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Characterization of the *Aspergillus nidulans nmrA* Gene Involved in Nitrogen Metabolite Repression

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The gene *nmrA* of *Aspergillus nidulans* has been isolated and found to be a homolog of the *Neurospora crassa* gene *nmr-1*, involved in nitrogen metabolite repression. Deletion of *nmrA* results in partial derepression of activities subject to nitrogen repression similar to phenotypes observed for certain mutations in the positively acting *areA* gene.

Fungi are capable of using a wide range of compounds as sources of nitrogen. Genes encoding enzymes and permeases required for nitrogen utilization are often regulated by specific induction mechanisms. In addition, they are usually subject to a general control mechanism, sometimes called nitrogen metabolite repression, according to which they are expressed at high levels only under conditions of nitrogen limitation. This enables readily assimilated nitrogen sources such as ammonium and glutamine to be used preferentially (for a review, see reference 26).

In all fungi investigated, a key feature of this regulatory mechanism is activation by regulatory proteins containing a DNA binding domain consisting of a four-cysteine, single zinc finger characteristic of the GATA family of transcription factors. In each case, loss-of-function mutations in the genes encoding these GATA factors result in reduced ability for growth on many different sole sources of nitrogen. *Saccharomyces cerevisiae* has two genes, *GLN3* and *NIL1*/*GAT1*, while in other fungi only a single gene has been found: *nit-2* in *Neurospora crassa*, *areA* in *Aspergillus nidulans*, *nut1* in *Magnaportha grisae*, and *nreA* in *Penicillium chrysogenum* (4, 6, 17, 18, 21, 25, 27, 34, 36). The basic model for nitrogen metabolite repression is that growth in the presence of preferred nitrogen sources results in the generation of one or more signals which antagonize activation of gene expression by GATA factors. Under nitrogen-limiting conditions, the activators activate the expression of genes involved in the use of nitrogen sources.

In *N. crassa*, the gene *nmr-1* has been found to be important for the response to nitrogen metabolite repression. Recessive mutations in this gene result in derepression of some nitrogen-controlled activities, suggesting a negative role for the gene (16, 31, 32, 37). Cloning and characterization of this gene have enabled studies of its role in nitrogen metabolite repression (19, 23, 24, 40). NIT2 and NMR1 were shown to interact by the use of the yeast two-hybrid system as well by in vitro assays. Two regions of NIT2 have been shown to be involved in interactions with NMR1, one in the conserved region adjacent to the zinc finger and one consisting of the 12 carboxyl-terminal residues. Mutations in both of these regions prevent interaction with NMR1 and result in derepressed phenotypes (39). These data strongly suggest that at least one component of nitrogen metabolite repression in *N. crassa* involves NMR1 interacting with NIT2 under nitrogen-sufficient conditions to prevent NIT2 binding to its recognition sequences and activating gene expression. Some in vitro binding studies support this model (39).

Extensive mutagenesis studies of *areA* in *A. nidulans* have shown that residues within the AreA DNA binding domain as well as in the carboxyl-terminal region lead to some degree of derepression for nitrogen-regulated activities (25, 29, 30, 35). In addition to the DNA binding domains, the carboxyl termini of NIT2, NreA and AreA, are highly conserved (29). Further, it has been shown that the deletion of sequences within the 3′ untranslated region of *areA* results in some derepression and that this correlates with a stabilization of *areA* mRNA in ammonium-grown mycelia (29). The sequences involved are conserved in the *P. chrysogenum nreA* homolog. The gene *nit-2* has been shown to complement *areA* loss-of-function mutations in *A. nidulans* (12). However, partial derepression of the various nitrogen-regulated activities was observed. The *nit-2* plasmid used may have lacked the necessary 3′ untranslated sequences (13). The *xprD1* mutation, isolated as leading to derepression of protease expression (7), is an inversion truncating *areA* such that the 3′ coding and untranslated regions of the *areA* mRNA are missing (2, 25). Truncation of *areA* as in the *xprD1* mutation would result in the loss of both mechanisms; this has been confirmed by the construction of appropriate double *areA* deletion mutations (29). Therefore there is strong evidence for two distinct mechanisms of modulation for *areA* and *nit-2* activity, protein-protein interactions affecting DNA binding and nitrogen regulation of mRNA stability.

Although no mutants with the predicted phenotype have been isolated, the data lead to the strong prediction that *A. nidulans* has a homolog of *nmr-1* of *N. crassa*. Expression of *nmr-1* in *A. nidulans* suggests that this is the case (Polley and Caddick as cited in reference 26). We have confirmed this by cloning the *nmr-1* homolog from *A. nidulans* and have found a central extended conserved region. Disruption of the gene results in partially derepressed phenotypes similar to those observed in *areA* mutants with alterations in the DNA binding domain and the carboxyl terminus.

Cloning and analysis of *nmrA*. A search of the *A. nidulans* expressed sequence tag (EST) database (32a) with the *N. crassa nmr-1* predicted protein sequence (accession no. P23762) re-

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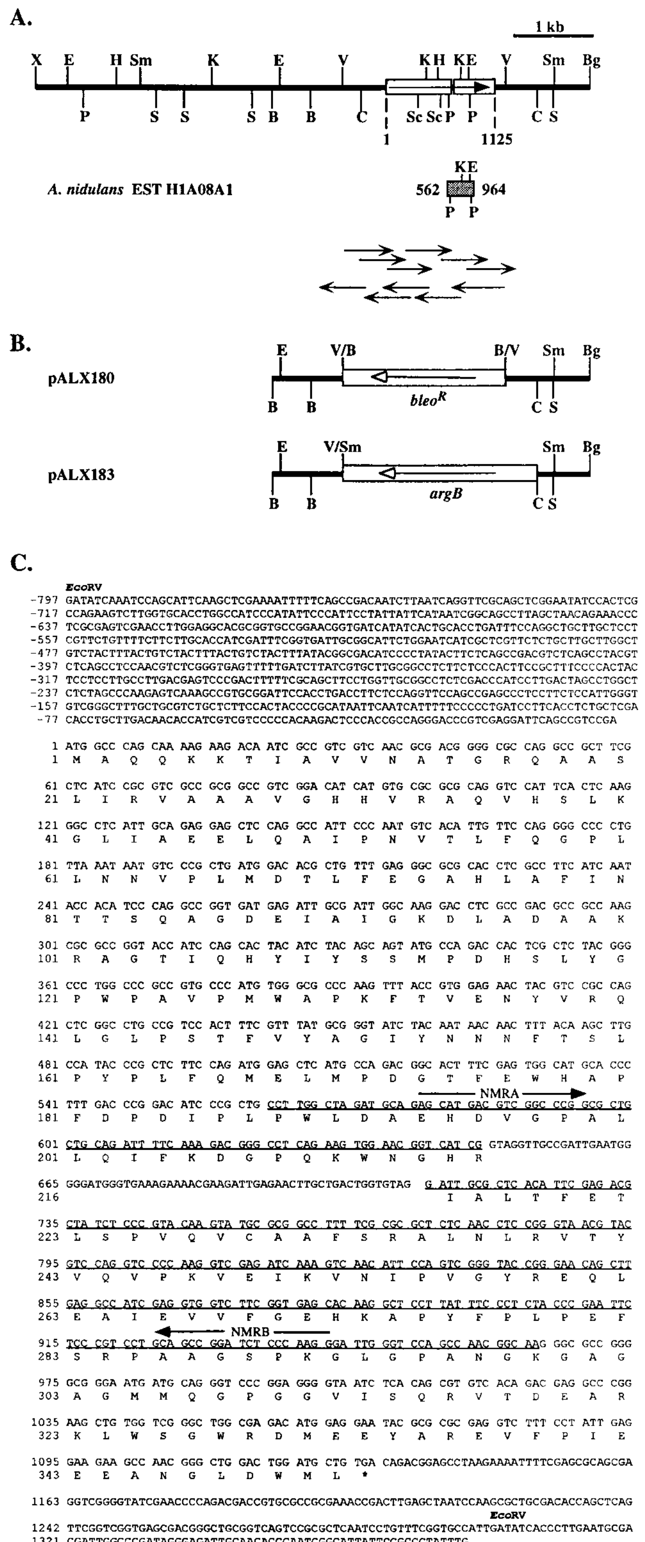

FIG. 1. (A) Restriction map of the 7-kb *Xba*I-*Bgl*II fragment containing the gene *nmrA*. The two exons of the coding region (open rectangles) and the direction of transcription (arrow) are shown. The flanking coordinates of the open reading frame relative to the translational start are shown below the map. The location and coordinates of the EST (hatched rectangle) are shown. The gene *nmrA* was sequenced with a combination of dye primer and dye terminator reactions on an ABI 377 sequencer by the strategy shown, with the direction and size of the arrows indicating the strand and length of sequence. Restriction sites are *Bam*HI (B), *Bgl*II (Bg), *Cla*I (C), *Eco*RI (E), *Eco*RV (V), *Hin*dIII (H), *Kpn*I (K), *Pst*I (P), *Sac*I (Sc), *Sal*I (S), *Sma*I (Sm), and *Xba*I (X). (B) Deletion constructs. pALX180, in which the *Eco*RV fragment spanning the entire coding region of *nmrA* as well as approximately 800 and 200 bp of 5′ and 3′ sequences, respectively, was replaced with an end-filled *Bam*HI fragment containing the bleomycin resistance gene from pAmPh520 (3), and pALX183, in which the *Cla*I-*Eco*RV fragment spanning the entire coding region of *nmrA* as well as approximately 800 bp from both 5′ and 3′ sequences was replaced with a *Cla*I-*Sma*I fragment containing the *A. nidulans* gene *argB* (38). The flanking sequences (solid line) and selectable markers (open rectangles) and their direction of transcription (arrow) are shown. (C) Nucleotide and conceptual protein sequence of *nmrA*. The region encompassed by the EST (underlined) and the oligonucleotide primers NMRA and NMRB (arrow) used for the PCR-generated *nmrA* probe are shown. The two *Eco*RV restriction sites are marked for reference to the map in panel A. Nucleotides are numbered with reference to the +1 at the start of the coding region.

vealed a sequence encoding a polypeptide fragment with extensive similarity. This allowed the design of primers for amplification from *A. nidulans* genomic DNA of a 383-bp sequence by PCR (Fig. 1A). Cloning and sequencing of this fragment confirmed homology with *nmr-1* and the presence of a 68-bp intron. Southern blot analysis of *A. nidulans* genomic DNA indicated that the amplified sequence was unique and allowed the identification of a 7-kb *Xba*I-*Bgl*II hybridizing fragment. This fragment was cloned into *Xba*I-*Bam*HI-digested pBLUESCRIPT SK+ (Stratagene, Inc.) by generating a partial genomic library and probing colony lifts with the PCR fragment. Restriction mapping confirmed that the arrangements of sites within the genome and the clone were identical (Fig. 1A).

Sequence determination of 2,173 bp flanking the PCR fragment indicated an open reading frame encoding a 352-amino-acid polypeptide which is interrupted by a single intron (Fig. 1C). Comparison of the predicted polypeptide with that of *N. crassa nmr-1* showed similarity extending throughout the sequence, with five regions of very high conservation (Fig. 2). However, the *N. crassa* sequence is extended at both the amino and carboxyl termini by 59 and 77 amino acids, respectively. It has been previously shown that the introduction of stop codons into *nmr-1*, leading to the loss of up to 77 carboxyl-terminal amino acids, does not appear to affect function, while function is abolished by the loss of 104 carboxyl-terminal amino acids (40). The former truncation removes all additional carboxyl-terminal amino acids of NMR1, while the latter removes the fifth highly conserved region between NMR1 and NmrA. In addition, protein-protein interaction studies have shown that the 45 amino-terminal amino acids of NMR1, not present in NmrA, are not required for binding to NIT2 and that the region from amino acids 118 to 284, spanning the three central conserved regions between NMR1 and NmrA, also binds NIT2, although the interaction is weaker (39). One insertion of 26 residues in NMR1 is not present in NmrA, and one insertion of 28 residues in NmrA is not present in NMR1. Inspection of the DNA sequences encoding these insertions indicate that they could have arisen by the mutation of intron splice sites (Fig. 1C) (40).

Characterization of *nmrA* deletion strains. Two different constructs were used to create *nmrA* deletions. In pALX180 the bleomycin resistance gene from Tn*5* expressed from the *N. crassa am* promoter (3) replaced the *nmrA* sequence (Fig. 1). A linear *Sac*I-*Kpn*I fragment containing the *nmrA*::*bleo*^{R} insert was used to transform *A. nidulans* MH3408 (*biA1 amdS*::*lacZ niiA4*), selecting for resistance to bleomycin. Approximately 20% of transformants showed some phenotypes characteristic of derepression of nitrogen-regulated activities (see below). In pALX183, *argB* (38) replaced the *nmrA* sequence (Fig. 1), and a *Not*I-*Kpn*I fragment containing the *nmrA*::*argB* insert was used to transform *A. nidulans* MH8826 (*yA1 pabaA1*

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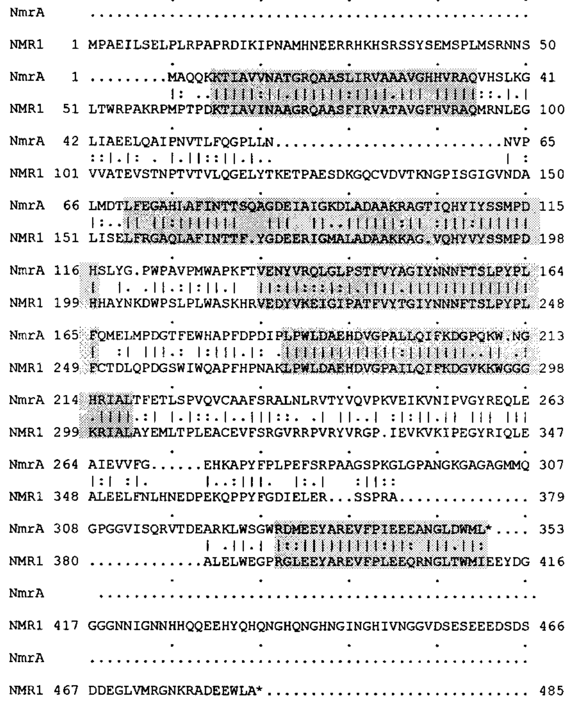

FIG. 2. Alignment of the *A. nidulans* NmrA and *N. crassa* NMR1 conceptual protein sequences. The alignment was generated with GAP from the Wisconsin package (version 8; Genetics Computer Group, Madison, Wis.) with the Dayhoff protein comparison weight matrix, a GAP weight of 3, and a length weight of 0.1. Identities between the two sequences are marked with vertical lines, and similarities are marked by colons. The five regions of highest identity are shaded. The two sequences show 60.8% identity and 75.5% similarity over their entire region.

argB1 amdA7), selecting for arginine prototrophy. Approximately 20% of transformants showed derepression phenotypes. Southern blot analysis of DNA isolated from transformants confirmed that, for each construct, the observed phenotypes correlated with replacement of *nmrA* sequences with *bleo*^{R} or *argB*$^+$ gene, respectively. One transformant from a single *nmrA* deletion event was isolated from each experiment and used for further characterization.

Various plate tests can be used to determine derepression of activities subject to nitrogen metabolite repression (reference 29 and references therein). The *nmrA* deletion transformants as well as an *xprD1*-containing strain were sensitive to the toxic effects of aspartylhydroxamate in the presence of ammonium, an indication of derepression of asparaginase (15) (Fig. 3). The *nmrA* deletion also resulted in slight sensitivity to thiourea in the presence of ammonium, but the sensitivity was not as great as that of the *xprD1*-containing strain (Fig. 3). Thiourea toxicity is an indicator of the activity of the *ureA*-encoded urea permease (28). Similarly, sensitivity to chlorate, a toxic analog of nitrate, in the presence of ammonium was observed in the *nmrA* deletion strain, but the sensitivity was intermediate between the *xprD1* and wild-type strains (Fig. 3). Tests for derepression of extracellular protease production by the observation of a halo of clearing of milk (0.5 to 1.0%) in the presence of ammonium indicated that the *nmrA* deletion strains, unlike *xprD1*-containing strains, were not detectably derepressed. These phenotypes were similar to those observed by Platt et al. (29) for *areA* mutant strains encoding AreA proteins truncated at the carboxyl-terminal end but encoding mRNA with an intact 3′ untranslated region and indicated partial derepression for some activities.

The *nmrA*::*argB* deletion strain was transformed with the bleomycin resistance-encoding plasmid pAmPh520 (3) together with a plasmid (pALX186) containing *nmrA* on an *Eco*RV fragment (Fig. 1) and selecting for bleomycin resistance. Approximately 50% of the transformants were found to be phenotypically *nmrA*$^+$ as shown by resistance to aspartylhydroxamate and chlorate in the presence of ammonium. This finding indicated that these phenotypes resulted from the deletion of *nmrA* and that this fragment is sufficient for *nmrA* function.

The gene *amdS*, encoding acetamidase, is subject to nitrogen metabolite repression (14). The deletion of *nmrA* was found to result in partial derepression of the expression of an *amdS*::*lacZ* reporter gene (11) with respect to both ammonium and glutamine (Table 1). Partial derepression was also observed in the presence of GABA as inducer, reflecting derepression of both *amdS* expression and GABA uptake via the *gabA*-encoded permease (1). Partial depression for nitrate reductase was also observed (Table 2), and the level of derepression was similar to that reported for the *areA* mutants encoding proteins truncated at the carboxyl terminus (29, 35).

The *areA102* mutation, resulting from an amino acid substitution in the zinc finger region, is a change in specificity mutation resulting in increased activation of some nitrogen-controlled activities and decreased activation of others (22, 25). A

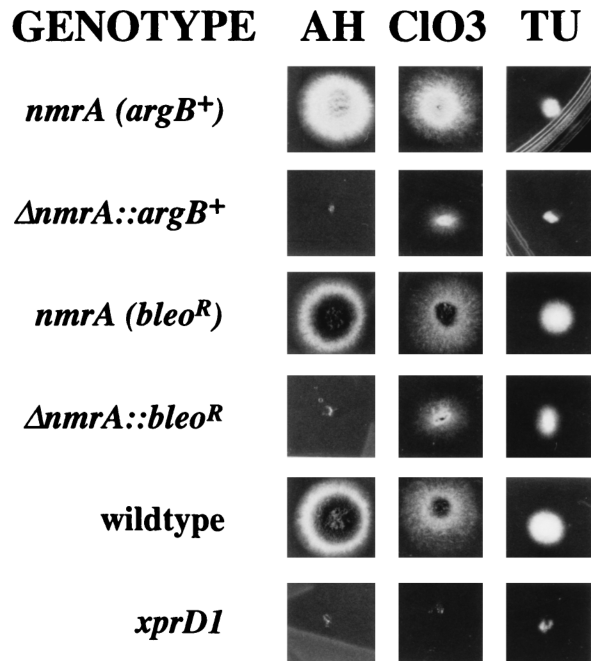

FIG. 3. Growth properties of *nmrA* deletion strains. Growth was scored for 2 to 3 days at 37°C on 1% glucose medium (9) containing 5 mM ammonium tartrate together with 200 mM potassium chlorate (ClO_3), 5 mM ammonium tartrate together with 5 mM D,L-β-aspartylhydroxamate (AH), and 2.5 mM ammonium tartrate with 10 mM thiourea (TU) with appropriate auxotrophic supplements. The Δ*nmrA*::*argB*$^+$ strain was generated by transformation of a strain whose genotype was *yA1 pabaA1 argB1 amdA7* with a gel-purified insert of pALX183 selecting for arginine prototrophy (Fig. 1B). The *nmrA*$^+$ (*argB*$^+$) control strain was an ArgB$^+$ transformant from the same transformation that did not result in the deletion of *nmrA*. The Δ*nmrA*::*bleo*^{R} strain was obtained by transformation of a strain whose genotype was *biA1 amdS*::*lacZ niiA4* with a gel-purified insert of pALX180 (Fig. 1B) selecting for resistance to bleomycin. The *nmrA*$^+$ (*bleo*^{R}) control strain was a bleomycin-resistant transformant from the same transformation that did not result in the deletion of *nmrA*. The genotypes of the strains designated wild type and *xprD1* were *biA1* and *biA1 amdS*::*lacZ xprD1 niiA4*, respectively. Genotypes and phenotypes were reported by Clutterbuck (5). The *xprD1* mutation results from an inversion truncating the *areA* gene (2, 25).

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TABLE 1. Effect of *nmrA* deletions on nitrogen metabolite repression of *amdS*::*lacZ* expression

Nitrogen source[a]	β-Galactosidase level (Miller units/min/mg of protein)[b]			
	nmrA	% Derepression[c]	Δ*nmrA*::*bleo*^{R}	% Derepression[c]
Ala	12.2 ± 1.3		13.7 ± 1.5	
NH_4	1.8 ± 0.4	15	4.6 ± 1.5	34
Gln	2.4 ± 0.2	20	6.3 ± 0.8	46
Ala + GABA	91.2 ± 7.3		147.4 ± 16.0	
Ala + GABA + NH_4	6.6 ± 1.5	7	48.2 ± 10.1	33

[a] Mycelium was grown for 16 h at 37°C in glucose minimal medium (9) containing in each case the following nitrogen sources: 10 mM L-alanine (Ala), 10 mM ammonium tartrate (NH_4), 10 mM L-glutamine (Gln), and 10 mM γ-aminobutyric acid (GABA).

[b] β-Galactosidase levels encoded by an *amdS*::*lacZ* translational fusion gene integrated by gene replacement at the *amdS* locus were assayed by the method of Davis et al. (11) and are expressed as means ± standard errors.

[c] The percentage of derepression is calculated as the level of enzyme activity under repressed conditions relative to that under nonrepressed conditions: NH_4 and Gln relative to the limiting nitrogen source Ala, Ala plus GABA plus NH_4 relative to Ala plus GABA.

cross between the *nmrA*::*bleo*^{R} deletion strain and an *areA102* strain resulted in *areA102* Δ*nmrA*::*bleo*^{R} double mutants which showed increased sensitivity to chlorate and thiourea in the presence of ammonium relative to the Δ*nmrA*::*bleo*^{R} single mutant and derepression for extracellular protease activity as shown by a halo of milk clearing in the presence of either ammonium or glutamine.

These results clearly indicate that one of the mechanisms for nitrogen metabolite repression is conserved between *N. crassa* and *A. nidulans*, namely protein-protein interactions between NMR1/NmrA and NIT2/AreA in the presence of sources of repression. The magnitude of the effects of deletion of *nmrA* on nitrogen metabolite repression is similar to that observed for deletion of the conserved 12 carboxyl-terminal amino acids of AreA (29). It is predicted that the effects of these mutations will not be additive in double mutants. Since deletion of *nmrA* does not result in complete derepression, this gene is unlikely to be involved in modulation of *areA* mRNA stability via sequences in the 3′ untranslated region (29). Therefore, it is predicted that double mutants containing an *nmrA* deletion and a deletion of the 3′ untranslated region of *areA* will show additive levels of derepression, as previously observed for the *xprD1*

TABLE 2. Effect of *nmrA* deletions on nitrogen metabolite repression of nitrate reductase expression

Nitrogen source[a]	Nitrate reductase level (mU/min/mg of protein)[b]			
	nmrA	% Derepression[c]	Δ*nmrA*::*argB*	% Derepression[c]
NO_3	66.5 ± 16.1		63.6 ± 11.2	
NO_3 + NH_4	6.2 ± 2.8	9	27.5 ± 6.3	43
NO_3 + Gln	4.7 ± 0.5	7	28.7 ± 4.6	45

[a] Mycelium was grown for 16 h at 37°C in glucose minimal medium (9) containing in each case the following nitrogen sources: 10 mM ammonium tartrate (NH_4) and sodium nitrate (NO_3).

[b] Nitrate reductase levels were assayed by the method of Cove (9) and are expressed as means ± standard errors.

[c] The percentage of derepression is calculated as the level of enzyme activity under repressed conditions relative to that under nonrepressed conditions: NO_3 plus NH_4 and NO_3 plus Gln relative to NO_3.

inversion mutation and for *areA* mutants with deletions of both the carboxyl terminus and the relevant 3′ untranslated sequences (29).

The nature of the signal or signals generated by nitrogen metabolites and the question of whether both mechanisms have any common components remain to be determined. Furthermore, the role of negatively acting factors of the GATA family as found in *S. cerevisiae* (8, 10, 33), and recently suggested to occur in filamentous fungi (20), needs to be investigated.

Nucleotide sequence accession number. The sequence for the *nmrA* gene has been deposited in GenBank under accession no. AF041976.

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