

A Mitogen-Activated Protein Kinase That Senses Nitrogen Regulates Conidial Germination and Growth in *Aspergillus fumigatus*

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We show that the mitogen-activated protein (MAP) kinase pathway that responds to osmotic stress in *Aspergillus fumigatus* is also involved in nutritional sensing. This MAP kinase regulates conidial germination in response to the nitrogen source and is activated upon starvation for either carbon or nitrogen during vegetative growth.

Mitogen-activated protein (MAP) kinases play a central role in regulating cellular homeostasis in microbial eukaryotes in response to environmental changes (3, 4, 7, 9–12, 15–18, 20). We have been studying the role of the MAP kinase SakA in *Aspergillus fumigatus*, an opportunistic human pathogen. We were interested in SakA because in other fungi homologues of this protein kinase have been shown to play a role in stress responses and pathogenesis (1, 2, 6, 8, 10, 13–15, 19, 21, 22). It is therefore possible that SakA plays a role in pathogenesis and response to immune cell action.

A *sakA* deletion strain was made by using *sakA* 5' and 3' flanking sequences that were amplified by PCR from genomic DNA (strain AF293) with oligonucleotides (Table 1) that incorporated specific restriction sites that facilitated cloning around the hygromycin phosphotransferase gene (Fig. 1A). The selective marker was first cloned into the EcoRI site of the plasmid Bluescript (5). Deletion of *sakA* was confirmed by Southern blot analysis (Fig. 1B), and initial experiments were conducted with three independent deletion strains to confirm that phenotypes were associated with deletion of the gene and not an unlinked mutation resulting from transformation. Specific oligonucleotide pairs were used to make PCR probes for the genes *msnA*, *ptpA*, *pbsA*, and *actin* with genomic DNA as the template (Table 1). Strain AF293 and those derived from it were used throughout these studies and cultured on minimal medium (MM; 70 mM NaNO₃, 7 mM KCl, 2 mM MgSO₄, 12 mM KPO₄ [pH 6.8], trace elements, 1% [wt/vol] glucose) or complete medium (CM), which is MM supplemented with yeast extract (0.1%, wt/vol), peptone (0.2%, wt/vol), and tryptone (0.1%, wt/vol) (Difco Laboratories, Detroit, Mich.). For experiments involving different nitrogen sources MM salts were prepared without sodium nitrate and the nitrogen source was added to a final concentration of 10 mM prior to sterilization. For nitrogen and carbon starvation experiments, mycelia were grown for 14 h at 37°C in yeast extract (0.5%, wt/vol)-glucose (1%, wt/vol) (YG) medium and washed with sterile water. The mycelium was then suspended in MM lack-

ing nitrogen or glucose and incubated for 30 min at 37°C. The control was maintained in YG.

We assessed the conservation of the SakA MAP kinase signaling pathway in *A. fumigatus* using Northern blotting to measure changes in the abundances of transcripts for the genes *msnA*, *ptpA*, *pbsA*, and, as a control, *actin*. We looked at these genes because the mRNAs for *msnA*, *ptpA*, and *pbsA* are known to increase in response to osmotic stress in other fungi (3, 9, 12). We found that the abundances of transcripts for *msnA*, *ptpA*, and *pbsA* all increased in response to increasing osmotic stress in the wild-type control strain but not in the *sakA* deletion (Δ *sakA*) strain (Fig. 1C). This demonstrates that the transcriptional response to osmotic stress is conserved in *A. fumigatus*. Similarly, we found levels of *sakA* transcripts increased in response to hydrogen peroxide, a response also seen in some fungi (data not shown) (3, 9, 12).

We next examined the growth response of actively growing germlings and conidia to increased osmolarity. We found that germlings of the Δ *sakA* strain undergo growth arrest in response to increased osmolarity, whereas conidia germinated in

TABLE 1. Oligonucleotides used during this study

Oligo-nucleotide	Sequence	Product size (kb)
sakA-1	GTTTTGACATCCTCATCTGTGCG	2.2
sakA-2	GTACGAATTCAGATTGCATCCTG	
sakA5'-1	TCTACCGCGGCAGGTTAAAGC	2.7
sakA5'-2	GATCTCAAAGCTAGTGGATGTTCAACG	
sakA3'-1	CCTTATCGATTGAAAACGTCTAATCAC	2.3
sakA3'-2	CTCTTCGGTACCCATGTATCCG	
ptp-5'	CATGAAGCTTGAATACAGCAATAAGCGC	0.84
ptp-3'	CTTTCAAAGCTTAAGACAAACTGGCTC	
msn-5'	GAACGAAGCTTGGAAAGATCTGGACG	0.54
msn-3'	AGCAAGCTTGTCTCGCTCGGAGAG	
pbs-5'	AACCAAGCTTGAAACTGTCAGATGCC	1.3
pbs-3'	TGCAAAGCTTTCTGCCTCTTGTAGGTGC	
actin 5'	GAAGTTGCTGCTCTCGTCATCGAC	1.7
actin 3'	GCACTTGCGGTGAACGATCGAAG	

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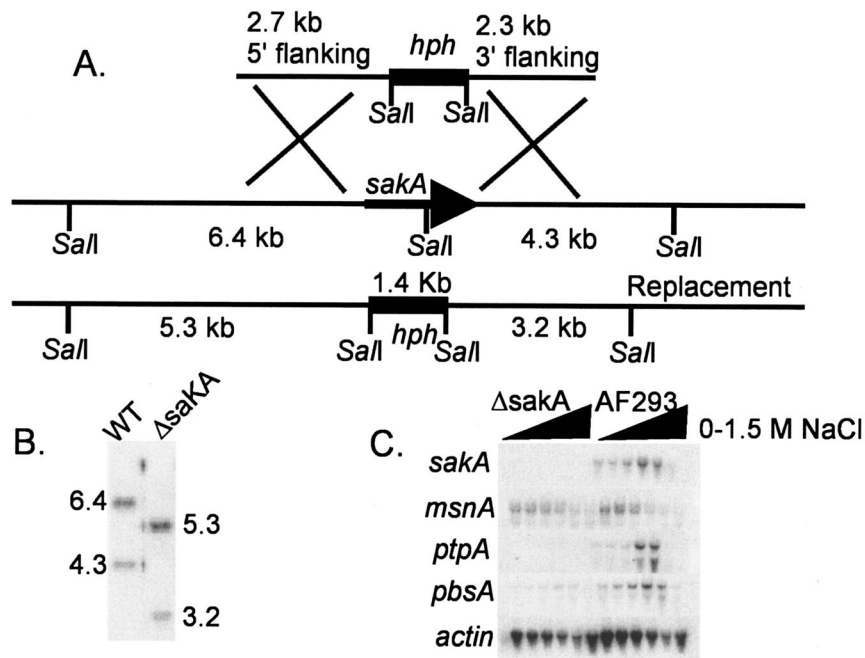


FIG. 1. Deletion of the *sakA* gene. (A) Strategy used to delete *sakA*. The *hph*-selective marker was flanked by DNA sequences from around *sakA*. Integration of the linear molecule by homologous recombination replaces *sakA* with *hph* in the chromosome. (B) Southern blot showing the wild-type and $\Delta sakA$ patterns of hybridization. One representative is shown for each. (C) Northern blots of RNA from the $\Delta sakA$ strain and AF293, the wild-type strain, exposed to increasing concentrations of NaCl. The first lane in each set had CM; lanes 2 to 6 had in addition 0.25, 0.5, 0.75, 1, and 1.5 M NaCl, respectively. The probes used for hybridization of each set of lanes are indicated on the left.

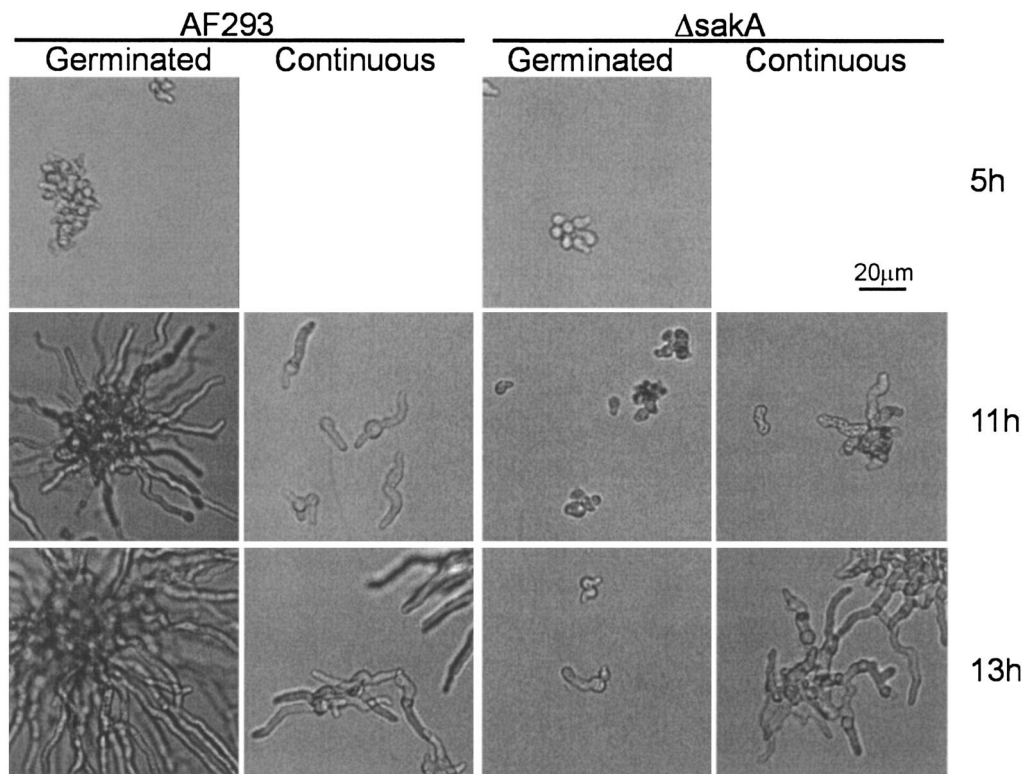


FIG. 2. Photomicrographs of the wild-type parental strain AF293 and a $\Delta sakA$ strain grown continuously (continuous) in CM with 1 M NaCl or shifted to CM with 1 M NaCl after 5 h of germination in CM (germinated). Fields of germings were also photographed after 11 and 13 h of growth. Scale bar, 20 μm .

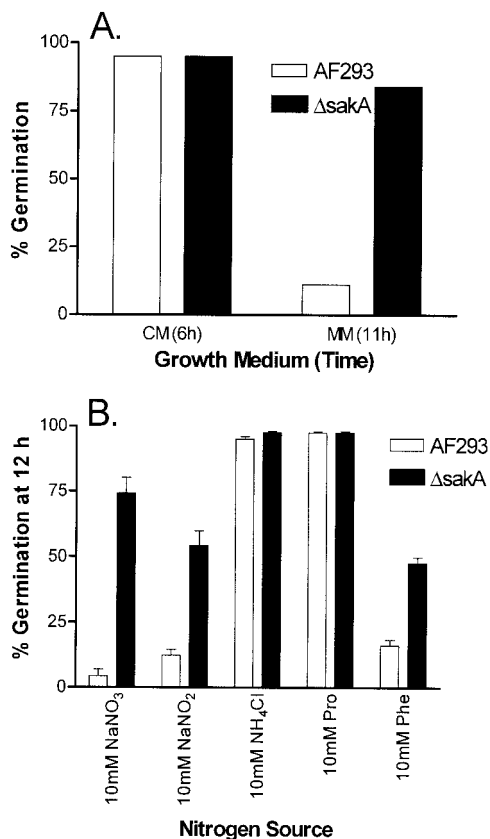


FIG. 3. Conidial germination is altered in the *sakA* deletion strain and is dependent on the nitrogen source. (A) Conidial germination in CM is the same for AF293 and *ΔsakA* strains after 6 h of growth. In contrast, on MM the AF293 conidia were significantly slower to germinate than those of the *ΔsakA* strain. (B) The germination of conidia depends on the nitrogen source in the medium.

high-osmolarity medium were able to grow hyphae but did so more slowly than the parental control, suggesting that signaling through this MAP kinase pathway in conidia is different from that in growing germlings (Fig. 2). We also observed that *ΔsakA* strains failed to produce a pigment secreted into the medium of plates by control strains (data not shown).

During the course of these initial studies, we found that conidia of the *ΔsakA* strain and the parent germinated differently on MM and CM (Fig. 3A). On CM the parental and *ΔsakA* strains germinated equally well. In contrast, on MM the wild-type strain germinated more slowly than the *ΔsakA* strain. A major difference between MM and CM is the inclusion of yeast extract, peptone, and tryptone, all sources of reduced nitrogen. We therefore tested germination of the wild-type and *ΔsakA* strains on MM containing different nitrogen sources (Fig. 3B). Conidia of the wild-type strain germinated more slowly than those of the *ΔsakA* strain on MM containing the poor nitrogen source sodium nitrate or sodium nitrite, but both strains germinated equally well on MM with the reduced-nitrogen source ammonium chloride or proline. When phenylalanine was used as a nitrogen source, both strains germinated less well, suggesting that this amino acid is less suitable as a nitrogen source. Interestingly, we found no difference in germination on a variety of carbon sources, including glycerol,

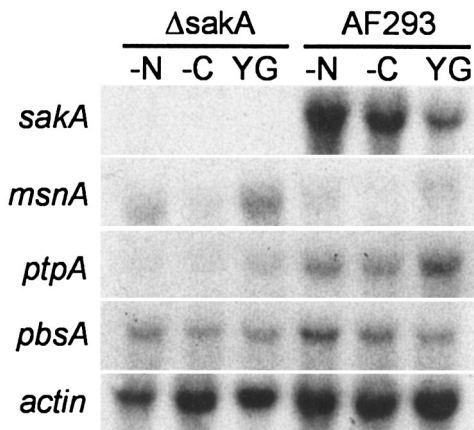


FIG. 4. *sakA* RNA levels change following starvation for nitrogen or glucose. Northern blots of RNA prepared from hyphae from *ΔsakA* and AF293 strains starved for nitrogen (lanes -N) or glucose (lanes -C) for 30 min. The probes used for hybridization of each set of lanes are indicated on the left.

acetate, sorbitol, and lactose (data not shown). These results indicate that Saka functions to negatively regulate conidial germination in response to less-preferred nitrogen sources but not in response to the carbon sources.

Finally, because of the newly discovered role for Saka in sensing nitrogen in the medium, we investigated the effect of starvation for carbon and nitrogen on transcriptional activation of the genes in this pathway (Fig. 4). When hyphae are transferred from rich medium to MM lacking either a nitrogen or carbon source, we find a robust activation of transcription for *sakA* but not for *msnA*, *ptpA*, or *pbsA* and no changes in the *ΔsakA* strain are observed.

In summary, the osmotic stress response pathway in *A. fumigatus* is conserved. We have shown that this MAP kinase pathway negatively regulates conidial germination and is activated in response to starvation for nitrogen or carbon sources.

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