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

Julio Avila, M. Dolores Pérez, Nélida Brito, Celedonio González, José M. Siverio*

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

Received 26 April 1995

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.

*Corresponding author. Fax: (34) (9) 603724.
E-mail: Jsiverio@ull.es

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 $MgCl_2$ 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 *Sau*3AI 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,

SSDI 0014-5793(95)00511-0

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 lambda 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 *Sal*I digestion. By restriction and Southern blot analysis it was found that all the inserts were overlapping and hybridized with YNRp. One of these phages (λJA13) contained three *Sal*I 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 *Sal*I *fragment of* λJA13 hybridized with a 481 bp *Hind*III–*Eco*RI fragment corresponding to the nitrite reductase coding region from *A. nidulans* (*niiA*) [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 b_5 [27] and NADPH cytochrome b_5 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.

```
GGATCCTGCT TGAAACAGGA GGCATCGAAC GAGCGTCCTA CCATTTTGCT TTCATTAGCT CTTTAGCAGC AATGATACAT CTAGTGCTAT ACTCTGAGAA GCTACGTCC  110

A GAACACCTTC TCAACTTGGA AACCTGTGTC ACGGCTCTGA AAATTTCCGG ACAACGATGG AAAAGGGGTG CACTGCAACT AGAGATGCTA TGTGAATTTT CGCAAAGC  218

CA CGGAATCAAT GTGCCAATTC TAGAAACGTT CTCGTCCAGG TGGACAAAAT TTTAATTTTA TTTCCGCTTG TTAAATTTTA TTCTCCGTCT TATCTTGCAG AGATACT  327

AAT ATAGTAGCTA CTAAAAGGAC TTCATGTAAT 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  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 CTT AGA CTC ACT GGT TCA CAC CCA TTC AAT TCT GAG CCG CCA CTG ACA AAG CTT TAT GAC  607
 D  N  F  V  A  R  D  P  D  L  L  R  L  T  G  S  H  P  F  N  S  E  P  P  L  T  K  L  Y  D   74

TCG GGG TTT CTC ACT CCA GTG AGT CTT CAC TTT GTG AGA AAC CAC GGC CCC GTT CCT TAC GTT CCT GAT GAA AAT ATT TTA GAC TGG GAA  697
 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  104

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 AAC AGA AGA AAG GAG CAG AAT ATG GTA AAG AAG GGA GCC GGT TTC AAT TGG GGA GCA GCT GGA ACA TCT ACT TCT  877
 M  V  C  A  G  N  R  R  K  E  Q  N  M  V  K  K  G  A  G  F  N  W  G  A  A  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 TCA GTC AAA TGG CTA AAG AAA CTA 1147
 E  W  L  H  P  D  H  G  K  P  L  R  V  V  I  P  G  V  I  G  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 AAT CAG CAG GTT ATC 1327
 D  D  R  W  W  K  D  E  R  Y  A  I  Y  D  L  N  L  Q  T  I  I  C  K  P  E  N  Q  Q  V  I  314

AAG ATT TCA GAG GAC GAG TAC GAA ATT GCA GGT TTT GGC TAC AAC GGA GGT GGA GTC AGA ATA GGC CGG ATT GAG GTC AGT CTT GAC AAA 1417
 K  I  S  E  D  E  Y  E  I  A  G  F  G  Y  N  G  G  G  V  R  I  G  R  I  E  V  S  L  D  K  344

GGG AAG AGT TGG AAA CTG GCA GAT ATA GAC TAT CCG GAA GAC AGA TAT AGG GAA GCA GGT TAC TTC AGA TTG TTT GGC GGA CTT GTG AAT 1507
 G  K  S  W  K  L  A  D  I  D  Y  P  E  D  R  Y  R  E  A  G  Y  F  R  L  F  G  G  L  V  N  374

GTT TGC GAC AGA ATG AGC TGC CTG TGC TGG TGT TTC TGG AAG CTC AAG GTT CCT CTT TCT GAA TTA GCA AGG TCA AAA GAT ATT CTC ATT 1597
 V  C  D  R  M  S  C  L  C  W  C  F  W  K  L  K  V  P  L  S  E  L  A  R  S  K  D  I  L  I  404

CGT GGC ATG GAT GAG CGT ATG ATG GTT CAG CCG CGC ACG ATG TAC TGG AAC GTA ACG TCC ATG CTG AAC AAC TGG TGG TAT CGA GTC GCC 1687
 R  G  M  D  E  R  M  M  V  Q  P  R  T  M  Y  W  N  V  T  S  M  L  N  N  W  W  Y  R  V  A  434

ATT ATC CGC GAG GGT GAG AGT CTT CGA TTT GAG CAT CCC GTG GTG GCC AAC AAG CCT GGC GGT TGG ATG GAT AGG GTC AAG GCA GAG GGT 1777
 I  I  R  E  G  E  S  L  R  F  E  H  P  V  V  A  N  K  P  G  G  W  M  D  R  V  K  A  E  G  464

GGA GAT ATT CTA GAT AAC AAT TGG GGA GAG GTG GAC GAC ACG GTC AAG CAG GCT GAA AGG AGG CCG CAT ATT GAT GAG GAT CTC GAG ATG 1867
 G  D  I  L  D  N  N  W  G  E  V  D  D  T  V  K  Q  A  E  R  R  P  H  I  D  E  D  L  E  M  494

ATG TGC AAC CGG GAG AAA ATG GAC GTC GTT ATC AAA TAT TCA GAG TTT GAA GCA CAC AAG GAC AGC GAG ACA GAG CCA TGG TTT GCT GTC 1957
 M  C  N  R  E  K  M  D  V  V  I  K  Y  S  E  F  E  A  H  K  D  S  E  T  E  P  W  F  A  V  524

AAA GGC CAA GTG TTC GAT GGT AGC TCG TAT CTG GAA GAC CAT CCA GGA GGG GCC CAG TCG ATC TTG ATG GTG AGC GGC GAA GAC GCA ACC 2047
 K  G  Q  V  F  D  G  S  S  Y  L  E  D  H  P  G  G  A  Q  S  I  L  M  V  S  G  E  D  A  T  554

GAC GAC TTC ATT GCA ATT CAC TCA TCT TTT GCT AAA AAG CTG CTC CCT TCC ATG CAC CTG GGA AGA CTC GAA GAG GTC AGT TCA GTT ACA 2137
 D  D  F  I  A  I  H  S  S  F  A  K  K  L  L  P  S  M  H  L  G  R  L  E  E  V  S  S  V  T  584

AAA GTG AAG TCT GTA GAG CAA AAT GTG AAG CGA GAA GTT TTG CTC GAT CCG CGA AAA TGG CAC AAG ATA ACG CTT GCA GAA AAA GAG GTT 2227
 K  V  K  S  V  E  Q  N  V  K  R  E  V  L  L  D  P  R  K  W  H  K  I  T  L  A  E  K  E  V  614

ATC TCT TCC GAC TCT AGA ATA TTC AAG TTC GAC CTG GAG CAT TCA GAA CAG CTT TCT GGT CTG CCA ACG GGT AAA CAC CTG TTT CTG AGG 2317
 I  S  S  D  S  R  I  F  K  F  D  L  E  H  S  E  Q  L  S  G  L  P  T  G  K  H  L  F  L  R  644

CTA AAA GAT TCA TCT GGC AAG TAT GTG ATG AGG GCA TAC ACC CCT AAA TCG AGC AAT TCT TTG CGG GGT CGT CTA GAG ATA TTG ATA AAG 2407
 L  K  D  S  S  G  K  Y  V  M  R  A  Y  T  P  K  S  S  N  S  L  R  G  R  L  E  I  L  I  K  674

GTT TAT TTC CCA AAT AGG GAA TAC CCC AAC GGC GGA ATT ATG ACA AAT CTT ATC GAA AAC CTC CAA GTG GGA AAC CAG ATC GAG GTC AAA 2497
 V  Y  F  P  N  R  E  Y  P  N  G  G  I  M  T  N  L  I  E  N  L  Q  V  G  N  Q  I  E  V  K  704

GGA CCT GTC GGC GAG TTT GAG TAT GC AAG TGC GGG CAC TGC AGT TTC AAT AAC AAG CCT TAT CAA ATG AAG CAT TTT GTT ATG ATC TCG 2587
 G  P  V  G  E  F  E  Y  V  K  C  G  H  C  S  F  N  N  K  P  Y  Q  M  K  H  F  V  M  I  S  734

GGA GGA TCG GGC ATC ACC CCA ACT TAC CAG GTT CTG CAA GCT ATT TTC AGC GAT CCC GAA GAT AGA ACA AGC GTT CAG TTA TTT TTT GGA 2677
 G  G  S  G  I  T  P  T  Y  Q  V  L  Q  A  I  F  S  D  P  E  D  R  T  S  V  Q  L  F  F  G  764

AAT AAA AAA GTT GAC GAT ATC CTG CTT CGA GAA GAG CTT GAC CAC ATA CAG GAA AAA TAT CCA GAA CAA TTC AAA GTT GAT TAC TCG CTG 2767
 N  K  K  V  D  D  I  L  L  R  E  E  L  D  H  I  Q  E  K  Y  P  E  Q  F  K  V  D  Y  S  L  794

TCG GAT CTG GAT CAT CTA CCG GAG AAT TGG AGC GGA GTG AGA GGC AGG TTA ACA TTC GAT ATT CTA GAC ACC TAT GTT CGG GGG AAA AAA 2857
 S  D  L  D  H  L  P  E  N  W  S  G  V  R  G  R  L  T  F  D  I  L  D  T  Y  V  R  G  K  K  824

ATG GGA GAG TAT ATG CTA CTG GTA TGT GGA CCG CCA GGA ATG AAC GGT GTG GTC GAA AAC TGG TGC AAT GCG CGC AAG TTG GAT AAA CAG 2947
 M  G  E  Y  M  L  L  V  C  G  P  P  G  M  N  G  V  V  E  N  W  C  N  A  R  K  L  D  K  Q  854

TAT GTA GTG TAC TTC TGACCAAGCA AAATCGAAAT CGAAACTATC AAGAAATGTG GGTATGTTGT AAATATCAAG AACAGGGGTA TCCGTTGCAA AGTCTCGATC  3052
 Y  V  V  Y  F                                                                                                        859

CATTTCGCCT CTCAAATAGT TTTGGTAGAC                                                                                      3082
```

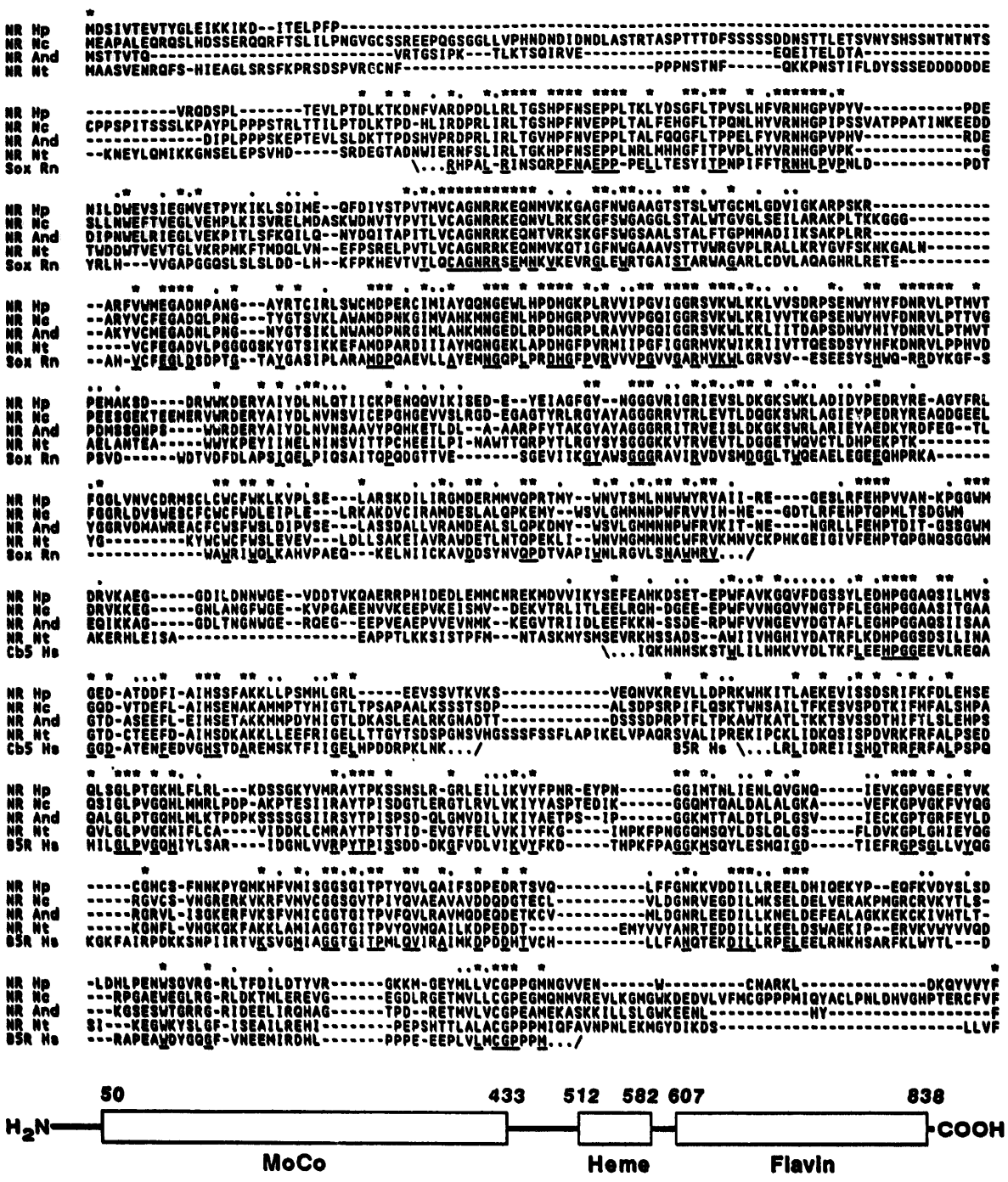

Fig. 2. NR proteins comparison. Alignment of the deduced amino acid sequences encoded by *YNR1* (*H. polymorpha*, Hp), *Nit-3* (*N. crassa*, Nc) *niaD* (*A. nidulans*, And), and *nia1* (tobacco, Nt) as well as the MoCo, heme and FAD domains corresponding to rat sulfite oxidase (Sox, Rn), human cytochrome 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 $NO_2^- \cdot min^{-1} \cdot mg^{-1}$ protein after incubation in nitrate for 2 h, while the recipient strain showed an activity of about 130 nmol $NO_2^- \cdot min^{-1} \cdot mg^{-1}$ 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 *Δynr1*::*URA3* mutant appears as an interesting tool to

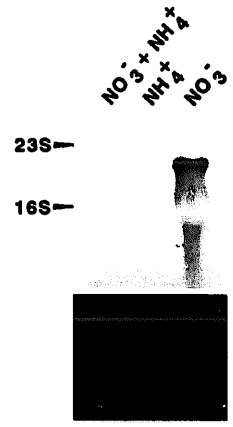

Fig. 3. Northern blot analysis. Total RNA (30 μg) from *H. polymorpha* (NCYC 495) grown in ammonium and then incubated for 2 h in nitrate plus ammonium, ammonium and nitrate were applied on each lane. The large and small ribosomal subunits are indicated by the arrows. The fragment *Xba*I–*Hin*dIII from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

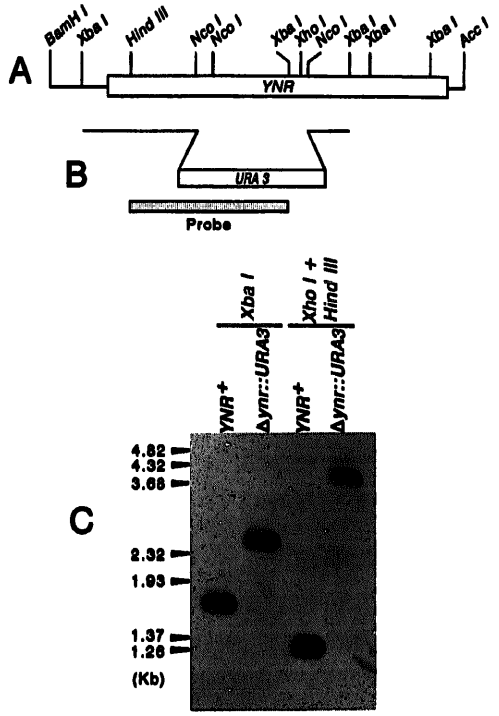

Fig. 4. Structure of *YNR1* region of *H. polymorpha*, strategy to construct ynr1 null mutants by disruption and Southern analysis of the disruptants. (A) Restriction map of the insert of the plasmid pJA5; the region corresponding to the *YNR1* gene is enclosed in a box. (B) Interruption of *YNR1*, the *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+*Hin*dIII and probed with the *Xba*I–*Hin*dIII 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.

Acknowledgements: We thank Carlos Gancedo (IIB, CSIC, Madrid, Spain) for advice and criticisms of the manuscript, J. Kinghorn (St Andrews, UK), E. Fernández (Córdoba, Spain) and C. Hollenberg (Düsseldorf, Germany) for providing NR probes and yeast strains. The help of L. Rodríguez and T. Ruiz (La Laguna, Spain) is also acknowledged. J.M.S. thanks K.D. Entian (Frankfurt, Germany), P. Greenway and M. Tully (Salisbury, UK) for initiating him in molecular biology techniques. This work was supported by Grants PB90-0842 and PB 91-0762 from Dirección General de Investigación Científica y Técnica (DGICYT).

References

[1] González, C. and Siverio, J.M. (1992) J. Gen. Microbiol. 138, 1445–1451.
[2] Guerrero, M.G., Vega, J.M. and Losada, M. (1981) Annu. Rev. Plant Physiol. 32, 169–204.
[3] Campbell, W.H. and Kinghorn, J.R. (1990) Trends Biochem. Sci. 15, 315–319
[4] Kinghorn, J.R. in: Molecular and Genetic Aspects of Nitrate Assimilation (Wray, J.L. and Kinghorn, J.R. Eds.) pp. 69–87, Oxford Scientific Publications.
[5] Okamoto, P.M., Fu, Y.-H. and Marzluf, G.A. (1991) Mol. Gen. Genet. 227, 213–223.
[6] Johnstone, I.L., McCabe, P.C., Greaves, P., Gurr, S.J., Cole, G.E., Brow, M.A.D., Unkles, S.E., Clutterbuck, A.J., Kinghorn, J.R. and Innis, M.A. (1990) Gene 90, 181–192.
[7] Unless, S.E., Campbell, E.I., Punt, P.J., Hawker, K.L., Contreras, R., Hawkins, A.R., Van den Hondel, C.A.M.J.J. and Kinghorn, J.R. (1992) Gene 111, 149–155.
[8] Banks, G.R., Shelton, P.A., Kanuga, N., Holden, D.W. and Spanos, A. (1993) Gene 131, 69–78.
[9] Crawford, N.M., Campell, W.H. and Davis, R.W. (1986) Proc. Natl. Acad. Sci. USA 93, 8073–8076.
[10] Cheng, C.H., Dewdeney, J., Kleinhofs, A. and Goodman, H.M. (1986) Proc. Natl. Acad. Sci. USA 83, 6825–6828.
[11] Vaucheret, H., Vincentz, M., Kronenberger, J., Caboche, M. and Rouzé, P. (1989) Mol. Gen. Genet. 216, 10–15.
[12] Fernández, E., Schnell, R., Ranum, L.P.W., Hussey, S.C., Silflow, C.D. and Lefebvre, P.A. (1989) Proc. Natl. Acad. Sci. USA 86, 6449–6453.
[13] Botstein, D., Falco, S.C., Stewart, S.E., Brennan, M., Scherer, S., Stinchcomb, D.T., Struhl, K. and Davis, R.W. (1979) Gene 8, 17–24.
[14] Faber, K.N., Haima, P., Harder, W., Veenhuis, M. and Ab, G. (1994) Curr Genet. 25, 305–310.
[15] Rothstein, R. (1985) in: DNA Cloning Volume II (Glober, D.M. Ed.), pp. 45–66, IRL press Oxford.
[16] Carlson, M. and Botstein, D. (1982) Cell 28, 145–154.
[17] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbord, NY.
[18] Engler-Blum, G., Meier, M., Frank, J. and Müller, G.A. (1993) Anal. Biochem. 210, 235–244.
[19] Sanger, F., Nicklen, S. and Coulson A.R. (1977) Proc. Natl. Acad. USA 74, 5463–5467.
[20] Cunningham, T.H. and Cooper, T.G. (1991) Mol. Cell. Biol. 11, 6205–6215.
[21] Fu, Y.-H. and Marzluf, G.A. (1990) Mol. Cell. Biol. 10, 1056–1065.

[22] Fu, Y.-H. and Marzluf, G.A. (1990) Proc. Natl. Acad. Sci. USA 87, 5331–5335.
[23] Jarai, G., Truong, H.-N., Daniel-Vedele, F. and Marzluf, G.A. (1992) Curr. Genet. 21, 37–42.
[24] Quesada, A., Galván, A. and Fernández, E. (1994) Plant J. 5, 407–419.
[25] Crawford, N.M. and Arst Jr., H.-N. (1993) Annu. Rev. Genet. 27, 115–146.
[26] Garrett, R.M. and Rajagopalan, K.V. (1994) J. Biol. Chem. 269, 272–276.
[27] Yoo, M. and Steggles, A.W. (1988) Biochem. Biophys. Res. Commun. 156, 576–580.
[28] Yubisui, T., Miyata, T., Iwanaga, S., Tamura, M., Yoshida, S., Takeshita, M. and Nakajima, H. (1984) J. Biochem. 96, 579–582.
[29] Truong, H.-N., Meyer, C. and Daniel-Vedele, F. (1991) Biochem. J. 278, 393–397.
[30] Okamoto, P.M. and Marzluf, G.A. (1993) Mol. Gen. Genet. 240, 221–230.
[31] Cannons, A.C, Barber, M. J. and Solomonson, L.P. (1993) J. Biol. Chem. 268, 3268–3271.