARRAATLAG---EAGSFGADMSTKLKLLGVDAVSGD-----	
H.p.	..***** GPKWLLA-----DTTKKFDVLVEFEDIDATYTKLLFTKDGKVMGLGILVGTSNYTKFSAMIR--KPLPKSPSELLIG--KSGEDDM---	451
A.n.	GKFLPQRFSAESIGAADFNREEPQVAKTYRDPFGQVYKYLTKDGKVMGLGILVGTSNYTKFSAMIR--KPLPKSPSELLIG--KSGEDDM---	
K.n.	-----AQGRTPGCQSYQHTGPPQVYKYLTKDGKVMGLGILVGTSNYTKFSAMIR--KPLPKSPSELLIG--KSGEDDM---	
H.p.	..***** EMLSDETQICSCHNITKGLVEAVKNG--CSSLADVKKCTKAGTACGGCEPTVKIIFEKELKGLGVSNLGVHFDYSKVDLFSLDVKNFRSFRVME	550
A.n.	DLDLSDTQICSCHNITKGLVEAVKNG--CSSLADVKKCTKAGTACGGCEPTVKIIFEKELKGLGVSNLGVHFDYSKVDLFSLDVKNFRSFRVME	
K.n.	AALPDGQICSCHNITKGLVEAVKNG--GMAALIKRTRKATGCGGCSALVQVMEYQLAQGGVEVKKDICEHPFWSRQETYLHVRVNHITFQQLIAR	
H.p.	..***** LQNDSSSGCEICKTIGSLSTLYNRHLKKEVHGLQDNDRYLGNIQDGTFSVVPFMSAGEITPEKLVSIGKIAKYGUYTKITGAQRDLDFGVKKS	650
A.n.	AGKCFDGLGCEICKTIGSLSTLYNRHLKKEVHGLQDNDRYLGNIQDGTFSVVPFMSAGEITPEKLVSIGKIAKYGUYTKITGAQRDLDFGVKKS	
K.n.	YG--QQHGCEVCKPLVASVLASCWNEYLLKPAHLPLQDNDRYFANIQDGTFSVVPFMSAGEITPEKLVSIGKIAKYGUYTKITGAQRDLDFGVKKS	
H.p.	..***** DLLKIKWDLNAGFESGQAYGKSLRNKSCVGSWCRYGIGDSVGLAVRLEERYKGIKIRSPHAKGG---VSGCVRDCAETHSKDFGLCAVSGG--FNII	744
A.n.	DLLDITWELVDAGMESGHRVAKSLRTVKS CVGTWCRFGVGD SVGMATRLQRYKSIRAPHKFGA---VSGCVRDCAETHSKDFGLCAVSGG--FNII	
K.n.	QLPAWRELAEAGFETGHAYGKSLRTVKS CVGSWCRYGVQDSTGLVATLEHRYKGLRAPHKFGA---VSGCTRECAEAQKDGIVTATEKG--WNLY	
Siroh	RENSMACVSFPCTCLMAEAERFLPSFDINIDNLMAGHGVSDHIVMRTGCPNGCGRAMLAELVGLVGAAPGRYNLH	
H.p.	..***** VGGN--GGMKPRHAQLLATNVRPDEVIPILDRIYIMFYITADRLQRTARWLENLDGGIEYLDKDVVIRDKLGICKDLEAQRHVAVAGYDDEWAKVSEKE	842
A.n.	VGGN--GGMKPRHSELLAKDVPPDEVIPILDRIYIMFYITADRLQRTARWLENLDGGIEYLDKDVVIRDKLGICKDLEAQRHVAVAGYDDEWAKVSEKE	
K.n.	VGGN--GGMKPRHADLFASDLDEATLIRSIDRLIMFYITADRLQRTSTWMDNLEGGVDYLRIMLEDLSLGIGEELEQEMARVVESSYQCEWQTITLNDPQR	
Siroh	LGGRIGTRIPR	
H.p.	..***** NPIFQFVNTFENQDCVEIVNERGQFPAMVPEKAANKFNEIRWSVSVQVCECSDPLAEAG--SSATVLVGDQIALFRTSKNELYCSQNMCGHRA	941
A.n.	RKYFQGFANTDETENVVEIVKEREQVRPTWPKDGANEDFKGHQWSLSWQPVIKADYFSDGPPAISANIKRGDTQLAIFKV--KGGYATQCMCPHKRT	
K.n.	LALFRSYVNSDEPDETQGRQLRGQPQLAPFAAQG---EPALPSRPQACLDLDAIPQ--QAGIGA---RLGERQIALFRFG--DQVYALDNLEPGSEA	
H.p.	..***** FVNLGGLSLEDSDKNYISCPMHKRNFLYS--GACQNDALSATTFVKE--ENGKVVAKLPPLKELDEVLTGTSKWKVTSQET-----EA	1023
A.n.	FVNLGGLSLEDSDKNYISCPMHKRNFLYS--GACQNDALSATTFVKE--ENGKVVAKLPPLKELDEVLTGTSKWKVTSQET-----EA	
K.n.	NVLSRGLGDAAGEPI--VISPLYQRIRLDRG--QCDGGEQ--AVRAWPVKV--ENGKV-----W-----	
H.p.	KQV--RKITTEKNLVDAKILLDW 1044	
A.n.	KRAGAKGIEGSKPTSPSNTIDW	
K.n.	-----VGNQQLLARAAS--	

Figure 3 NiR protein sequence comparison

Alignment of the deduced amino acid sequences of the NiR proteins encoded by *YNI1* (*H. polymorpha*; H.p.), *niIA* (*A. nidulans*; A.n.) and *nasB* (*K. pneumoniae*; K.n.), as well as the region corresponding to Fe-S and sirohaem (Siroh) domain from *E. coli* sulphite reductase. Identities between the NiR proteins are indicated by asterisks above the sequences. The sequence GXGXG in the putative domain involved in nucleotide binding is doubly underlined. Residues showing identity in cysteine-containing CXXXXXC₁GCXXXC sequences between NiRs and sulphite reductase are underlined.

trations of nitrite and nitrate tested, up to 1 mM nitrite and 5 mM nitrate (results not shown).

The lack of NiR activity in the mutant strain and Southern analysis of the WT strain confirmed that *H. polymorpha* contains a single copy of *YNI1* (Figure 1c).

The effects of the NiR null mutation on *YNI1* expression and NR activity were determined. The mutant ($\Delta yni1::URA3$) possesses about 50 % of the NR activity of the recipient (WT) strain (Figure 6). However, Northern blot analysis showed that the expression of *YNI1* is at the same level in both strains (Figure 4c). The decrease in NR activity in the mutant could be due to the inactivation of NR by nitrite, as has been described in *H. anomala* [39]. However, in the present case the decrease in NR activity could also be due to a decrease in translation of the *YNI1* transcript. Although this was not observed, an increase of *YNI1*

expression might also be expected in the NiR null mutant because of the release of *YNI1* repression exerted by reduced nitrogen sources, which are absent from a NiR null mutant with nitrate as nitrogen source, such as described in plants [12]. From the Northern blot results (Figure 4c) it can be concluded that the two genes are independently transcribed, because mutation of *YNI1* located upstream of *YNI1* does not affect *YNI1* expression. In the *YNI1* null mutant, *YNI1* expression shows the same pattern as in the WT (Figure 4c), indicating that NiR does not have a regulatory role in the expression of nitrate assimilation genes. In contrast, it has been reported that mutations affecting NR-encoding genes modify the expression pattern of nitrate assimilation genes [37,40,41].

In summary, the cloning of *YNI1* contributes to an improved knowledge of nitrate assimilation in yeast, where few data have

Figure 4 Levels of *YNI1* and *YNR1* expression

Total RNA (10 µg) was prepared from cells grown in ammonium salts, washed and then incubated at 10 mg (wet weight) of cells/ml for 2 h in different nitrogen sources, and Northern blots were carried out. *YNI1* and *YNR1* indicate the probe used. At the bottom of each panel the RNA samples stained with ethidium bromide are shown. (a) Levels of *YNI1* expression in different nitrogen sources: ammonium salts (NH_4^+), nitrate (NO_3^-) and nitrite (NO_2^-). The relative molecular size of the *YNI1* transcript is about 3.18 kb. (b) Levels of *YNR1* and *YNI1* at different nitrite concentrations and at 5 mM nitrate. (c) Expression of *YNR1* in the WT and $\Delta\text{yni1}::\text{URA3}$ strains.

been reported so far. Moreover, it has permitted us to construct a NiR null mutant that can be used in biochemical studies of NiR structure–function relationships by expressing either the *YNI1* gene or its counterparts from other sources in the yeast *H. polymorpha* $\Delta\text{yni1}::\text{URA3}$ strain.

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Figure 5 NiR activity and nitrite extrusion in the WT and NiR null mutant ($\Delta\text{yni1}::\text{URA3}$) strains

WT and mutant cells were grown in 5 mM ammonium salts, washed, incubated at 10 mg (wet weight) of cells/ml for 2 h in a nitrogen-free medium buffered with Mes/Tris at pH 5.5 (Mes 25 mM), and then 5 mM nitrate was added (indicated by the arrow). NiR activity was determined in the WT (○) and mutant (●). Nitrite extrusion was also measured in the WT (△) and mutant (▲).

Figure 6 NR activity in the WT and NiR null mutant ($\Delta\text{yni1}::\text{URA3}$) strains

WT and mutant cells were grown in 5 mM ammonium salts, washed, incubated at 10 mg (wet weight) of cells/ml for 2 h in nitrogen-free medium, and then 5 mM nitrate was added (indicated by the arrow). NR activity was determined in the WT (■) and mutant (□).

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