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Hansenula polymorpha NMR2 and *NMR4*, two new *loci* involved in nitrogen metabolite repression

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Abstract

In the yeast *Hansenula polymorpha* (*Pichia angusta*) nitrate assimilation is tightly regulated and subject to a dual control: nitrogen metabolite repression (NMR), triggered by reduced nitrogen compounds, and induction, elicited by nitrate itself. In a previous paper [Serrani, F., Rossi, B. and Berardi, E (2001) Nitrogen metabolite repression in *Hansenula polymorpha*: the *nmrl-l* mutation. Curr. Genet. 40, 243–250], we identified five *loci* (*NMR1-NMR5*) involved in NMR, and characterised one of them (*NMR1*), which likely identifies a regulatory factor. Here, we describe two more mutants, namely *nmr2-1* and *nmr4-1*. The first one possibly identifies a regulatory factor involved in nitrogen metabolite repression by various nitrogen sources alternative to ammonium. The second one, apparently involved in ammonium assimilation, probably has sensor functions.

Keywords: Nitrate assimilation; Nitrogen metabolite repression; *NMR* genes; *Hansenula polymorpha*

1. Introduction

Sensing and responding to a continuously changing environment is crucial for the survival of microorganisms. To access adequate nutrient supply, cells have evolved sophisticated signal transduction pathways that sense different environmental factors, such as nitrogen compounds. The targets of these transduction pathways are often transcriptional factors that mediate the tuning of gene expression, in order to maximise organisms' fitness in a given environment [1].

Nitrogen is one of the main elements of all living organisms, and most prokaryotic and eukaryotic microorganisms are capable of using a wide variety of nitrogen compounds. Accordingly, they have evolved a complex of controlling mechanisms to maintain an adequate level of nitrogen in the cells and to ensure the preferential use of certain nitrogen compounds, such as ammonium, glutamine and glutamate (i.e., primary nitrogen sources), over certain others, such as nitrate, nitrite, amides, purines and most amino acids (i.e., secondary nitrogen sources). When primary nitrogen sources are not available at adequate levels, one or more secondary sources can be used by a highly regulated process that often requires the transcriptional induction of structural genes followed by de novo synthesis of pathway-specific catabolic enzymes and permeases.

In most of the nitrate-utilising organisms, nitrate assimilation requires two different signals: the first one is a global signal indicating the absence of preferred nitrogen sources; the second one is a pathway-specific signal indicating the presence of nitrate itself [2, and references therein].

The regulation of nitrate assimilation is well-characterised in filamentous fungi such as *Aspergillus nidulans* and *Neurospora crassa*. In these organisms, nitrate

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induction is mediated by pathway-specific positive regulators: NirA in *A nidulans* and NIT4 in *N. crassa* [3–10]. Ammonium repression involves more complex mechanisms acting upon the activation of various pathways required for the utilisation of secondary nitrogen sources. The DNA-binding global activators AreA of *A. nidulans* and NIT2 of *N. crassa* [11–31] are zinc-finger transcription factors of the GATA-type, playing a central role in such mechanisms. In addition, the negatively acting regulators NmrA and NMR1 bind to AreA and NIT2 to help modulation of their activities and nitrogen metabolite repression [32–39]. As yet, the nature of the signalling molecules defining the nitrogen state of the cell with respect to nitrogen metabolism is an open question. It is traditionally believed that, in *A. nidulans* and *N. crassa*, the intracellular glutamine concentration is crucial for this definition [40,2]. Recent evidence, however, suggests that, in addition to a glutamine-dependent signal, a second signalling mechanism exists that responds to intracellular glutamate concentration [41–43,1]. The identity of the receptor(s) that sense the nitrogen state is also unknown. In *Saccharomyces cerevisiae*, Mep2p, one of the three known ammonium transporters [44], is required for low-ammonium sensing [45]. Although an amino-acid permease, Ssy1p [46] and a tRNA ($Gln\text{-}tRNA^{CUG}$; [47]), appears to be implicated in amino-acid sensing, their involvement in general nitrogen metabolism has never been demonstrated [48]. So far, in *S. cerevisiae* the TOR cascade is the only signalling mechanism acting on nitrogen metabolism [49–52, for review].

The methylotrophic yeast *Hansenula polymorpha* (syn. *Pichia angusta*) is able to use various compounds as nitrogen source, including ammonium, methylamine, glutamate and glutamine, nitrate and nitrite. *H. polymorpha* nitrate assimilation is subject to a dual control: nitrogen metabolite repression, triggered by reduced nitrogen compounds (e.g., ammonia, glutamine and glutamate) and specific induction mechanisms, elicited by nitrate itself [53,1]. In this yeast, nitrate assimilation is similar to that in other organisms. Nitrate enters the cell through a high-affinity nitrate transporter (*YNT1*; [54]), and is then converted to ammonia via two successive reductive steps catalysed by nitrate reductase (*YNR1*; [55]) and nitrite reductase (*YNI1*; [56]). The aforementioned genes are closely clustered with two transcriptional activator genes, namely *YNA1* and *YNA2*, belonging to the fungal C6 zinc-cluster family [57–60]. The DNA-binding domain of this family is a $Zn(II)_2Cys_6$ binuclear cluster, shared – among others – by the well-known *S. cerevisiae* transcriptional activator Gal4p [61–65], and by the well-characterised *A. nidulans* NirA and *N. crassa* NIT4, specifically involved in nitrate metabolism [3,4,10].

In *H. polymorpha*, mutations in the *NMR1* locus bring about de-repression of nitrate assimilation in the presence of glutamate, but not of glutamine [1], suggesting that a glutamine-depending signalling circuit may coexist with a glutamate-depending one.

To further investigate the mechanisms controlling nitrogen metabolite repression in *H. polymorpha*, here we describe two other mutants (*nmr2-1, nmr4-1*) defective in NMR [1]. Our data indicate that, whereas *NMR2* is likely to be a regulatory gene, possibly encoding a member of the glutamine-dependent signalling mechanism, *NMR4* appears to be more directly involved in ammonium assimilation and sensing.

2. Materials and methods

2.1. Strains

All strains used in this work were derivatives of *H. polymorpha* homothallic haploid NCYC 495. The NCYC 495 derivative strain *leu1-1* (L1; [66]) was used as a control strain throughout this work. MV13-23 (*leu1-1 mam2-1*) is an L1 derivative mutant strain impaired in methylamine utilisation (Mam^-), with wild-type methylamine repression (Mre^+) and ammonium repression (Are^+; [1]). This strain was used to isolate the Mre^-, Are^- mutants used in this work and listed below. The mutants FM-32 (*leu1-1 mam2-1 nmr1-1*), FM-49 (*leu1-1 mam2-1 nmr2-1*) and FM-101 (*leu1-1 mam2-1 nmr4-1*) are three of the Are^- Mre^- mutants isolated in our laboratory [1]. FM-32B3 (*leu1-1 nmr1-1*), FM-49A1 (*leu1-1 nmr2-1*) and FM-101B (*leu1-1 nmr4-1*) are three *MAM2* ($methylamine^+$) segregants deriving from mutants FM-32, FM-49 and FM-101. L1 (*leu1-1*) and A11 (*ade11-1*; [66]) are also Are^+ strains used to obtain all *NMR/nmr* diploids.

The *Escherichia coli* strain MC1061 [*hsdR mcrB araD139D (araABC-leu) 7679 dlacX74 galU galK rpsL thi*] was used for routine plasmid preparation.

2.2. Media

YPD contained 1% yeast extract, 2% peptone and 2% glucose. Minimal media contained 2% glucose, 0.2% yeast nitrogen base without amino acids and ammonium sulphate (Difco, Detroit, MI, USA) and, as appropriate, leucine 0.006% or 0.004% adenine was added. Moreover various nitrogen sources were added: MA1 plates contained 38 mM ammonium sulphate, MA2 plates 50 mM ammonium chloride, MLA plates 100 μM ammonium chloride, MMa plates 37 mM methylamine, MN2 plates 50 mM sodium nitrate, MG plates 50 mM glutamine, MGlu plates 50 mM glutamate. When nitrate was used in combination with another nitrogen compound in flask, both concentrations were 50 mM, with the exception of urea (10 mM) and arginine (12.5 mM). If required, aspartate-β-hydroxamate (0.96 mM) was added to MA2, MG and MGlu

plates. When necessary, 1.6% agar was added. ME containing 2% malt extract was used to perform mating and sporulation.

2.3. Transformants

H. polymorpha FM-49A1 and FM-101B were transformed by the LiAc method of Berardi and Thomas [67]. The integrative vector pGP β-1 was provided by J. Siverio. It harbours the promoter region of *YNR1* fused to the reporter gene *LacZ*, and the *LEU2* gene of *H. polymorpha* as selectable marker [68].

2.4. Growth tests

Growth tests were performed in MA2, MN2, MLA, MG and Mglu media. Plate growth of FM-32B3 (*nmr1-1*), FM-49A1 (*nmr2-1*) and FM-101B (*nmr4-1*) was evaluated in comparison with a control strain by spot assay. Strain suspensions in water were normalised with respect to cell density (2×10^8 cell ml^{-1}), and equal volumes of cell suspensions (4 μl of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions) were spotted onto the plates and incubated at 37 °C for 3 d (5 d for MLA). Growth of L1, FM-32B3, FM-49A1 and FM-101B in liquid media was evaluated by OD_{600} measures. Flasks were incubated at 37 °C and shaken at 220 rpm. Growth was followed for 25 or 35 h.

2.5. Cultures

All cultures were grown in 250-ml flasks with 50 ml medium, using an orbital incubator at 37 °C and 200 rpm. After 15 h pre-cultivation in YPD, the cells were washed with sterile water and inoculated (10^6 cells ml^{-1}) in a minimal medium with the appropriate nitrogen source (see Tables 1–5). After 15 h cultivation (late-exponential phase) enzyme activities were assayed.

2.6. Biochemical methods

Crude extracts were obtained by glass bead vortexing (three times, 30 s each time; 1 vol. Sigma No. G-9268 beads: 1 vol. cells) in suitable extraction buffer [1,54,69]. Protein concentration of crude extract was determined using a Bio-Rad (Hercules, CA, USA) kit (Ref. 500-0006; with bovine serum albumin as standard). NAD(P)H:NR (EC 1.6.6.2), amine oxidase (Amo, EC 1.4.3.4), β-galactosidase, arginase (EC 3.5.3.1) and glutamine synthetase (EC 6.3.1.2) activities were all assayed spectrophotometrically as already specified [1,54]. NADPH:GDH (EC 1.6.6.2) activity was assayed as described [69].

2.7. Toxic analogues

Growth of FM-32B3 (*nmr1-1*), FM-49A1 (*nmr2-1*) and FM-101B (*nmr4-1*) on aspartate-β-hydroxamate (a

Table 1
NADPH:nitrate reductase (NR) activity of *H. polymorpha* mutants FM32-B3 (*nmr1-1*), FM-49A1 (*nmr2-1*), FM-101B (*nmr4-1*) and control strain L1 grown to late-exponential phase in minimal medium containing nitrate plus a second nitrogen source

Second nitrogen source	Serrani et al. [1]		This work	
	L1	FM-32B3 (*nmr1-1*)	FM-49A1 (*nmr2-1*)	FM-101B (*nmr4-1*)
None	20.8 ± 4.3	20.8 ± 1.6	35.0 ± 2.1	17.5 ± 6.7
50 mM Ammonium	ND	22.6 ± 2.2	18.7 ± 0.6	12.5 ± 0.4
50 mM Methylamine	0.6 ± 0.3	10.7 ± 1.6	28.1 ± 4.9	11.3 ± 0.4
50 mM Urea	ND	16.2 ± 0.3	24.2 ± 1.7	ND
50 mM Glutamine	ND	ND	16.0 ± 3.5	ND
50 mM Glutamate	ND	8.3 ± 1.2	37.6 ± 7.4	ND
50 mM Asparagine	4.0 ± 3.0	2.5 ± 1.8	11.0 ± 1.9	4.0 ± 0.7
50 mM Aspartate	ND	10.5 ± 0.4	27.0 ± 3.9	12.0 ± 0.0
50 mM Arginine	4.7 ± 0.0	3.8 ± 0.2	27.9 ± 0.6	0.5 ± 0.4

Data are mean values (±standard error) of three separate experiments and are expressed as nmol NADPH oxidised min^{-1} (mg protein)$^{-1}$. ND, not detectable.

Table 2
Glutamine synthetase activity of *H. polymorpha* mutants FM32-B3 (*nmr1-1*), FM-49A1 (*nmr2-1*), FM-101B (*nmr4-1*) and control strain L1 grown to late-exponential phase in minimal medium containing ammonium or glutamine

Nitrogen source	Serrani et al., [1]		This work	
	L1	FM-32B3 (*nmr1-1*)	FM-49A1 (*nmr2-1*)	FM-101B (*nmr4-1*)
Ammonium	143 ± 26	417 ± 40	251 ± 34	115 ± 12
Glutamine	75 ± 12	77 ± 16	103 ± 17	85 ± 19

Data are mean values (±standard error) of three separate experiments and are expressed as nmol L-glutamate γ-monohydroxamate min^{-1} (mg protein)$^{-1}$.

Table 3
NADPH:glutamate dehydrogenase activity of *H. polymorpha* mutants FM32-B3 (*nmr1-1*) FM-49A1 (*nmr2-1*), FM-101B (*nmr4-1*) and control L1 grown to late exponential phase in minimal medium containing ammonium, glutamate or nitrate

Nitrogen source	This work			
	L1	FM-32B3 (*nmr1-1*)	FM-49A1 (*nmr2-1*)	FM-101B (*nmr4-1*)
50 mM Ammonium	490 ± 92	696 ± 291	1527 ± 454	637 ± 27
50 mM Glutamate	120 ± 44	744 ± 51	1876 ± 337	42 ± 1
50 mM Nitrate	2957 ± 718	4500 ± 767	3776 ± 900	2761 ± 511

Data are mean values (±standard error) of three separate experiments and are expressed as nmol NADPH oxidised min^{-1} (mg protein)$^{-1}$.

Table 4
Amine oxidase activity of *H. polymorpha* mutants FM-32B3 (*nmr1-1*), FM-49A1 (*nmr2-1*), FM-101B (*nmr4-1*) and control strain L1 grown to late-exponential phase in minimal medium containing different nitrogen sources

Nitrogen source	This work			
	L1	FM-32B3 (*nmr1-1*)	FM-49A1 (*nmr2-1*)	FM-101B (*nmr4-1*)
Methylamine	9.4 ± 2.4	6.1 ± 0.9	8.3 ± 2.3	11.5 ± 1.1
Methylamine + ammonium	ND	1.5 ± 0.1	ND	ND
Ammonium	ND	ND	ND	ND

Data are mean values (±standard error) of three separate experiments and are expressed as μmol oxidised 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonate) min^{-1} (mg protein)$^{-1}$. ND, not detectable.

Table 5
Arginase activity of *H. polymorpha* mutants FM-32B3 (*nmr1-1*), FM-49A1 (*nmr2-1*), FM-101B (*nmr4-1*) and control strain L1 grown to late-exponential phase in minimal medium containing different nitrogen sources

Nitrogen source	Serrani et al. [1]		This work	
	L1	FM-32B3 (*nmr1-1*)	FM-49A1 (*nmr2-1*)	FM-101B (*nmr4-1*)
Arginine	2.4 ± 0.1	2.1 ± 0.6	3.7 ± 0.6	4.1 ± 0.4
Arginine + ammonium	0.3 ± 0.1	1.5 ± 0.2	4.1 ± 0.2	1.1 ± 0.1
Ammonium	ND	0.1 ± 0.1	ND	0.2 ± 0.1

Data are mean values (±standard error) of three separate experiments and are expressed as μmol urea min^{-1} (mg protein)$^{-1}$. ND, not detectable.

toxic analogue of asparagine; 0.96 mM) plates was evaluated in comparison with a control strain by spot assay followed by 2- or 3-day incubation at 37 °C.

3. Results and discussion

3.1. Genetic characterisation of two nitrogen metabolite repression mutants

H. polymorpha mutants FM-49 (*leu1-1 mam2-1 nmr2-1*) and FM-101 (*leu1-1 mam2-1 nmr2-1*) were obtained in our laboratory as described in [1]. These mutants harbour the recessive pleiotropic mutations *nmr2-1* and *nmr4-1*, respectively, associated with the distinctive phenotype denoted Are$^-$ (*A*mmonium *Re*pression, i.e., NR activities in medium containing ammonium plus nitrate). They also show chlorate sensitivity (Clo^s) on ammonium-plus-nitrate plates and, since they derive from a methylamine$^-$ (Mam$^-$) strain (MV13-23; *mam2-1*, [1]), they are not able to grow on methylamine as sole nitrogen source. Genetic analyses have indicated that the Mam$^-$ trait of our mutants could be segregated independently of the Are$^-$ trait and that such phenotype is due to a single recessive mutation inherited in a Mendelian fashion [1]. Both, FM-49 and FM-101 were crossed sequentially with A11 (*ade11*) and L1 (*leu1*) to obtain diploids and then second-generation segregants. Two of them, denoted FM-49A1 (*leu1-1 nmr2-1*) and FM-101B (*leu1-1 nmr4-1*) have been used throughout. After crossing them with A11, diploids were isolated and sporulated. Random analyses of 250 spores from each diploid confirmed a 1:1 ratio of Are$^+$, Are$^-$ phenotypes, establishing that the deregulated Are$^-$ phenotype of FM-49A1 and FM-101B is due to single mutations (*nmr2-1* and *nmr4-1*, respectively).

3.2. NR activity in the presence of reduced nitrogen compounds

Preliminary experiments on late-exponential cells grown on nitrate demonstrated that, whereas FM-101B NR activities are comparable with the control strain, FM-49A1 NR activities are clearly higher than the control ones (not shown). Upon cultivation in media containing nitrate plus a second nitrogen source some

differences between these mutants and the control emerged. Table 1 shows that in *nmr2-1*, nitrogen metabolite repression (NMR) of NR activity is damaged in all tested conditions. Indeed, in nitrate-containing media additioned with either ammonium or methylamine, glutamate, glutamine, arginine, asparagine, aspartate or urea, FM-49A1 (*nmr2-1*) exhibits NR activities comparable with or higher than those revealed in fully induced conditions (growth on nitrate). In the same conditions, the control strain L1 never showed any NR activity.

Mutant FM-101B (*nmr4-1*) acted differently. Table 1 shows that *nmr4-1* releases NMR of NR activity only when the reduced nitrogen source is ammonium or methylamine. On aspartate plus nitrate medium, only a slight de-repression is observed.

These data suggest that *NMR2* and *NMR4* are involved at different levels of the NMR regulative circuit. The first one could act as a general transcription factor, similarly to *A. nidulans* AreA, *N. crassa* NIT2 or *S. cerevisiae* Gln3p. In this case, according to the de-repressing phenotype of *nmr2-1* mutation, the mutant should also be defective in repression of other ammonium-repressing activities as in the case of certain mutant *areA*^{d} alleles [70–72]. Nevertheless, this kind of mutation should have a semi-dominant phenotype, but it is not the case of *nmr2-1*. Thus, it seems more reasonable to hypothesise that *NMR2* has a role similar to the one of the negatively acting proteins *A. nidulans* NmrA, *N. crassa* NMR1 or their orthologue *S. cerevisiae* Ure2p, which inhibit AreA, NIT2 and Gln3p, respectively [32–39,73–76]. Mutation in proteins involved in TOR signalling cascade acting on nitrogen metabolism could also be argued [52, and references therein]. Even in these cases defects in repression of other ammonium repression activities are possible. The second mutant is more likely to act as a sensor also participating in ammonium assimilation. These assumptions are reinforced by the results described in the following sections.

3.3. Heterologous β-galactosidase activities

In order to investigate whether the alterations characterising mutants FM-49A1 (*nmr2-1*) and FM-101B (*nmr4-1*) acted over the *YNR1* promoter, we transformed these mutants, and a control strain, with an integrative vector harbouring the *YNR1* promoter fused to a reporter gene (*E. coli LacZ*; [68]). The expression of β-galactosidase activities of those transformants grown with different nitrogen sources mirrors the NR activities previously determined (not shown), i.e., null or low activities on ammonium and methylamine, and increments of activities in media containing both nitrate and one of those reduced nitrogen compounds. These results seem to exclude allelism of *nmr2-1* and *nmr4-1* with *YNR1*.

3.4. Growth on different nitrogen sources

The ability of mutants FM-49A1 and FM-101B to grow on various nitrogen sources was tested. Mutant FM-49A1 (*nmr2-1*) had slow growth on all tested nitrogen sources (Fig. 1). Interestingly, FM-101B (*nmr4-1*) showed a significantly reduced growth rate only on media containing ammonium, but on media with glutamine or glutamate was able to grow as well as the control strain (Fig. 1). Moreover, the growth of mutant FM-101B on medium containing a limited concentration of ammonium was quite similar to the growth on a normal concentration of this ion. These data suggest a possible role of *NMR4* in ammonium sensing and/or transport mechanisms. Involvement of *nmr4-1* in nitrogen metabolite repression could act through two possible ways: if *NMR4* functions as an ammonium transporter (or as a positive regulator of it), its mis-function could cause the lowering of the intracellular ammonium concentration below the repressing threshold. Consequentially, it could determine the release of the repression of secondary nitrogen source assimilation [70,71,77]. In the case in which *NMR4* acts as a sensor of external ammonium concentration, mutations in this *locus* could simulate low-ammonium condition and, again, lead to release of repression and to assimilation of secondary nitrogen sources.

3.5. Further characterisation

All the phenotypic studies presented so far cannot exclude that the Nmr^{-} phenotype of our mutants, and especially of *nmr4-1*, is due to leaky mutations in the glutamine synthetase (GS) or in the glutamate dehydrogenase (NADPH-GDH) gene. Indeed, glutamine and/or glutamate are thought the be crucial molecules for signalling the nitrogen nutritional status; in addition, Are^{-} mutants, impaired on GS activity or NADPH-GDH activity, have been described in *A. nidulans* and in *N. crassa.* While showing a leaky weak growth on ammonium plates, these mutants exhibit wild-type phenotype on media containing glutamine or glutamate as nitrogen source [78–86]. In order to test this possibility we analysed the GS and the NADPH-GDH activities of our mutants on different nitrogen sources. As illustrated in Tables 2 and 3, our experiments led us to rule out this possibility, since the GS and the NADPH-GDH activities of both mutants were comparable or even higher than those found in the control strain. Interestingly, *nmr2-1* was characterised by high GS and NADPH-GDH activities in all tested conditions. These results suggest once more a regulatory role for *NMR2.* This gene seems to affect nitrogen metabolism at different levels, as found in some *ure2* mutations in *S. cerevisiae* [75,76,86, for review].

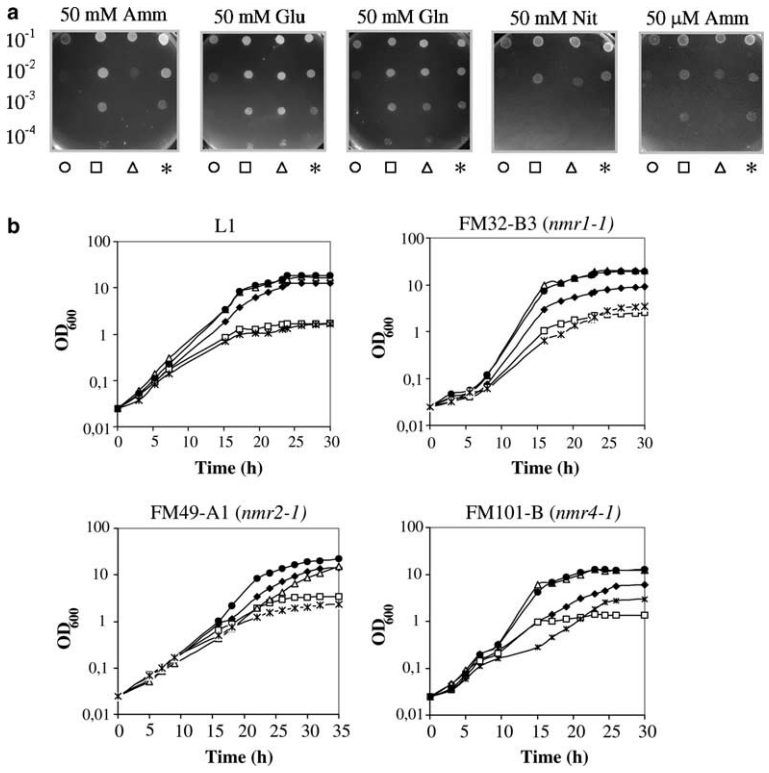

Fig. 1. Growth tests of *H. polymorpha nmr* mutants and control on various nitrogen sources: (Panel a) spot assays, as described in Section 2. (□) F32-B3 (*nmr1-1*), (○) FM49-A1 (*nmr2-1*); (△) FM101-B (*nmr4-1*); (✻) L1 (control); Amm, ammonium; Glu, glutamate; Gln, glutamine; Nit, nitrate. (Panel b) Growth in liquid, as described in Section 2. Cells were pre-grown in YPD, washed and incubated (37 °C, 200 rpm) into 250 ml-flasks containing 50 ml–medium with the following nitrogen sources: (△) 50 mM glutamine; (●) 50 mM glutamate; (◆) 50 mM ammonium; (□) 100 μM ammonium; (✻) 50 mM nitrate. Cell density was monitored spectrophotometrically (600 nm) until stationary phase was reached (30–35 h). The *y*-axis has a logarithmic scale.

3.6. Other ammonium-repressed activities

The FM-49A1 and FM-101B phenotype clearly suggests different but evident changes in repressing mechanisms of nitrate assimilation. In particular, our data indicate that *NMR2* is part of a regulative circuit whereas *NMR4* seems to take part in ammonium assimilation and sensing. Do *nmr2-1* and *nmr4-1* affect also other ammonium-repressible assimilating enzymes, as in the case of *nmr1-1* [1]? Methylamine oxidase plays a central role in alkylated amine utilisation; it is induced by methylamine and repressed in the presence of ammonium [87,88]. Our results revealed that neither *NMR2* nor *NMR4* are involved in the repression of this gene (Table 4), again suggesting the seemingly different role of *NMR1*, *NMR2* and *NMR4* in NMR.

We then tested two other activities that in wild-type are subject to ammonium repression, namely arginase (Table 5) and asparaginase (Fig. 2). In arginine plus ammonium, FM-49A1 (*nmr2-1*) showed arginase activities that are about 10-fold higher than those of the control, indicating a possible role of *NMR2* in ammonium repression of arginine catabolism. In the same medium, FM-101B had an activity 2- to 3-fold higher than the control (similar data were previously reported for *nmr1-1* mutation, [1]). These results suggest that *nmr4-1* only slightly affects arginase repression. In aspartate β-hydroxyamate plates of L1, FM-49A1, FM-101B and FM-32B3 we evaluated asparaginase activity in the presence of ammonium, glutamine or glutamate [89]. Growth delays of FM-49A1 and FM-101B on these plates was only marked in the presence of ammonium, suggesting that both *nmr2-1* and *nmr4-1* affect the ammonium repression of asparagine utilisation. This trait of *nmr2-1* and *nmr4-1* marks another difference with *nmr1-1*, which is not involved in the control of asparagine metabolism [1].

Results described in this section confirm our previous hypothesis, suggesting that while *NMR4* takes part in ammonium assimilation and sensing, *NMR2*, as well as *NMR1* [1], is part of a negative regulatory response to reduced nitrogen compounds. However, *nmr2-1* causes a more general de-regulated phenotype, affecting NMR of NR in all conditions tested (Table 1) and ammonium repression of arginase and asparaginase (Table 5 and Fig. 2). In particular, NR activities in *nmr2-1* on nitrate plus glutamine, asparagine and arginine are clearly higher

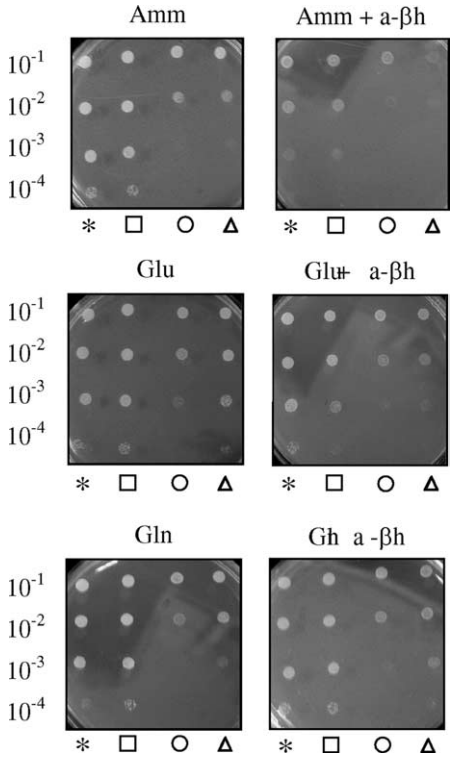

Fig. 2. Effects of aspartate β-hydroxamate (a-βh) on growth of *H. polymorpha* mutants: (□) FM32-B3 (*nmr1-1*); (○) FM49-A1 (*nmr2-1*); (△) FM101-B (*nmr4-1*); (✻) L1 (control) on ammonium, glutamate or glutamine (repressing nitrogen sources: Amm, ammonium; Glu, glutamate; Gln, glutamine). Plates without β-hydroxamate were used for comparison.

than in *nmr1-1*, which shows activities similar to the control strain [1].

It is interesting to note that only *NMR1* is involved in the control of utilisation of alkylated amines [1].

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