

Cloning and disruption of the *YNR1* gene encoding the nitrate reductase apoenzyme of the yeast *Hansenula polymorpha*

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Abstract The nitrate reductase gene (*YNR1*) from the yeast *H. polymorpha* was isolated from a lambda EMBL3 genomic DNA library. As probe a 350 bp DNA fragment synthesized by PCR from *H. polymorpha* cDNA was used. By DNA sequencing an ORF of 2,577 bp was found. The predicted protein has 859 amino acids and presents high identity with nitrate reductases from other organisms. Chromosomal disruption of *YNR1* causes inability to grow in nitrate. Northern blot analysis showed that *YNR1* expression is induced by nitrate and repressed by ammonium.

Key words: Yeast; Nitrate reductase; Nitrate; *Hansenula*

1. Introduction

The methylotrophic yeast *Hansenula polymorpha* is able to use nitrate as sole nitrogen source [1]. Nitrate is reduced to nitrite by nitrate reductase (NR) and nitrite to ammonium by nitrite reductase. Assimilatory NRs possess three different conserved domains involved in binding of molybdopterin (MoCo), heme-iron and FAD cofactors [2,3]. Several genes are responsible for the production of an active NR [4]: (a) the gene coding the NR apoenzyme; (b) the genes involved in the synthesis of the MoCo; (c) regulatory genes involved in the expression of the NR gene. Assimilatory NR genes have been isolated from filamentous fungi [5–8], plants [9–11] and algae [12] but not from yeasts. Due to the sophistication level of molecular genetic techniques in yeast, the cloning of the gene *YNR1* encoding NR in this organism offers great possibilities to study further the nitrate assimilation pathway and the regulation of this enzyme. NR null mutant yeast strains would be suitable as hosts to express in vitro mutagenized NR genes, homologous or heterologous, to study the structure-function relationship of NR protein as well as the expression of its gene.

In this work we describe the isolation and initial characterization of the gene encoding NR in the yeast *H. polymorpha*.

2. Materials and methods

2.1. Yeast strains

H. polymorpha strains NCYC 495 and HMI-39 *leu1-1 ura3* were used to construct a lambda EMBL3 genomic DNA library and to perform gene disruption, respectively.

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Abbreviations: *YNR1*, nitrate reductase-encoding gene; NR, nitrate reductase; MoCo, molybdopterin cofactor; ORF, open reading frame; PCR, polymerase chain reaction; YNRp, homologous probe synthesized by PCR.

2.2. Synthesis of a homologous *YNR1* probe by PCR

Two degenerated oligonucleotides were designed on the basis of protein sequence similarity of NRs from *N. crassa*, *A. nidulans* and *A. niger* [5–7]. The degenerated oligonucleotide mixtures were: 5'-ATGATGAA(C/T)AA(C/T)(C/T)(C/G)(A/G/C/T)TGGTT-3' and 5'-CC(A/G/C/T)CC(A/G/C/T)GG(G/A)TG(A/G/C/T)CC(T/C)TC-3'. These oligonucleotides were used to prime a PCR with cDNA synthesized from total RNA from *H. polymorpha* grown in nitrate. The cDNA was synthesized from 20 µg of total RNA using the Gene Amp RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT, USA). PCR was run as follows: 100 ng of cDNA, 2 µM each primer, 100 µM each dNTP, 2 mM MgCl₂ and 1 U *Taq* polymerase in a volume of 50 µl. The mixture was incubated 5 min at 94°C and then subjected to 35 cycles according to the following program: 1 min at 95°C, 1 min at 45°C and 1 min at 72°C, and a final cycle of 15 min at 72°C. The amplification products of about 350 bp were fractionated on an agarose gel and ligated in the pGEM-T vector system (Promega Corp., Madison, WI, USA). The ligated fragment was sequenced and the deduced protein sequence had high similarity with the corresponding region of the fungal NRs. The DNA fragment of about 350 bp amplified by PCR was used as homologous probe (YNRp) to screen a lambda EMBL3 *H. polymorpha* genomic DNA library.

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

Genomic DNA from *H. polymorpha* was partially digested with *Sau3A*I and separated on agarose gel electrophoresis. DNA fragments of average size 15 kb were isolated from the agarose gel by electroelution and ligated into the *Bam*HI site of EMBL3 DNA phage. The resulting recombinant DNA was encapsidated in vitro with a lambda DNA packaging kit (Promega Corp. Madison, WI, USA).

2.4. Gene disruption

The strategy followed to disrupt *YNR1* is shown in Fig. 4b. In the plasmid pJA5 harboring a fragment *Bam*HI–*Acc*I which contains the *YNR1* coding region, a *Nco*I–*Nco*I fragment of 868 bp was replaced by a 1,100 bp *Hind*III–*Hind*III fragment from YEp24 [13] containing the *S. cerevisiae* *URA3* gene. The *Nco*I ends from the plasmid and the *Hind*III ends from the *URA3* gene were filled up with the Klenow fragment of the DNA polymerase before ligation. The resulting plasmid, pJS2, digested with *Xba*I produced a fragment containing the *URA3* marker flanked by *YNR1* regions that was used to electrotransform the *H. polymorpha* HMI-39 (*ura*-, *leu*-) strain [14]. Afterwards, cells were plated on synthetic medium consisting of 0.67% yeast nitrogen base w/o amino acids supplemented with 0.23 mM leucine and 2% glucose. Cells which were prototroph for uracil were further screened in a medium consisting of 0.17% yeast nitrogen base without amino acids and ammonium sulfate supplemented with 0.23 mM leucine and 10 mM nitrate as nitrogen source. From 450 colonies screened, two were unable to grow on nitrate. These were selected and the disruption checked by Southern analysis.

2.5. Nucleic acid isolation

Yeast DNA was isolated according to [15], but harvesting the cells in the early exponential phase of growth. Total yeast RNA was isolated as in [16] and fractionated by electrophoresis on formaldehyde agarose gel. Lambda DNA was isolated as described in [17].

2.6. Southern and Northern blot analysis

Southern and Northern blot analysis were carried out as described in [17,18]. Nitrocellulose membranes (Schleicher and Schuell, Dassel,

Germany) and positively charged nylon membranes (Boehringer, Mannheim, Germany) were used in Southern and Northern blot analysis, respectively. Nucleic acids were fixed to the filter by ultraviolet radiation. The probes were labeled with the digoxigenin system from Boehringer Mannheim. The detection method used in Southern blot analysis was the ECL chemiluminiscent system (Amersham, Madrid, Spain), using an anti-digoxigenin conjugated to peroxidase. In the case of Northern blot analysis the detection was carried out with the CDP-Star system (Boehringer, Mannheim, Germany), using an anti-digoxigenin conjugated to alkaline phosphatase.

2.7. DNA sequencing

Bluescript phagemid plasmids from Stratagene (Heidelberg, Germany, USA) were used.

Exonuclease III unidirectional deletions and single strand DNA were prepared following the manufacturer's indications (Promega Corp. Madison, WI, USA). DNA was sequenced on single strand by the dideoxy chain termination method [19], using Sequenase (U.S.B., Cleveland, OH, USA). The sequence was performed on both strands. Multiple protein sequence alignments were carried out with the CLUSTAL V program. The nucleotide sequence data reported in this paper will appear in the EMBL data base under Accession Number Z49110.

3. Results and discussion

3.1. Molecular cloning of the nitrate reductase gene (*YNR1*)

The high similarity between amino acid sequences of fungal NRs permitted the design of a mixture of oligonucleotides to synthesize a *YNR1* homologous probe by PCR. A DNA fragment of 350 bp (*YNRp*) synthesized by PCR from *H. polymorpha* cDNA, the translated sequence of which had a high similarity with the NR genes from filamentous fungi, was used as probe to screen a λ EMBL3 *H. polymorpha* genomic DNA library. From 15,000 phage plaques screened, 17 were found to be positives. DNA was isolated from 10 of them, releasing an average insert length of 12 kb from the lambda arms by *SaI* digestion. By restriction and Southern blot analysis it was found that all the inserts were overlapping and hybridized with *YNRp*. One of these phages (λ JA13) contained three *SaI* fragments of 1.6, 3.4 and 7.4 kb. The fragment of 7.4 kb hybridized with *YNRp* and was subjected to further restriction enzymes and Southern analysis. A *Bam*HI–*Acc*I fragment of about 3 kb, that hybridized with *YNRp*, was cloned in Bluescript KS⁺ (pJA5, Fig. 4a) and sequenced. The DNA sequence revealed an ORF of 2,577 bp encoding a putative protein of 859 amino acids (Fig. 1) with a mass of 98.5 kDa and high amino acid sequence homology with NR sequences from other fungi [5,6] and from tobacco [11].

In the 5' non-coding region of *YNR1* a putative TATA element could be located at 59 bp upstream of the initiation translation codon. In addition, several regions containing the core sequence GATA, implicated in binding of GATA family proteins, some of them related with the regulation of expression of genes involved in utilization of nitrogen sources [20], are present in the same region (Fig. 1). One of these proteins is the one encoded by the *nit-2* gene from *N. crassa* [21]. It has been shown that this protein is able to bind in vitro to promoters of different genes at regions containing the core GATA sequence

[22,23]. The possible participation of these GATA sequences in *YNR1* regulation remains to be elucidated.

The 3.4 kb *SaI* fragment of λ JA13 hybridized with a 481 bp *Hind*III–*Eco*RI fragment corresponding to the nitrite reductase coding region from *A. nidulans* (*niaA*) [6]; suggesting that the nitrite reductase and NR encoding genes are very close in the *H. polymorpha* genome. The clustering of genes involved in nitrate assimilation occurs in some organisms like *A. nidulans* [6] *A. niger* [7] *U. maydis* [8] and the alga *C. reinhardtii* [24]. However, in *N. crassa* as well as in plants the genes involved in nitrate assimilation are unlinked [25].

3.2. Comparison of the putative NR protein sequence encoded by *YNR1* with NRs from other organisms

The putative NR encoded by *YNR1* shares a high similarity with other NRs such as that encoded by *Nit-3* (*N. crassa*) [5], *niaD* (*A. nidulans*) [6] and *nia1* (tobacco) [11] (Fig. 2). The similarity is specially high in the MoCo, heme and FAD binding regions. These regions have been identified by similarity with the mammalian protein regions of sulfite oxidase [26], cytochrome *b₅* [27] and NADPH cytochrome *b₅* reductase [28], proteins that contain the MoCo, heme and FAD domains, respectively. These cofactor binding regions are located in the sequence encoded by *YNR1* in a lineal way (Fig. 2) with the MoCo region near the N-terminus, followed by the heme-Fe binding region and the FAD-NADPH binding region at the C-terminus, in a similar way to that described for other NRs [3].

3.3. *YNR1* expression

In order to determine *YNR1* expression, total RNA from cells grown in ammonium and then transferred for 2 h to nitrate, nitrate plus ammonium, or ammonium was isolated. By Northern blot analysis it was determined that the *YNR1* gene is expressed in cells transferred to nitrate while cells transferred to ammonium did not present detectable *YNR1* transcripts (Fig. 3). In the case of cells transferred to ammonium plus nitrate only a faint band was visible on the film (not detectable in the picture). Therefore the expression of *YNR1* correlates well with the NR activity described in *H. polymorpha* grown in nitrate, ammonium or nitrate plus ammonium [1] indicating that the levels of NR activity are mainly regulated at transcriptional level. The effect of nitrate and ammonium on *YNR1* expression is similar to that described in filamentous fungi [5–8].

3.4. Chromosomal disruption of *YNR1*

The strategy followed to disrupt *YNR1* is depicted in Fig. 4b. The chromosomal copy of the *YNR1* gene was replaced by the *YNR1* gene carrying a *S. cerevisiae* gene *URA3* insertion. The transformants carrying the disrupted chromosomal copy were unable to grow in nitrate. Southern analysis of the disruptant strains confirmed the correct replacement of the chromosomal *YNR1* copy (Fig. 4c). The disrupted strains showed a

Fig. 1. Nucleotide sequence of the *H. polymorpha* *YNR1* gene and the deduced amino acid sequence. In the coding region, the zones overlined indicate the primers used for PCR. The 350 bp region comprised between both primer was used as probe to screen the lambda EMBL3 *H. polymorpha* genomic DNA library. In the 5' non-coding region the TATA element is underlined and the core sequences GATA overlined.

GATGCTCGCT	TGAACAGGA	GGCATCGAAC	GAGCGTCTTA	CCATTTTGCT	TTTATTAGCT	CTTTAGCAGC	AATGATACAT	CTAGTGCTAT	ACTCTGAGAA	GCTACGCTC	110																				
A	GAACACCTTC	TCAACTTGA	AACCTGTGT	ACGGCTCTGA	AAATTTCCGG	ACAACGATGG	AAAAAGGGTG	CAGTCAACT	AGAGATGCTA	TGTGAATTTT	CGCAAGC	218																			
CA	CGGAATCAAT	GTGCCAATC	TAGAACGTT	CTCGTCCAGG	TGGACAAAAT	TTTAATTTTA	TTTCGGCTTG	TTAAATTTTA	TTCTCGGTCT	TATCTTGCAG	AGATACT	327																			
AAT	AGAGTAGCTA	CTAAAAGGAC	TTTATGTAAT	TTCTCAAGAA	TTCCGGATAG	TCGTA	ATG	GAT	TCT	ATT	GTC	ACT	GAG	GTA	ACC	TAT	GGT	CTG	GAG	ATC	427										
							M	D	S	I	V	T	E	V	T	Y	G	L	G	E	I	14									
AAK	AAA	ATC	AAA	GAT	ATC	ACG	GAG	CTA	CCT	TTT	CCA	GTC	AGG	CAA	GAC	TCT	CCT	CTT	ACC	GAG	GTG	CTT	CCA	ACA	GAT	CTG	AAG	ACC	AAA	517	
K	K	I	K	D	I	T	E	L	P	F	P	V	R	Q	D	S	P	L	T	E	V	L	P	T	D	L	K	T	K	44	
GAT	AAT	TTT	GTC	GCT	AGA	GAT	CCT	GAC	CTT	LTT	AGA	CTC	ACT	GGT	TCA	CAC	CCA	TTT	AAT	TCT	GAG	CCG	CCA	CTG	ACA	AAG	CTT	TAT	GAC	607	
D	N	F	V	A	R	D	P	D	L	C	R	L	T	T	S	H	P	F	N	S	E	P	L	T	K	L	Y	D	G	74	
TCG	GGG	TTT	CTC	ACT	CCA	GTG	AGT	CTT	CAC	TTT	GTG	AGA	AAC	CAC	GGC	CCG	GTT	CCT	TAC	GTT	CCT	GAT	GAA	AAT	TTA	GAC	TGG	GAA	692		
S	G	F	L	T	P	V	S	L	H	F	V	R	N	H	G	P	V	P	Y	V	P	D	E	N	I	L	D	W	E	10	
GTT	TCA	ATT	GAA	GGG	ATG	GTT	GAA	ACG	CCT	TAT	AAA	ATC	AAA	TTG	TCA	GAC	ATA	ATG	GAG	CAG	TTT	GAT	ATC	TAT	TCA	ACC	CCC	GTT	ACT	787	
V	S	I	E	G	M	V	E	T	P	Y	K	I	K	L	S	D	I	M	E	Q	F	D	I	Y	S	T	P	V	T	134	
ATG	GTC	TGC	GCT	GGA	ANC	AGA	AGA	AAG	GAG	CAG	AAT	ATG	GTA	AAG	AGA	GGA	GCC	GGT	TTC	AAT	TGG	GGA	GCA	GCT	AGA	ACA	TCT	ACT	T	877	
M	V	C	A	G	A	R	R	K	E	Q	N	M	V	K	K	G	A	G	F	N	W	G	A	G	G	T	S	T	S	164	
GTT	TGG	ACA	GGA	TGC	ATG	CTT	GGA	GAT	GTA	ATA	GGC	AAG	GCT	AGA	CCA	TCA	AAG	AGA	GCA	AGA	TTC	GTA	TGG	ATG	GAG	GGT	GCA	GAT	AAT	967	
L	W	T	G	C	M	L	G	D	V	I	G	K	A	R	P	S	K	R	A	R	F	V	W	M	E	G	A	D	N	194	
CCG	GCA	AAC	GGC	GCA	TAC	CGC	ACC	TGT	ATC	CGC	TTA	AGC	TGG	TGT	ATG	GAC	CCT	GAA	CGG	TGC	ATC	ATG	ATC	GCA	TAC	CAG	CAG	AAC	GGC	1057	
P	A	N	G	A	Y	R	T	C	I	R	L	S	W	C	M	D	P	E	R	C	I	M	I	A	Y	Q	Q	N	G	224	
GAG	TGG	TTG	CAT	CCT	GAC	CAT	GGA	AAG	CCC	CTT	CGA	GTA	GTA	ATC	CCC	GGT	GTT	ATT	GGT	GGA	CGA	GTA	GTC	AAA	TGG	CTA	AAG	AAA	CTA	1147	
E	W	L	H	P	D	H	G	K	P	C	R	V	V	I	C	CCG	G	V	I	GGT	G	R	S	V	K	W	L	K	K	L	254
GTA	GTG	AGC	GAT	CGG	CCG	TCT	GAA	AAT	TGG	TAT	CAT	TAT	TTT	GAT	AAT	CGG	GTT	CTT	CCA	ACC	ATG	GTG	ACG	CCA	GAG	ATG	GCT	AAA	AGT	1237	
V	V	S	D	R	P	S	E	N	W	Y	H	Y	F	D	N	R	V	L	P	T	M	V	T	P	E	M	A	K	S	284	
GAC	GAC	AGG	TGG	TGG	AAA	GAC	GAG	CGA	TAT	GCC	ATA	TAT	GAT	CTG	AAC	TTG	CAA	ACG	ATC	ATT	TGC	AAG	CCC	GAA							

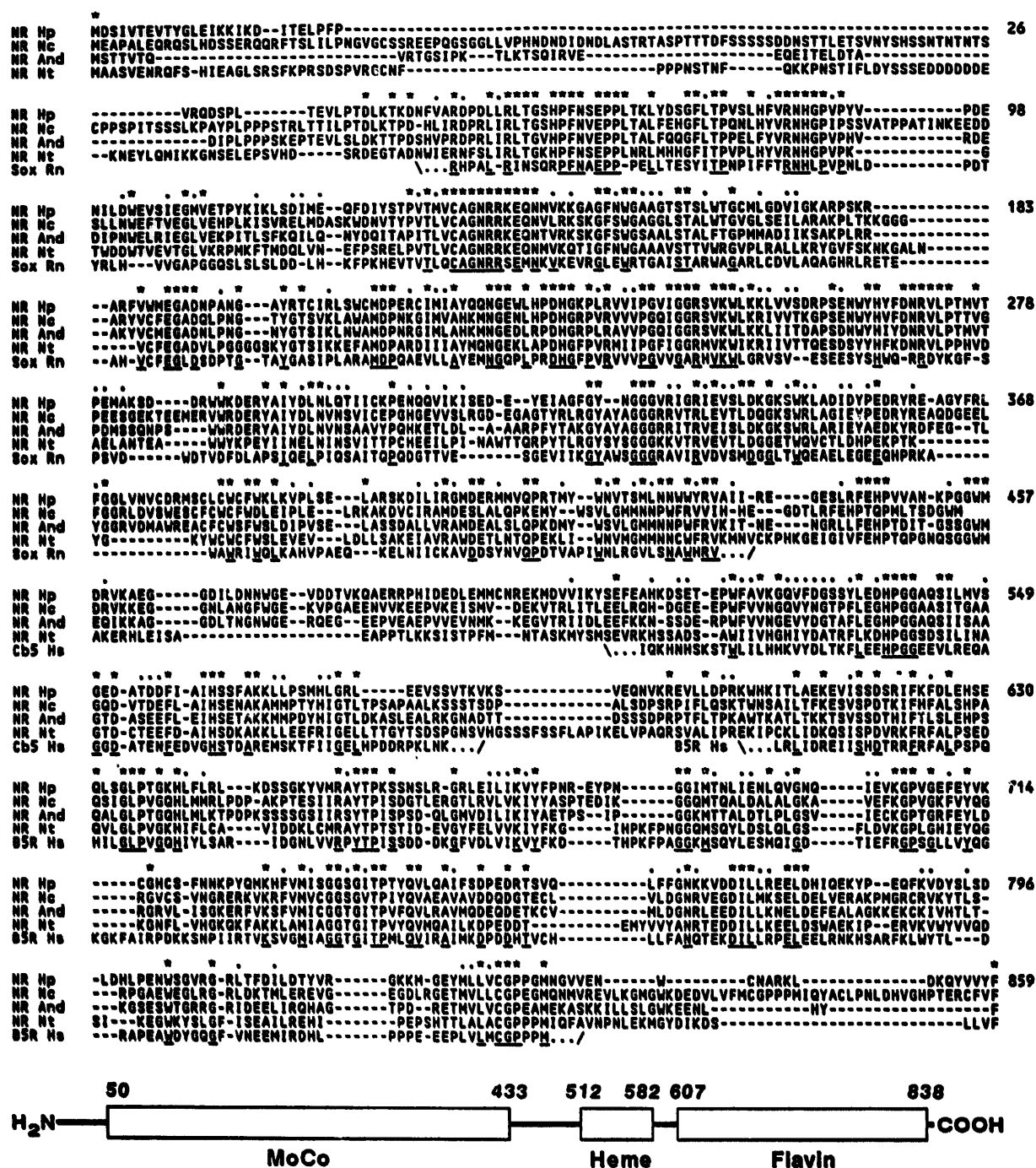


Fig. 2. NR proteins comparison. Alignment of the deduced amino acid sequences encoded by *YNR1* (*H. polymorpha*, Hp), *Nit-3* (*N. crassa*, Nc) *niaD* (*A. nidulans*, And), and *nial* (tobacco, Nt) as well as the MoCo, heme and FAD domains corresponding to rat sulfite oxidase (Sox, Rn), human cytochrome *b*₅ (Cb5, Hs) and human NADH-cytochrome *b*₅ reductase (B5R, Hs), respectively. The similarities between the NR proteins are indicated on the top of the sequences. At the bottom, the similarity between all the NR and the MoCo, heme and FAD domains, corresponding to sulfite oxidase, cytochrome *b*₅ and NADH-cytochrome *b*₅ reductase respectively, are indicated.

NR activity of less than 10 nmol NO₂ · min⁻¹ · mg⁻¹ protein after incubation in nitrate for 2 h, while the recipient strain showed an activity of about 130 nmol NO₂ · min⁻¹ · mg⁻¹ protein in the same conditions. The lack of NR activity in the interrupted strain as well as the Southern analysis of the wild type strain, indicate that *H. polymorpha* only contains one *YNR1* copy.

It was curious that from 450 transformants scored (prototrophs for uracil) only two were unable to grow in nitrate. This very low frequency of targeted integration remains unclear, since use of the *H. polymorpha* *URA3* gene raised the targeted integration up to 80% (K.N. Faber, personal communication).

The *Aynr1::URA3* mutant appears as an interesting tool to

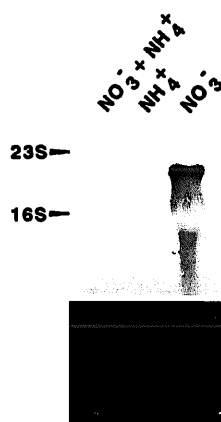


Fig. 3. Northern blot analysis. Total RNA (30 µg) from *H. polymorpha* (NCYC 495) grown in ammonium and then incubated for 2 h in nitrate plus ammonium, ammonium and nitrate were applied on each lane. The large and small ribosomal subunits are indicated by the arrows. The fragment *Xba*I–*Hind*III from *YNR1* coding region was used as probe. At the bottom of the figure are the same samples stained with ethidium bromide.

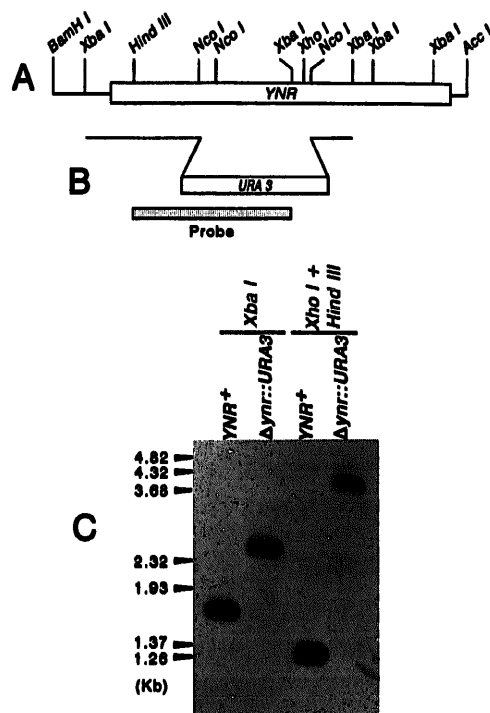


Fig. 4. Structure of *YNR1* region of *H. polymorpha*, strategy to construct *ynr1* null mutants by disruption and Southern analysis of the disruptants. (A) Restriction map of the insert of the plasmid pJA5; the region corresponding to the *YNR1* gene is enclosed in a box. (B) Interruption of *YNR1*, the *Nco*I–*Nco*I fragment was replaced by *S. cerevisiae* *URA3* gene (see section 2 for details of the construction). (C) Southern analysis of the genomic interruption. DNA from the recipient strain (*YNR1*) and from the disruptant (*ynr1::URA3*) were digested with *Xba*I or *Xho*I+*Hind*III and probed with the *Xba*I–*Hind*III fragment shown in the hatched box.

express NR encoding genes to study structure-function relationships of NR by site directed mutagenesis. This approach cannot be carried out in *S. cerevisiae* due to its lack of MoCo [29] and has been performed up to now by expressing mutated NR encoding genes either in *N. crassa* NR deficient mutants by non targeted integrative transformation or in *E. coli* [30,31]. The advantages of *H. polymorpha* are the possibility of transformation by replicative plasmids as well as targeted integration, which offer wider possibilities for gene manipulation.

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