Nitrate reduction and the isolation of Nit[−] mutants in *Hansenula polymorpha*

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***Hansenula polymorpha* (syn. *Pichia angusta*) is able to grow on nitrate as sole nitrogen source. Nitrate reductase (NR) assays, optimized in crude extracts from nitrate-grown cells, revealed that NR preferentially used NADPH, but also used NADH, as electron donor and required FAD for maximum activity. NR activity was present in nitrate-grown and nitrite-grown cells, and was absent in cells grown in ammonium, glutamate and methylamine. Addition of reduced nitrogen compounds to nitrate-grown cells led to loss of NR activity, even if added with nitrate. Under nitrogen starvation, NR activity was not observed; however, following growth on nitrate, NR activity is maintained in the absence of nitrate. Increases but not decreases in NR activity were dependent on protein synthesis. Conditions for chlorate selection were optimized, and Nit[−] (nitrate[−]) mutants were isolated. Some of these mutants showed reduced or absent NR activity. Sixty-one NR[−] mutants revealed the monogenic recessive nature of their lesions and were grouped in 10 complementation classes. These mutants will be used in gene cloning experiments aimed at identifying structural and regulatory elements involved in the first step of nitrate reduction.**

Keywords: nitrate assimilation, nitrate reductase, Nit[−] mutants, chlorate resistance, *Hansenula polymorpha*

INTRODUCTION

In the biosphere, nitrate assimilation is the major pathway converting inorganic nitrogen to organic forms. It has been estimated that more than 2×10^4 megatons of organic nitrogen per year are produced by nitrate assimilation in a variety of organisms, including bacteria, fungi, algae and plants (Guerrero *et al.*, 1981). As pointed out by Solomonson & Barber (1990), as much as 25% of the energy of photosynthesis is consumed in driving nitrate assimilation. Therefore, interest in the nitrate assimilation pathway has been steadily growing, especially in relation to crop yield improvements and to the worldwide concern over the use of nitrate fertilizers, costly in terms of production and in terms of ecological and toxicological impact.

Nitrate assimilation consists of a two-step reduction of nitrate to ammonia, catalysed by the sequential action of nitrate reductase (NR) and nitrite reductase (NiR). NR catalyses the two-electron reduction of nitrate to nitrite; it is a complex multicentre enzyme that in eukaryotes

uses NAD(P)H as electron donor and contains FAD, ferrohaem and molybdopterin prosthetic groups. It is considered to be a limiting factor for growth, development and protein production in all nitrate-assimilating organisms. NiR catalyses the six-electron reduction of nitrite to ammonia; it is also a multicentre enzyme that in plants uses reduced ferredoxin as electron donor, whilst in bacteria and fungi uses NAD(P)H (Campbell & Kinghorn, 1990, and references therein).

The genetics and molecular biology of nitrate assimilation have been studied mostly in plants, filamentous fungi and bacteria (Wray & Kinghorn, 1989, and reviews therein). Despite being considerably advantageous as experimental models, yeasts have been neglected in these studies, perhaps, as noted by Hipkin (1989), because of the tendency to equate yeast with *Saccharomyces cerevisiae*, a species unable to assimilate nitrate or nitrite. However, a large number of nitrate-assimilating yeasts exist (Barnett *et al.*, 1984), and various investigations have shown that the enzymological layout of their nitrate metabolism is similar to that of the other nitrate-assimilating eukaryotes (Hipkin, 1989, and references therein). The yeast *Hansenula polymorpha*

Abbreviations: NiR, nitrite reductase; NR, nitrate reductase.

(syn. *Pichia angusta*) promises well as a model for studying nitrate assimilation, since it is amenable to genetic analysis, transformation and chromosome engineering (*vide* Berardi, 1997). This ascomycete has long served as a model to study peroxisome function (Veenhuis, 1992; Waterham & Cregg, 1997) and more recently is being employed for heterologous protein expression (e.g. Berardi, 1997; Faber *et al.*, 1996). To understand the nitrate assimilation of *H. polymorpha* it is important to identify and characterize genetic factors involved in this process. As yet, the screening and isolation of nitrate assimilation mutants in this yeast have not been attempted, although the isolation of genes involved in this metabolism has been reported (Avila *et al.*, 1995; Brito *et al.*, 1996; Pérez *et al.*, 1997). Here we describe the isolation and characterization of 10 complementation groups of *H. polymorpha* mutants with reduced or undetectable NR activities. It is likely that these mutants identify structural and regulatory elements involved in the first step of nitrate reduction.

METHODS

Strains. All are derivatives of *Hansenula polymorpha* homothallic haploid NCYC 495. M6 (*met6-1*) and L1 (*leu1-1*) are Nit⁺ (nitrate)⁺ strains used as 'control' strains. M6 was used to isolate all Nit⁻ mutants; L1 served to obtain all *nit*⁺/*nit* diploids.

Media. YPD contained 2% glucose, 1% yeast extract and 2% peptone. ME contained 2% malt extract. All the following minimal media contained 2% glucose, 0.2% yeast nitrogen base without amino acids and ammonium sulphate (Difco); when necessary, 0.006% leucine or 0.002% methionine was added. In addition, MA1 contained 38 mM ammonium sulphate, MA2 contained 50 mM ammonium chloride, MC contained 1.8 mM sodium nitrite + 0.3–0.8 M potassium chlorate, MN contained 50 mM sodium nitrate, MN1 contained 8 mM potassium nitrate, MN2 contained 25–200 mM sodium nitrate, MN3 contained 1.8 mM sodium nitrite, MG contained 4 mM sodium glutamate, MMA contained 10 mM methylamine, MH contained 0.7 mM hypoxanthine, MAs contained 4 mM aspartate and MMo contained 50 mM sodium nitrate + 200 mM sodium molybdate. When necessary, 1.6% agar was added.

Induction and repression experiments. All cultures were grown in 250 ml flasks with 100 ml medium, using an orbital incubator at 37 °C and 220 r.p.m. After 15 h pre-cultivations in YPD, 5×10^5 M6 or Nit⁻ mutants ml⁻¹ were washed and inoculated in MG flasks (induction experiments) or MN2 flasks (repression experiments). After 15 h cultivation (late-exponential phase), a different nitrogen source (or combinations of sources) was added (nitrite, 1 mM or 10 mM; methylamine, 10 mM; others, 50 mM); when necessary, 100 mg cycloheximide mg⁻¹ was also added. One of the flasks received no nitrogen addition ('negative control'). Cell number (direct count) and NR activities were determined at fixed intervals.

NR assays. Extraction buffer (100 mM potassium phosphate buffer, pH 7.4; 20 µM FAD; 1 mM EDTA; 1 mM PMSF) and reaction mixture (see below) were after González & Siverio (1992). Crude extracts were obtained by glass bead vortexing in extraction buffer. Protein concentrations were determined using a Bio-Rad kit (catalogue no. 500-0006; BSA as standard).

NAD(P)H:NR (EC 1.6.6.2) activities of crude extracts were assayed spectrophotometrically (340 nm; Varian 'Cary 3' spectrophotometer) by monitoring the nitrate-dependent NAD(P)H oxidation in reaction mixtures containing crude extract (0.10–0.15 mg protein), 100 mM potassium phosphate buffer, pH 7.4, 1 mM PMSF, 10 mM potassium metabisulphite, 0.2 mM NAD(P)H and 20 mM sodium nitrate. The plots of NAD(P)H absorbance against time were linear for a few minutes. Unless otherwise specified, the assay temperature was 37 °C. The difference between the rate of NAD(P)H oxidation in the presence and absence of nitrate was used to calculate the NR activity. Specific activities are expressed as nmol NAD(P)H oxidized min⁻¹ (mg protein)⁻¹.

Mutant isolation. Chlorate-resistant (Clo^r) mutants were obtained by positive selection on MC plates (no mutagens used; 10^7 M6 cells per plate; 37 °C for 15 d). To isolate Nit⁻ mutants, Clo^r mutants were purified, replica-plated onto MA1, MN and MN3 plates, incubated for 2–4 d and then grouped according to their growth on these media. To isolate NR⁻ mutants, all Nit⁻ mutants that failed to grow on nitrate plates but retained growth on nitrite were cultivated in YPD flasks, washed and inoculated (10^6) in MA2 flasks. After 15 h incubation, the cells were washed, transferred to MN2 flasks for 3 h and then subjected to NR assays.

Crosses and sporulations. These were done onto ME plates, as described by Gleeson & Sudbery (1988).

RESULTS

Assay conditions and electron donor

After optimizing the NR assay conditions in crude extracts of nitrate-grown M6 cells, we were able to obtain a quick and sensitive assay based on the measurement of the initial rate of NAD(P)H oxidation associated with the enzymic nitrate reduction. We would like to point out that our continuous monitoring assay allows one to verify the linearity of NAD(P)H concentration against time, and is therefore very accurate. Kinetic methods to measure NR activity in yeasts are not new: Bruinenberg *et al.* (1983) described a spectrophotometric assay for *Candida utilis*. However, all our attempts to reproduce this assay with *H. polymorpha* strains were unsuccessful. We found that the NR of *H. polymorpha* is bispecific, i.e. uses both NADPH and NADH as electron donors [NAD(P)H:NR; 1.6.6.2]. However, NADPH was more effective than NADH as electron donor: after 3 h cultivation, the NADPH-dependent NR activity is approximately threefold higher than the NADH-dependent activity. Potassium metabisulphite, a NiR inhibitor, was essential. Adding FAD and PMSF (a protease inhibitor) to the extraction buffer resulted in higher activities (approx. twofold), suggesting a stabilizing role of these compounds during extraction and storage. NADPH:NR activities were also studied as a function of the reaction temperature. Maximal activities are observed around 40 °C, which is consistent with the optimal growth temperature of *H. polymorpha* (40–42 °C). In the interval 35–45 °C, the activities are nearly maximal (>95%), whereas at 30 and 50 °C the activities are lower than 85% of maximum. Since NR stability in the extracts may vary with the temperature, these observations are just indicative.

Growth on different nitrogen sources

H. polymorpha was able to grow on media with all the nitrogen sources tested, i.e. ammonium, nitrate, nitrite, glutamate, aspartate, methylamine and hypoxanthine. The exponential growth rates on all the sources were similar, with the exception of nitrite, which appeared to be a poor nitrogen source (data not shown). After pre-cultivation in minimal ammonium medium (MA), significant lag phases were only observed on nitrite (approx. 7 h), nitrate (approx. 1 h) and methylamine (approx. 2 h).

Appearance of the NR activity

Preliminary experiments on cells harvested during the late-exponential phase of growth demonstrated high NR activities on nitrate media, low NR activities on nitrite media and no activities on all other media (data not shown), suggesting that, as in other organisms, the nitrate metabolism of *H. polymorpha* is an inducible system responding mainly to nitrate. Time-course NR measurements of cells transferred to nitrate media after ammonium or glutamate pre-cultivation revealed progressive increases of NR activities after nitrate was made available (Fig. 1a). Increasing the nitrate concentration in the medium led to significant depressions of the induction kinetics and of the maximum NR activity (Fig. 1b). During these experiments, the growth of the cell population was shown to be equal to 0.1 $\Delta\text{OD h}^{-1}$ (25 mM flasks) or 0.05 $\Delta\text{OD h}^{-1}$ (all other flasks). The appearance of NR activity in response to nitrate is suppressed by cycloheximide (Fig. 1b).

To study the effect of nitrate on NR induction, we set up various flask cultures using glutamate, an NR-non-inducing nitrogen source. During the late-exponential phase of growth, when the residual glutamate in the medium was approaching exhaustion, the desired nitrogen sources were added and the NR activities were then measured at fixed intervals. The results, summarized in Fig. 1(a), revealed that nitrate in combination with glutamate or nitrite retains a very weak inducing effect, whereas in combination with ammonium or methylamine it loses its inducing effect. During these experiments, the growth of the cell population was shown to be equal to 0.5 $\Delta\text{OD h}^{-1}$ (ammonium + nitrate and glutamate + nitrate) or 0.4 $\Delta\text{OD h}^{-1}$ (methylamine + nitrate) or 0.02 $\Delta\text{OD h}^{-1}$ (nitrite + nitrate). Similar experiments with no nitrogen additions revealed that nitrogen-starved cells after growth on ammonium or glutamate did not contain measurable NR activities during the time considered (not shown).

Disappearance of the NR activity

We also investigated the effect that various repressing nitrogen sources, alone or in combination with nitrate, exerted over NR activity. *H. polymorpha* was grown in nitrate medium (MN2) until late-exponential phase,

Fig. 1. (a) Effect of the addition of different nitrogen sources on the NADPH:NR activity of crude extracts of *H. polymorpha* M6 pre-grown with 4 mM glutamate as sole nitrogen source. Nitrate (50 mM) + another nitrogen source were added in the late-exponential phase (zero time); ○, 50 mM nitrate; ▽, 50 mM nitrate + 1.9 mM nitrite; △, 50 mM nitrate + 37 mM methylamine; □, 50 mM nitrate + 50 mM ammonium; ◇, 50 mM nitrate + 50 mM glutamate. The experiments were done in triplicate without significant differences. (b) Appearance of NADPH:NR activity in response to the addition of nitrate, alone or with cycloheximide, at different concentrations in crude extracts of *H. polymorpha* M6 pre-grown with 4 mM glutamate as sole nitrogen source. Nitrate was added in the late-exponential phase; □, 25 mM nitrate; ◇, 50 mM nitrate; △, 100 mM nitrate; ▽, 200 mM nitrate; ○, 50 mM nitrate + 100 µg cycloheximide ml⁻¹. The experiments were done in triplicate without significant differences; a similar pattern was obtained with ammonium pre-cultivation.

when the residual nitrate in the medium was approaching exhaustion and the NR activities were around 10 nmol min⁻¹ (mg protein)⁻¹. After adding the various nitrogen compounds, we measured the NR activities at fixed intervals. The results showed that adding nitrate alone leads to significant increases of NR activities (approx. threefold in 6 h), whilst adding nitrite (alone or with nitrate) causes a growth arrest or a long lag phase, probably due to toxic effect, followed by a slow induction of NR up to 15–20 nmol min⁻¹ (mg protein)⁻¹. All other nitrogen sources, even in combination with nitrate, trigger a loss of NR activities. However, in the presence of glutamate + nitrate, this loss is preceded by a small increase of NR activity (approx. twofold in 2 h).

Table 1. Complementation matrix representing 20 Nit^- mutants of *H. polymorpha* crossed in all pairwise combinations

+, Crosses which grow on nitrate medium, indicating complementation; –, crosses which do not grow on nitrate medium, indicating non-complementation. NADPH:NR activities of control strain (M6) and of the 20 Nit^- mutants are also reported (last column).

	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8	CP9	CP10	CP11	CP12	CP13	CP14	CP15	CP16	CP192	CP193	CP221	CP222	Activity*
CP1	–	+	+	–	+	+	+	+	+	+	–	–	–	+	+	+	+	+	–	+	3.5
CP2		–	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	2.0
CP3			–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	3.7
CP4				–	+	+	+	+	+	+	–	–	–	+	+	+	+	+	–	+	ND
CP5					–	+	–	–	+	+	+	+	+	+	–	+	+	+	+	+	ND
CP6						–	+	–	+	+	+	+	+	+	–	+	+	+	+	+	ND
CP7							–	+	+	+	+	+	+	+	+	+	+	+	+	+	ND
CP8								–	+	+	+	+	+	+	–	+	+	+	+	+	ND
CP9									–	+	+	+	+	+	+	+	+	+	+	+	2.8
CP10										–	+	+	+	+	+	+	+	+	+	+	ND
CP11											–	–	–	+	+	+	+	+	–	+	ND
CP12												–	–	+	+	+	+	+	–	+	ND
CP13													–	+	+	+	+	+	–	+	ND
CP14														–	+	+	+	+	+	+	ND
CP15															–	+	+	+	+	+	ND
CP16																–	+	+	+	–	ND
CP192																	–	+	+	+	ND
CP193																		–	+	+	ND
CP221																			–	+	ND
CP222																				–	ND
M6																					27.0

* NADPH:NR activity was determined in crude extracts of these strains grown in ammonium medium for 15 h, washed, transferred to nitrate medium for 3 h and then subjected to NR assays. The results are expressed as nmol NADPH oxidized min^{-1} (mg protein^{-1}); ND, not detected. The experiments were done in triplicate without significant differences.

In general, the observed losses are more rapid with ammonium than with glutamate or methylamine. Interestingly, these losses of activities are also observed if cycloheximide is added. If no nitrogen compounds were added, under the interval of time considered no significant losses of the initial NR activity were observed, unless cycloheximide was added.

Isolation of Clo^r mutants

According to the principles described by Åberg (1947), if NR is expressed on chlorate plates, toxicity phenomena may impede cell growth (see also Cove, 1976). Mutants with reduced NR activities can survive, as long as a suitable nitrogen source is supplied. Therefore, it is crucial that this nitrogen source could allow NR expression. As previously described, nitrite allows measurable NR activities. In preliminary experiments, we established that the optimal nitrite concentration for growth on plates of *H. polymorpha* M6 is 1.8 mM. On nitrite medium with 0.3–0.8 M chlorate, *H. polymorpha* M6 showed no growth (chlorate-sensitive; Clo^s). Therefore, we selected Clo^r mutants on this medium. After 15 d incubation, several dozen colonies were obtained on each plate, their numbers being negatively correlated to the chlorate concentration. We purified many colonies onto YPD plates and re-tested them on chlorate medium to verify whether the acquisition of the Clo^r trait was permanent. All colonies tested retained this trait after YPD cultivation. Two thousand Clo^r colonies were identified and stored for further analyses.

Identification of NR activity mutants

Two hundred of the Clo^r mutants were tested for growth on MA, MN1 and MN3 and 180 mutants were identified with $\text{Nit}^- \text{Nii}^+$ (nitrite)⁺ phenotype. This phenotype suggested that, as expected from the selection procedure (chlorate plates with nitrite as sole nitrogen source), in these mutants only the first step of nitrate assimilation (nitrate reduction) is impaired. We tested the NR activity of 20 of these mutants after 3 h in nitrate medium and found strongly decreased NR activities in all of them (NR^- phenotype). Most of these mutants have no NR activity, and the others show 10–100-fold reductions (Table 1). These data indicated that most, if not all, the $\text{Nit}^- \text{Nii}^+$ mutants have structural or regulatory lesions which lead to reduction of NR activities.

Genetic analyses

Each of the 20 NR^- mutants (*met6*, Nit^-) was crossed with strain L1 (*leu1*, Nit^+) and diploids were selected onto MA plates. All resulting diploids showed chlorate sensitivity (Clo^s) on MC plates, as well as good growth on nitrate medium (Nit^+). They were sporulated and random analyses of approximately 200 spore populations revealed a 1:1 ratio of Nit^+ and Nit^- phenotypes from each population. Twenty-five Nit^- spore products from each population were further examined. They revealed that in all cases the Clo^r , Nit^- and NR^- phenotypes co-segregated. Taken together, these data indicated that the phenotype of each of the 20 mutants

Table 2. Complementation matrix representing 41 Nit⁻ mutants of *H. polymorpha* crossed with 10 'tester' mutants in all pairwise combinations

+, Crosses which grow on nitrate medium, indicating complementation; —, crosses which do not grow on nitrate medium, indicating non-complementation.

Nit ⁻ mutant	Tester mutant									
	CP1	CP2	CP6	CP7	CP192	CP193	CP3	CP9	CP10	CP14
CP101	—	+	+	+	+	+	+	+	+	+
CP103	+	+	+	—	+	+	+	+	+	+
CP104	—	+	+	+	+	+	+	+	+	+
CP109	—	+	+	+	+	+	+	+	+	+
CP110	+	+	—	+	+	+	+	+	+	+
CP111	+	+	—	+	+	+	+	+	+	+
CP112	+	+	+	—	+	+	+	+	+	+
CP138	—	+	+	+	+	+	+	+	+	+
CP141	+	+	+	—	+	+	+	+	+	+
CP143	+	+	+	—	+	+	+	+	+	+
CP145	—	+	+	+	+	+	+	+	+	+
CP158	—	+	+	+	+	+	+	+	+	+
CP160	—	+	+	+	+	+	+	+	+	+
CP167	+	+	+	+	—	+	+	+	+	+
CP168	+	+	+	+	—	+	+	+	+	+
CP171	+	+	+	+	—	+	+	+	+	+
CP172	+	+	+	+	—	+	+	+	+	+
CP185	+	—	+	+	+	+	+	+	+	+
CP189	+	+	+	—	+	+	+	+	+	+
CP190	+	—	+	+	+	+	+	+	+	+
CP194	+	+	—	+	+	+	+	+	+	+
CP195	+	+	+	—	+	+	+	+	+	+
CP196	+	—	+	+	+	+	+	+	+	+
CP205	+	+	+	—	+	+	+	+	+	+
CP206	+	+	—	+	+	+	+	+	+	+
CP207	—	+	+	+	+	+	+	+	+	+
CP208	—	+	+	+	+	+	+	+	+	+
CP209	+	+	+	—	+	+	+	+	+	+
CP210	+	+	+	—	+	+	+	+	+	+
CP231	+	+	+	—	+	+	+	+	+	+
CP232	—	+	+	+	+	+	+	+	+	+
CP235	—	+	+	+	+	+	+	+	+	+
CP236	+	+	+	—	+	+	+	+	+	+
CP237	+	+	+	+	—	+	+	+	+	+
CP238	+	+	+	—	+	+	+	+	+	+
CP239	+	+	+	—	+	+	+	+	+	+
CP247	—	+	+	+	+	+	+	+	+	+
CP248	+	+	+	—	+	+	+	+	+	+
CP250	+	+	+	—	+	+	+	+	+	+
CP251	+	+	—	+	+	+	+	+	+	+
CP252	+	+	+	—	+	+	+	+	+	+

was due to a single recessive mutation inherited in a Mendelian fashion.

Each of the 20 mutants was subjected to complementation analysis. Firstly, a set of *leu1*-marked derivatives of each Nit⁻ mutant was obtained. Secondly, each mutant was crossed with each *leu1* derivative and prototrophic diploids were selected onto minimal me-

dium. Thirdly, diploid colonies were tested for complementation of Nit⁻ mutations by their ability to grow on nitrate medium. This complementation analysis showed that the 20 mutations under study represented 10 complementation groups (Table 1). Finally, one 'tester' for each group was crossed with each of 41 additional Nit⁻ mutants. The analysis of these crosses (Table 2) reveals that each mutant can be ascribed to one of the

Table 3. Group distribution (1–10) of the 61 Nit[−] mutants analysed in this study

1	2	3	4	5	6	7	8	9	10
CP1	CP2	CP6	CP7	CP192	CP193	CP3	CP9	CP10	CP14
CP4	CP16	CP5	CP103	CP167					
CP11	CP222	CP8	CP112	CP168					
CP12	CP185	CP15	CP141	CP171					
CP13	CP190	CP110	CP143	CP172					
CP221	CP196	CP111	CP189	CP237					
CP101		CP194	CP195						
CP104		CP206	CP205						
CP109		CP251	CP209						
CP138			CP210						
CP145			CP231						
CP158			CP236						
CP160			CP238						
CP207			CP239						
CP208			CP248						
CP232			CP250						
CP235			CP252						
CP247									

previously defined groups. Table 3 summarizes the group distribution of the 61 Nit[−] mutants analysed.

Mutant growth on different nitrogen sources

The growth capabilities of the Nit[−] mutants on different nitrogen sources (glutamate, aspartate, methylamine, hypoxanthine) were assayed by streaking one tester for each group on appropriate plates and incubating at 37 °C for 5 d. The results show that on all nitrogen sources, the testers grow as well as the control strain, indicating that the mutations under study affect specifically the nitrate pathway. All testers were also incubated on nitrate medium with the addition of sodium molybdate, but growth was never observed.

DISCUSSION

In *H. polymorpha*, NR activity is induced by nitrate, as long as ammonium or other reduced nitrogen sources are absent. These nitrogen sources repress NR activity. Loss of NR activity from nitrate-grown cells is stimulated by ammonium or other reduced nitrogen sources, whether or not nitrate is present. Although the dynamics of repression are different for the different compounds, NR activity is never induced in the absence of nitrate. Taken together, these results indicate that in *H. polymorpha* the synthesis of NR is subjected to dual control: repression mechanisms elicited by ammonium (and other reduced nitrogen compounds) and induction phenomena stimulated by nitrate (and to a lesser extent by nitrite). These data mirror the RNA blot analysis of the *H. polymorpha* YNR1 gene (Avila *et al.*, 1995). The repressing stimuli overcome the inducing stimuli, i.e. when presented with nitrate and ammonium *H.*

polymorpha utilizes ammonium in preference to nitrate. These indications suggest that in this respect *H. polymorpha* NCYC 495 is similar to *Aspergillus nidulans* (Cove, 1979), *Neurospora crassa* (Garrett & Amy, 1978), *Hansenula wingei* ATCC 28162 (Jones *et al.*, 1987), *Sporobolomyces roseus* (Ali & Hipkin, 1985) and *H. polymorpha* CBS 4732 (González & Siverio, 1992) but differs from *H. wingei* CBS 2432 (González & Siverio, 1992), *Candida nitratophila* (Cannons *et al.*, 1986) and *Hansenula anomala* (González & Siverio, 1992) since in these yeasts ammonium + nitrate cultures result in measurable NR activities (e.g. in *C. nitratophila*, 25 % of the fully induced activity of nitrate-grown cells; Cannons *et al.*, 1986) and in the concomitant utilization of the two nitrogen sources. It is interesting to note that increases but not decreases in NR activity were dependent on protein synthesis. These observations can be explained by assuming the co-existence of two modes of control. A long-term, coarse mechanism, dependent on protein synthesis, could regulate the synthesis of NR in response to the nitrogen source (interestingly, in two other *Hansenula* species, the appearance and disappearance of NR correlates well with the syntheses and losses of NR protein; González & Siverio, 1992). This mechanism is likely to act at the transcriptional level, as indicated by the fact that the NR-coding gene of *H. polymorpha* is transcriptionally regulated (Avila *et al.*, 1995). A short-term, fine mechanism, independent of protein synthesis, could account for the observed NR activity losses in the presence of cycloheximide. This mechanism could rapidly inhibit nitrate assimilation in response to the addition of ammonium by acting early in the pathway, i.e. at the uptake and/or NR level. Although the nature of this action is obscure, reversible or irreversible inactivation

of NR is a likely mechanism; it is noteworthy that *in vivo* inactivation of NR in another *Hansenula* species has been reported (Minagawa & Yoshimoto, 1984).

H. polymorpha differs from *S. roseus* and *C. nitratophila* in that removal of ammonium from ammonium-grown cultures does not bring about 'gratuitous' NR activities (Ali & Hipkin, 1985; Cannons *et al.*, 1986). Thus in this yeast, NR activity does not seem to be strongly regulated by derepression. However, following growth on nitrate, NR activity is present in nitrogen-starved cells and may be sustained for at least 6 h in the absence of nitrate, as long as protein synthesis is active. Thus cycloheximide seems to inhibit the synthesis of a system involved in the NR inactivation (or perhaps in weak NR derepression). If protein synthesis is inhibited by cycloheximide, the presence of nitrate results in the maintenance of the existing NR levels, suggesting that nitrate plays a role in protecting NR from the supposed inactivation mechanisms.

Mutants defective in NR activity were isolated following chlorate selection procedures. We observed that wild-type *H. polymorpha* is sensitive to high levels of potassium chlorate (0.3 M and higher) on medium allowing NR expression. After optimizing the procedure, we were able to isolate Clo^r mutants, many of which were also Nit⁻. This fact indicated that these mutants could be isolated with a simple positive selection procedure. During this study, we confined the genetic dissection of the nitrate metabolism to the NR activity, and therefore concentrated on 61 Nit⁻ mutants. These mutants, shown to be due to a mutation in a single recessive gene, were grouped in 10 complementation classes, revealing a complex set of genes involved in the NR activity of *H. polymorpha*. The high number of classes defined indicates that the procedure adopted is very effective in identifying the diverse genes of this set. It is likely that both structural and regulatory lesions are present in our collection. By analogy with other organisms, it is possible to speculate that, apart from the NR structural gene (*YNR1*; Avila *et al.*, 1995), one or more nitrate transporters can be mutated. In this case, we must assume that lack of uptake would impede NR induction (it is noteworthy that recently the *YNT1* gene encoding a nitrate transporter of *H. polymorpha* has been cloned and its disruption has been shown to cause inability to grow in nitrate and, to a certain extent, reductions in NR activity; Pérez *et al.*, 1997). Additionally, the Mo prosthetic group biosynthetic genes as well as regulatory elements, if mutated, may lead to the NR⁻ phenotype. Some of these can be NR-specific; others can be involved in the regulation of more than one pathway. To further our understanding of these mutants, we tested them on a number of nitrogen sources and found that none of the mutants show reduced capabilities of utilization of diverse nitrogen sources alternative to ammonium. Thus it is plausible that in these mutants, lesions have occurred to genes playing major roles in the regulation of nitrate metabolism but not in the regulation of other nitrogen utilization pathways. Furthermore, none of the mutants

tested showed reduced growth on hypoxanthine medium and in none of these mutants was it possible to rescue the Nit⁻ phenotype in media with high Mo concentration, thus excluding a possible role of these genes in the biosynthetic pathway leading to the molybdopterin prosthetic group, since this prosthetic group is shared with hypoxanthine-xanthine dehydrogenase, involved in hypoxanthine utilization.

Our large collection of *H. polymorpha* Nit⁻ mutants (including some with altered NiR activity not described in this report), together with the availability of efficient transformation systems and replicating vectors for this yeast, will allow the cloning of *H. polymorpha* NIT genes by functional complementation. The predicted amino acid sequences of NIT products, along with biochemical and physiological information obtained from the mutants, should provide useful insight into the role of specific NIT products in nitrate assimilation. Gene isolation experiments aimed at identifying these structural and regulatory products are under way, together with genetic work aimed at characterizing more Nit⁻ mutants.

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