

Genetic Analysis of the TOR Pathway in *Aspergillus nidulans*

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We identified five genes encoding components of the TOR signaling pathway within *Aspergillus nidulans*. Unlike the situation in *Saccharomyces cerevisiae*, there is only a single Tor kinase, as in plant and animal systems, and mutant phenotypes suggest that the TOR pathway plays only a minor role in regulating nitrogen metabolism.

The TOR signaling pathway is a well-conserved mechanism controlling cell growth in eukaryotes, especially in response to nutrient availability (16, 23). One of the roles of the two Tor kinases (Tor1p and Tor2p) in *Saccharomyces cerevisiae* is to repress the transcription of genes subject to nitrogen metabolite repression (3, 6). In nitrogen-rich conditions, TOR signaling prevents nuclear accumulation of Gln3p, maintaining it within a cytoplasmic complex with Ure2p (3), preventing activation of a suite of nitrogen utilization genes. The major regulator of nitrogen metabolism in the filamentous fungus *Aspergillus nidulans*, the GATA factor AreA (19), is a homologue of Gln3p and a second partially redundant transcription factor, Gat1p. Despite detailed molecular and genetic characterization of nitrogen metabolism in *A. nidulans* (4), a TOR pathway has not been described or implicated in nitrogen signaling. However, one putative component of the pathway has been identified previously (9). We adopted a genetic approach to identify components of this pathway in *A. nidulans*, selecting mutants resistant to the immunosuppressive and antifungal compound sirolimus (rapamycin) that first revealed the TOR pathway in *S. cerevisiae* (13). The mutant phenotypes are consistent with the TOR signaling pathway having a minor role in *A. nidulans* nitrogen signaling.

Sirolimus causes growth inhibition in yeasts and filamentous fungi, including *Aspergillus fumigatus* (26). In *A. nidulans*, sirolimus has fungistatic activity, causing severely reduced growth and conidiation (Fig. 1). Mutations in *areA* do not alter sirolimus sensitivity, whereas *S. cerevisiae* *gln3 gat1* double mutants show partial resistance (3). Unlike for *S. cerevisiae*, where sirolimus treatment in a nitrogen-rich medium results in rapid expression of genes subject to nitrogen metabolite repression, such as the general amino acid permease gene *GAP1* and the glutamine synthase gene *GLN1* (3, 6), we did not detect induction of AN5678.2 (homologue of *S. cerevisiae* *GAP1*), *glnA* (glutamine synthase), *areA*, or *mepA* (ammonium permease) in Northern blot analysis over a 30-min time course (data not shown). This indicates that, unlike in *S. cerevisiae*, sirolimus does not override nitrogen metabolite signaling in *A. nidulans*.

In *S. cerevisiae*, sirolimus toxicity occurs via complex forma-

tion with the prolyl-isomerase-like protein FKBP12 (14). Specific mutations in *FPR1*, encoding FKBP12, *TOR1*, and genes encoding two downstream components of the TOR pathway, *TIP41* (17) and *TAP42* (10), result in distinct sirolimus-resistant phenotypes. We used UV irradiation of both haploid (*yA2 pabaA1; areA49*) and diploid (*pabaA1/suA1adE20 yA1 adE20; acrA1; galA1; pyroA4; acuA303; sB3; nicB8; riboB2*) *A. nidulans* strains to select mutants resistant to sirolimus (200 ng/ml). The sirolimus resistance phenotypes identified in the haploid strains segregated normally in crosses, and the mutants selected were associated with either linkage group (LG) I or LG II. Sirolimus resistance in all six diploid strains tested was associated with LG I, but it proved impossible to obtain sirolimus-resistant haploids from these diploid strains, suggesting that the mutant alleles disrupt an essential function.

To identify the mutations within the sirolimus-resistant strains, we used a combination of bioinformatics, PCR, and sequencing. Assembly and annotation of the public *A. nidulans* genome sequence proceeded during the course of our work. BLAST searches using protein sequences from other organisms submitted to *A. nidulans* databases aided identification.

A single Tor kinase gene homologue on LG I which we named *torA* (accession no. AJ717403) was identified in *A. nidulans*. Based on reverse transcription (RT)-PCR, the putative protein is 2,385 amino acids long and contains all the domains associated with Tor function (Fig. 2A). Full disruption of Tor activity is likely to be lethal, as has been shown in other fungi, including *S. cerevisiae* (20) and *Fusarium fujikuroi* (Sabine Teichert and Bettina Tudzynski, personal communication). PCR amplification and sequencing of the sirolimus-resistant diploid *torA3* strain revealed a single point mutation at position 5751 that would result in Pro replacing Ser-1871 within the highly conserved sirolimus-binding domain (8). The dominant nature of the allele indicates that the mutant protein must retain some aspects of its function in the presence of sirolimus. Its apparent lethality in the haploid suggests either multimerization, where the heterodimer retains partial function in the presence of sirolimus, or that the wild-type Tor kinase retains essential sirolimus-tolerant functions affected by this mutation. In *S. cerevisiae* and mammalian systems, there is evidence for sirolimus-sensitive and -insensitive functions, but Tor multimerization has not been observed (15, 20, 21, 25, 29).

An *FPR1* homologue was identified on LG II and named *fprA* (accession no. AJ717400). The predicted protein se-

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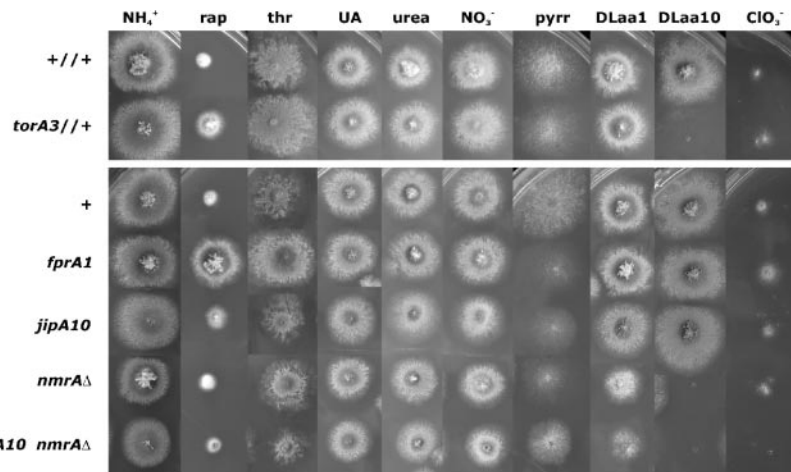


FIG. 1. Growth tests for the wild type and sirolimus-resistant mutants. The effects of sirolimus on the growth of the wild-type (+/+) and *torA3* heterozygote (*torA3*/+) diploids, as well as the wild-type (+), *fprA1*, *jipA10*, *nmrAΔ*, and *jipA10 nmrAΔ* haploid strains, are shown. Nitrogen source utilization was tested by growing the strains on supplemented minimal media containing NH_4^+ , threonine (thr), uric acid (UA), urea, NO_3^- , or 2-pyrrolidone (pyr) at 10 mM. Sirolimus (rap) was added at a final concentration of 200 ng/ml to supplemented minimal medium containing 10 mM NH_4^+ . Nitrogen metabolite derepression was assessed using chlorate (ClO_3^-) at 500 mM or DL-aspartic acid β -hydroxamate at either 1 mM (DLaa1) or 10 mM (DLaa10) or on minimal medium supplemented with 10 mM NH_4^+ .

quence was confirmed by sequencing a wild-type transcript obtained by RT-PCR (Fig. 3). The *fprA* alleles of four haploid sirolimus-resistant mutants were sequenced. In *fprA1*, the splicing signal TAG is mutated to ACG (intron three, positions 533 to 535), probably leading to replacement of the eight C-terminal residues of the protein. In *fprA2*, nucleotides 778 to 1202 are replaced by 87 nucleotides derived from LG VI, disrupting the putative sirolimus-binding pocket of the protein. In *fprA3*, a deletion of 5 bp (positions 549 to 553) near the stop codon resulted in a frameshift, with the mutant protein extending 41 amino acids beyond the original C terminus, presumably

resulting in a conformation change that prevented sirolimus binding. Finally, a single T→C transition at position 485 in *fprA7* altered one of three invariant residues (Phe-100→Ser) required for interaction between FKBP12-sirolimus and Tor kinase (22, 28).

The sirolimus-resistant mutations selected in the haploid strain and located on LG I are within *jipA* (9), the *A. nidulans* homologue of *S. cerevisiae* *TIP41*. PCR and sequencing of the *jipA* coding region in two mutants showed that single nucleotide insertions had caused frameshift mutations, truncating the putative protein after 92 (*jipA5*) and 49 (*jipA10*) residues (Fig. 2B).

Growth tests were used to assess the effects of mutations in *torA*, *fprA*, and *jipA* on nitrogen metabolism (Fig. 1). The heterozygous diploid *torA3/torA*⁺ showed increased sensitivity to 10 mM DL-aspartic acid β -hydroxamate in the presence of NH_4^+ , indicating partial derepression of the asparaginase gene *ahrA* (11, 27). Both *fprA*[−] and *jipA*[−] showed weak pleiotropic effects on several nitrogen sources and had severely reduced growth on 2-pyrrolidone. The *jipA*[−] strains showed marginally reduced growth irrespective of the nitrogen sources tested. The *fprA1* and *torA3* diploid strain showed enhanced growth on threonine. Interestingly, in *S. cerevisiae* FKBP12 has a role in aspartokinase feedback regulation of the threonine biosynthetic pathway (2).

A. nidulans does not have an orthologue of yeast *URE2* (12). However, a structurally unrelated protein, NmrA, is known to interact with and inhibit AreA (1, 24) and therefore has similar function to Ure2p, which represses Gln3p. In *S. cerevisiae*, activity of the phosphatase Sit4p, required to release Gln3p from Ure2p, is inhibited by an interaction with Tap42p as part of the TOR network (3). Within the *A. nidulans* genome, we identified *tapA* (accession no. AJ717402) and *sitA* (accession no. AJ717404), homologues of *TAP42* and *SIT4*, respectively, and confirmed their expression and intron structure using RT-PCR. As this arm of the TOR signaling network is conserved in *A. nidulans*, it was important to determine whether JipA

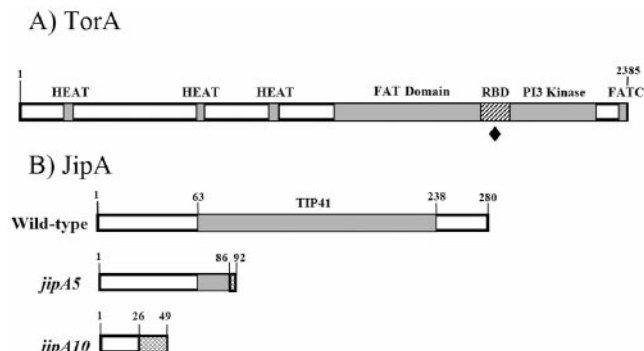


FIG. 2. Domain map of the predicted TorA and JipA proteins. (A) TorA contains three HEAT repeats (177 to 217, 701 to 732, and 985 to 1025), a FAT domain (1241 to 1818), a sirolimus-binding domain (1819 to 1931), a phosphatidylinositol 3-kinase domain (1932 to 2272), and a FATC domain (2353 to 2385). The position of the Ser-to-Pro substitution of TorA3, which lies within the sirolimus-binding domain, is indicated (◆). (B) JipA (accession no. AJ717401) has a conserved central region (shaded region), conforming to the TIP41 domain of *S. cerevisiae*. Frameshift mutations in both *jipA5* and *jipA10* introduce premature stop codons, resulting in 92- and 49-amino-acid peptides, respectively. The mutant sequence is indicated (cross-hatched box). In the *jipA5* strain, 24 residues of the TIP41 domain are present, whereas in the *jipA10* strain the truncation is introduced before the domain.



FIG. 3. The *A. nidulans* FKB12 gene homologue *fprA* shares high levels of homology with homologues from *Cryptococcus neoformans*, *S. cerevisiae*, *Homo sapiens*, *Mus musculus*, and *Drosophila melanogaster*. Amino acid residues marked with asterisks represent the hydrophobic sirolimus- and FK506-binding pocket. The three amino acid residues marked with arrows represent the surface residues known to interact with the TOR proteins.

effected nitrogen signaling through NmrA. We therefore constructed *jipA10 nmrAΔ* double mutants. These were sirolimus resistant, had reduced growth on nitrogen sources equivalent to *jipA10* single mutants, were hypersensitive to DL-aspartic acid β-hydroxamate, and were more sensitive to chlorate than either parental strain (Fig. 1). Results of plate tests for nitrite reductase activity were intermediate to those obtained with single mutants (data not shown). Deletion of *nmrA* results in asparaginase and nitrate reductase derepression (1), but poor growth observed on 2-pyrrolidone suggested that NmrA has a positive effect on the expression of some genes (Fig. 1). One interpretation is that NmrA may remain associated with AreA when it is active. The lack of a clear epistatic relationship suggests that JipA does not act through NmrA. Double mutants containing *jipA10* and *areA* alleles lacking specific domains (N terminus, *areAΔ2-389* [5]; C terminus, *areAΔ743-864* [24]; and C-terminal GATA domain and consensus nuclear export signal, *areA120* [7, 24]) also failed to give clear epistatic relationships (data not shown), indicating that TOR signaling is not effected specifically through any one of these regions.

Both *jipA⁻* and *fprA⁻* strains showed their most severe nitrogen utilization phenotypes on 2-pyrrolidone. Northern analysis was used to measure expression levels of the utilization genes *lamA* and *lamB* (18). Expression of both genes was significantly lower in *jipA10* than the wild type over a 2-h time course after transfer from NH₄⁺ to 2-pyrrolidone (Fig. 4). However, only *lamA* expression was significantly reduced in *jipA10* and *fprA1* strains when induced by β-alanine, which is a good nitrogen source for both strains. This is consistent with the defects in 2-pyrrolidone utilization being due, in part, to reduced transcription of *lamA*.

We have therefore identified five genes encoding components of the TOR signaling pathway within *A. nidulans*, namely, *torA*, *fprA*, *jipA*, *sitA*, and *tapA*, using a combination of mutant selection, database searching, PCR, and sequencing. The conservation of these components and the presence of only a single Tor kinase make *A. nidulans* a good model for the situation in animals and plants. There is no clear evidence to suggest TOR signaling acts through AreA. However, the mutant phenotypes suggest that TorA, FprA, and JipA play a minor role in nitrogen metabolism, which in the case of *jipA⁻*

and *fprA⁻* strains results in reduced transcription of at least one gene involved in nitrogen assimilation (*lamA*).

Nucleotide sequence accession numbers. Sequences from this study were submitted to GenBank and assigned accession no. AJ717400, AJ717401, AJ717402, AJ717403, and AJ717404.

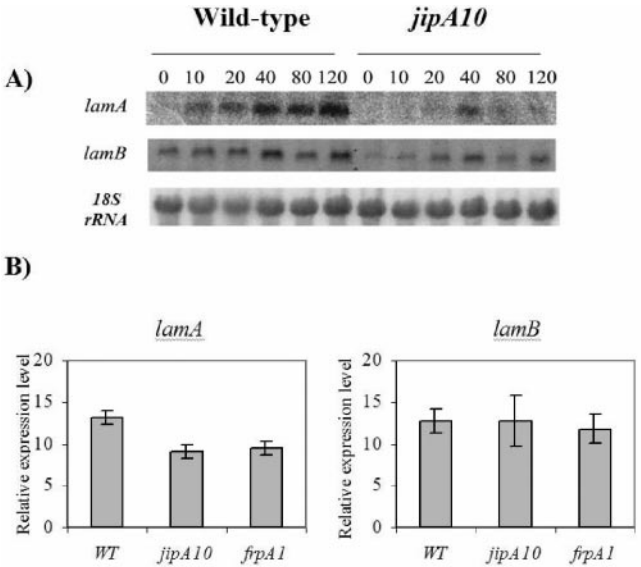


FIG. 4. Expression of *lamA* and *lamB* in wild-type (WT), *jipA10*, and *fprA1* strains. (A) Northern analysis of *lamA* and *lamB* transcription in wild-type and *jipA10* strains over a 2-h time course after transfer from NH₄⁺ to 2-pyrrolidone as the sole nitrogen source. The level of the 18S rRNA transcript is shown as a control. Quantification of triplicate blots revealed a significantly reduced expression for both genes in the mutant strain. (B) *lamA* and *lamB* expression was also examined for wild-type, *jipA10*, and *fprA1* strains after growth on β-alanine as the sole nitrogen source for 4 h. Expression was induced >100-fold by β-alanine compared with L-alanine, which was used as a noninducing control (data not shown). Graphs illustrate the expression levels of *lamA* and *lamB* relative to 18S rRNA as an average (± standard error) from three separate experiments quantified using a phosphorimager. *lamA* expression is significantly lower in the two mutant strains than in the wild type.

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