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Environmental Suppression of *Neurospora crassa cot-1* Hyperbranching: a Link between COT1 Kinase and Stress Sensing

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***cot-1* mutants belong to a class of *Neurospora crassa* colonial temperature-sensitive (*cot*) mutants that exhibit abnormal polar extension and branching patterns when grown at restrictive temperatures. *cot-1* encodes a Ser/Thr protein kinase that is structurally related to the human myotonic dystrophy kinase which, when impaired, confers a disease that involves changes in cytoarchitecture and ion homeostasis. When grown under restrictive conditions, *cot-1* cultures exhibited enhanced medium acidification rates, increased relative abundance of sodium, and increased intracellular glycerol content, indicating an ion homeostasis defect in a hyperbranching mutant. The application of ion transport blockers led to only mild suppression of the *cot-1* phenotype. The presence of increased medium NaCl or sorbitol, H_2O_2, or ethanol levels significantly suppressed the *cot-1* phenotype, restored ion homeostasis, and was accompanied by reduced levels of cyclic AMP-dependent protein kinase (PKA) activity. The *cot-1* phenotype could also be partially suppressed by direct inhibition of PKA with KT-5720. A reduced availability of fermentable carbon sources also had a suppressive effect on the *cot-1* phenotype. In contrast to the effect of extragenic *ropy* suppressors of *cot-1*, environmental stress-related suppression of *cot-1* did not change COT1 polypeptide expression patterns in the mutant. We suggest that COT1 function is linked to environmental stress response signaling and that altering PKA activity bypasses the requirement for fully functional COT1.**

In filamentous fungi, growth proceeds by extension of hyphal tips and branching. A defect in the *Neurospora crassa* colonial temperature-sensitive 1 gene (*cot-1*) confers a defect in hyphal extension that results in colonial growth at 32°C and above but normal growth and morphology, similar to that of the wild type, at or below 28°C (8). The *cot-1* gene encodes a Ser/Thr protein kinase (51), and the defect in a *cot-1* mutant has been mapped to a single base change resulting in a His-to-Arg substitution at amino acid 351 within the catalytic domain of the kinase (17). Antibodies raised against COT1 detect in *N. crassa* extracts a predominant 73-kDa polypeptide whose abundance is constant under all growth temperatures tested, while an additional 67-kDa polypeptide is present in extracts obtained from the wild type and the *cot-1* mutant grown at permissive temperatures but is almost undetectable in extracts obtained from the *cot-1* mutant grown under restrictive conditions (above 32°C). The *N. crassa* COT1 Ser/Thr protein kinase is related to the Rho kinase subfamily. COT-1 kinase highly resembles the *Drosophila* Warts kinase (23), the fission yeast Orb6 gene product (46), the budding yeast Cbk1 protein (4), and the human myotonic dystrophy kinase (DMPK) (25). Dysfunction or partial inactivation of these kinases leads to a dramatic change in the morphology of the cells or tissues of the organisms.

Myotonic dystrophy, the most prevalent muscular disorder in adults, occurs in individuals carrying a defect in the DMPK-encoding gene. The disease involves changes in cytoarchitecture and ion homeostasis (2). Reports have demonstrated that myotonia occurs with abnormalities of the muscle membrane, along with various channel abnormalities involving Na^+, K^+, and Ca^{2+} channels (7, 25, 33). Furthermore, the effective reduction of myotonia by use of ion channel blockers has been reported (45). It has also been suggested that the increased steady-state mRNA levels of Na^+ or K^+ ATPase may alter the regulation of the osmotic balance within the mouse eye lens (42).

Growing hyphae, like other cells, maintain a membrane potential and ionic gradients for transporting solutes in and out of the cell and establishing and maintaining apical organization, morphogenesis, and growth (11). The mechanistic aspects of sodium involvement in hyphal growth have been demonstrated by the identification and characterization of several sodium ATPases in *N. crassa* (3). The importance of proton efflux, rather than Na^+, in maintaining a pH gradient and a membrane potential has funneled much of the attention to analyses of the plasma membrane ATPase (11). In *Saccharomyces cerevisiae*, regulation of the plasma membrane ATPase ENA1 has been shown to be complex and can involve calcineurin, the high-osmolarity glycerol mitogen-activated protein kinase, and the cyclic AMP-dependent protein kinase A (PKA) pathways (18, 20, 26, 38). PKA, which can respond to general stresses (44), plays an important role in osmotic adaptation in parallel to mitogen-activated protein kinase (27, 31). Structural components of these pathways in different fungi have been identified. However, even though many of the components are highly conserved, some of their functions or regulatory mechanisms may differ (12, 13, 19, 34, 37).

In this article, we report on changes that occur in ion homeostasis in the *N. crassa cot-1* mutant, demonstrate morpho-

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logical suppression of the *cot-1* phenotype by imposing changes on the fungal membrane transport machinery and introducing osmotic or other environmental stresses, and compare the effects of environmental and genetic suppression on the abundance of immunodetectable COT1. We also show that the *cot-1* phenotype can be suppressed by inhibition of PKA, suggesting a functional link among COT1, PKA, and the cellular response to environmental stress.

MATERIALS AND METHODS

***N. crassa* strains, media, and growth.** *N. crassa* wild-type strains 74-OR23-1A and FGSC 987, *cot-1* (FGSC 4065), *ro-1* (FGSC 4531), *ro-3* (FGSC 43), *ro-1 cot-1* (36), and *ro-3 cot-1* (36) strains, and strain POP6 (17) were used throughout this study. Procedures used for fungal growth and other manipulations were described by Davis (11). Strains were grown in either liquid or on solid (supplemented with 1.5% agar) Vogel's medium with 1.5% (wt/vol) sucrose, unless stated otherwise. When required, diethylestilbestrol (DES; 50 μM), amiloride (150 to 200 μM), oubain (600 to 800 μM), KT-5720 (75 μM), H_2O_2 (7 mM), 8-bromoadenosine-cyclic AMP (cAMP) (1 to 5 mM), or ethanol (7.5%, vol/vol), each purchased from Sigma (St. Louis, Mo.), was added to the medium.

Protein extraction and immunoblotting. *N. crassa* mycelial samples were frozen in liquid nitrogen, pulverized, and suspended in lysis buffer (1 M sorbitol, 10 mM HEPES [pH 7.5], 5 mM EDTA, 5 mM EGTA, 5 mM NaF, 0.1 M KCl, 0.2% Triton X-100, complete protease inhibitor mixture [Roche Applied Science, Mannheim, Germany]). The samples were homogenized by 10 strokes of pestle A in a Dounce homogenizer. The homogenates were centrifuged for 40 min at 10,000 × *g*, and the supernatants were recovered and stored at −70°C until analysis. Proteins were separated by sodium dodecyl sulfate–7.5 or 10% polyacrylamide gel electrophoresis. Western blotting was performed by standard procedures (41). Antibodies used throughout this study included αCOT (17) and goat peroxidase-coupled secondary antibody (Amersham Biosciences, Freiburg, Germany).

Glycerol determination was performed as described by Ben-Amotz and Avron (1), with slight modifications. Proteins were extracted in the presence of HEPES (10 mM, pH 7.5) buffer (10 mM KH_2PO_3, 5 mM EGTA, 5 mM NaF, complete protease inhibitor mixture), which does not interfere with the detection of polyols. Protein extracts, brought to a final volume of 0.2 ml, were incubated with 0.2 ml of periodate reagent (65 mg of sodium periodate dissolved in 100 ml of 6% acetic acid containing 7.7 g of ammonium acetate) for 5 min at room temperature. The reaction was carried out in the presence of 0.625 ml of acetylacetone reagent (1.0 ml of acetylacetone in 99 ml of isopropanol) at 40 to 50°C for 15 to 20 min. After the samples had cooled, the optical densities at 410 nm were measured and compared to that of a glycerol standard (Sigma).

PKA activity assays. Protein extracts from *N. crassa* cultures were prepared from mycelial samples which had been frozen in liquid nitrogen and subsequently ground (twice, for 30 s each time, at 4,000 rpm) with 0.5-mm glass beads in a FastPrep FP120 bead beater (Savant, Farmingdale, N.Y.) in the presence of PKA extraction buffer (50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 5 mM EGTA, 10 mM NaF, 0.1 M KCl, 0.5% Triton X-100, 0.2% sodium dodecyl sulfate). The homogenates were centrifuged for 40 min at 10,000 × *g*, and the supernatants were recovered for PKA activity assays. PKA activity was determined on the basis of Kemptide phosphorylation, as measured by using a PepTag kit (Promega, Madison, Wis.) with minor modifications of the manufacturer's instructions (the PKA activator was diluted 10-fold, and the enzymatic reaction was carried out at 37°C).

Microscopy. For light microscopy, samples were viewed with a Zeiss Axioscope microscope. Photographs were taken with Fujichrome 100-ASA film. For X-ray microanalysis (9), hyphae were mounted on Ocolon mounts and transferred to the low-vacuum stage of a JEOL 5410LV scanning electron microscope equipped with a Link ISIS (Oxford Instruments, Oxfordshire, United Kingdom) energy-dispersive X-ray detector. This form of analysis provided an indication of the relative abundances of different ions within a scanned region.

RESULTS

Ionic balance is altered in a *cot-1* background. Defects in DMPK and DMPK-related proteins have been shown to be associated with ionic imbalance. As proton efflux is the key regulatory mechanism for the maintenance of membrane potential in fungi and is in concert with the balance of other ions, we first determined whether changes occur in proton efflux during the growth of *cot-1* cultures at restrictive temperatures. Changes in medium pH were used as an indication of alterations in proton efflux. Vogel's medium was inoculated with either *cot-1* or wild-type conidia and cultured on a rotary shaker for 20 h at 25°C. The cultures were then shifted to growth at 37°C, and the medium pH was measured periodically. Even though the medium pH decreased during the time course of the experiment, no significant difference in the rates of medium acidification between wild-type and *cot-1* cultures at the different time points was observed (Fig. 1A). However, a significant increase in the accumulation of wild-type culture biomass was measured relative to *cot-1* culture biomass. Because at the restrictive temperature *cot-1* culture growth was almost completely inhibited, the final *cot-1* culture biomass amounted to only about 25% that of the wild type (Fig. 1A). Thus, we concluded that the relative contribution of *cot-1* culture biomass to medium acidification is significantly higher than that of the wild type and that this finding is a clear indication of increased proton efflux by the mutant.

As proton efflux appears to be irregular in *cot-1* cultures grown under restrictive conditions, we tested the effect of several ion pump inhibitors on *cot-1* culture proton efflux and morphology. DES is a potential inhibitor of H^+ ATPases in different cell types (10). When the growth medium was amended with 50 μM DES (a concentration that did not significantly alter biomass accumulation), the extent of medium acidification by wild-type cultures was reduced to 0.5 to 0.6 pH unit, as measured 6 h after the temperature shift, whereas wild-type control cultures acidified the medium by 0.8 to 1.0 pH unit over the same growth period (Fig. 1B). The effect of DES on medium acidification by *cot-1* cultures was more dramatic (over the same time course, the pH was reduced by only 0.25 to 0.35 U). Furthermore, the *cot-1* phenotype was partially suppressed (Fig. 1C). Consequently, we also detected a slight increase in *cot-1* culture biomass.

We determined the influence of two other ion pump inhibitors on the medium acidification and morphology of *cot-1* cultures under nonpermissive conditions. Both inhibitors—amiloride (an Na^+ or H^+ antiporter inhibitor) (5) and oubain (an Na^+ or K^+ ATPase inhibitor) (48)—affected the extent of culture acidification (acidification over the 6-h growth period following the temperature shift was 0.4 to 0.45 pH unit). In addition, mild suppression of *cot-1* culture hyperbranching was evident in the growth cultures (Fig. 1C). If proton imbalance were the reason for the morphological defects observed in *cot-1* cultures, then blocking of proton or cation pumps might be able to suppress these defects. Even though these inhibitors had a significant effect on fungal cell proton efflux, induction of growth and elongation of *cot-1* hyphae with the addition of DES were very minor, and the effects of amiloride and oubain were even more subtle. We therefore concluded that the *cot-1* phenotypic defect most likely is not a result of proton imbalance.

To determine whether the increased proton efflux was accompanied by a change in the abundance of intracellular ions in the *cot-1* mutant, we performed X-ray microanalysis of hyphae. The relative abundances of phosphorus, sulfur, potassium, and sodium in wild-type and *cot-1* strains were moni-

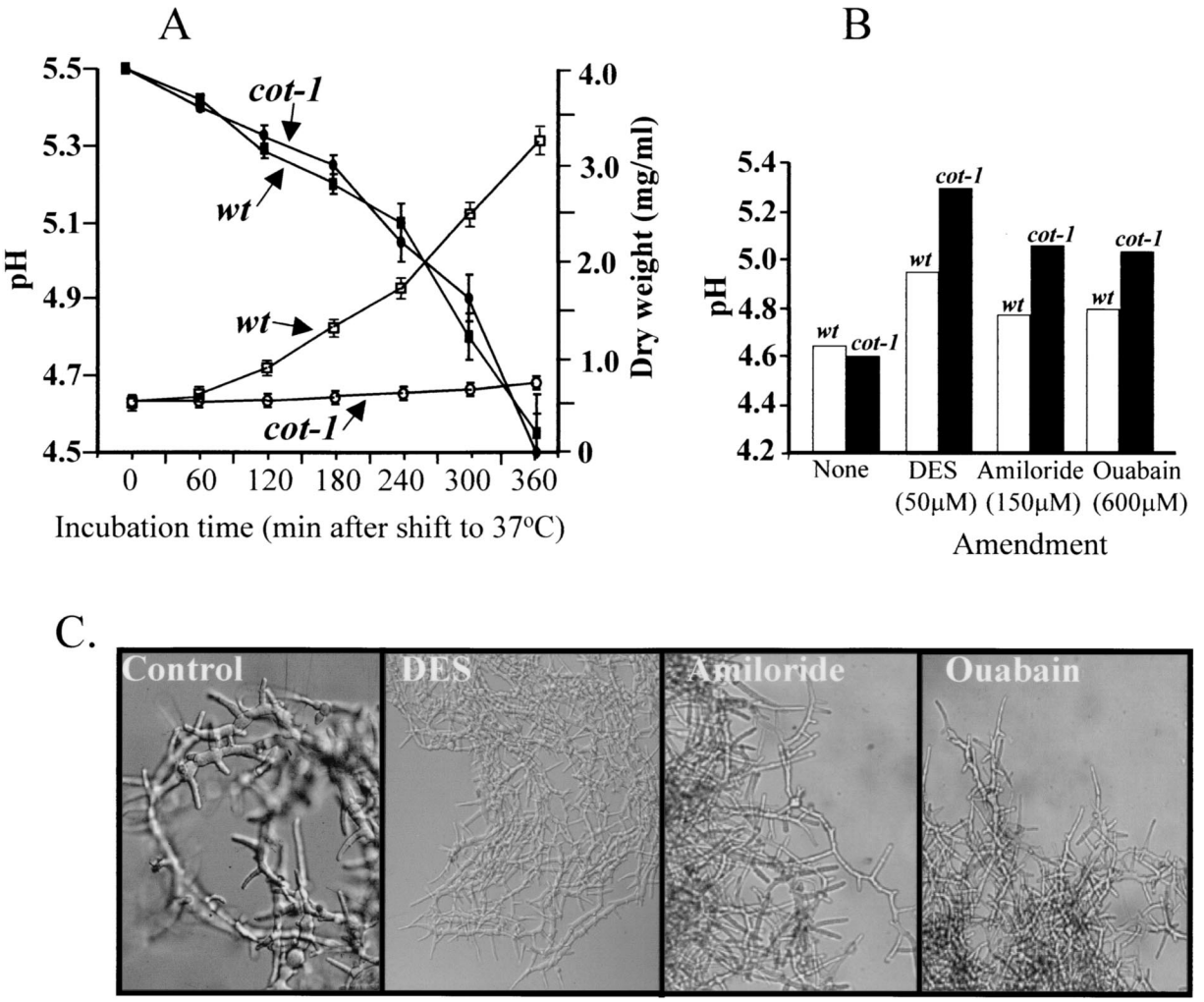

FIG. 1. Growth of *N. crassa* strains under different conditions. (A) Medium acidification and biomass accumulation during the growth of *N. crassa* wild-type (*wt*) and *cot-1* strains in Vogel's minimal salts medium. Error bars indicate standard deviations. (B) Medium pH of *wt* or *cot-1* cultures 6 h after a shift to 37°C in the presence of DES, amiloride, or ouabain. (C) Effect of DES, amiloride, or ouabain on *cot-1* culture morphology. Pictures were taken 8 h after the cultures were shifted from permissive (25°C) to restrictive (37°C) growth conditions (inhibitor concentrations were as shown in panel B).

tored. When the strains were grown at 25°C, the relative amounts of all analyzed ions were similar (data not shown). However, a clear decrease (two- to fourfold) in the relative abundance of sodium ions was observed in *cot-1* strains following a 6-h shift from 25 to 37°C (Table 1). No significant change in the relative abundances of the other ions was detected.

To determine whether overcoming the sodium deficiency would affect *cot-1* culture morphology, the growth medium was amended with NaCl at final concentrations ranging from 0.25

TABLE 1. Relative abundances of four elements in hyphae of *N. crassa* wild-type (*wt*) and *cot-1* strains grown at 37°C

Element	Mean ± SD % relative abundance in the presence of the following amendment for the indicated strain[a]:							
	None		50 mM DES		1 M NaCl		1.25 M sorbitol	
	wt	*cot-1*	*wt*	*cot-1*	*wt*	*cot-1*	*wt*	*cot-1*
P	35.0 ± 7.0	35.0 ± 7.0	35.0 ± 7.0	35.0 ± 7.0	30.0 ± 6.0	45.0 ± 9.0	35.0 ± 7.0	35.0 ± 7.0
S	12.5 ± 2.5	12.5 ± 2.5	12.5 ± 2.5	12.5 ± 2.5	10.0 ± 2.0	15.0 ± 3.0	10.0 ± 2.0	10.0 ± 2.0
K	50.0 ± 10.0	50.0 ± 10.0	45.0 ± 9.0	45.0 ± 9.0	10.0 ± 2.0	8.0 ± 2.0	45.0 ± 9.0	45.0 ± 9.0
Na	8.0 ± 2.0	3.0 ± 1.0	9.5 ± 2.0	4.5 ± 1.0	50.0 ± 10.0	30 ± 6.0	15.0 ± 3.0	10.0 ± 2.0

[a] Determined by X-ray microanalysis.

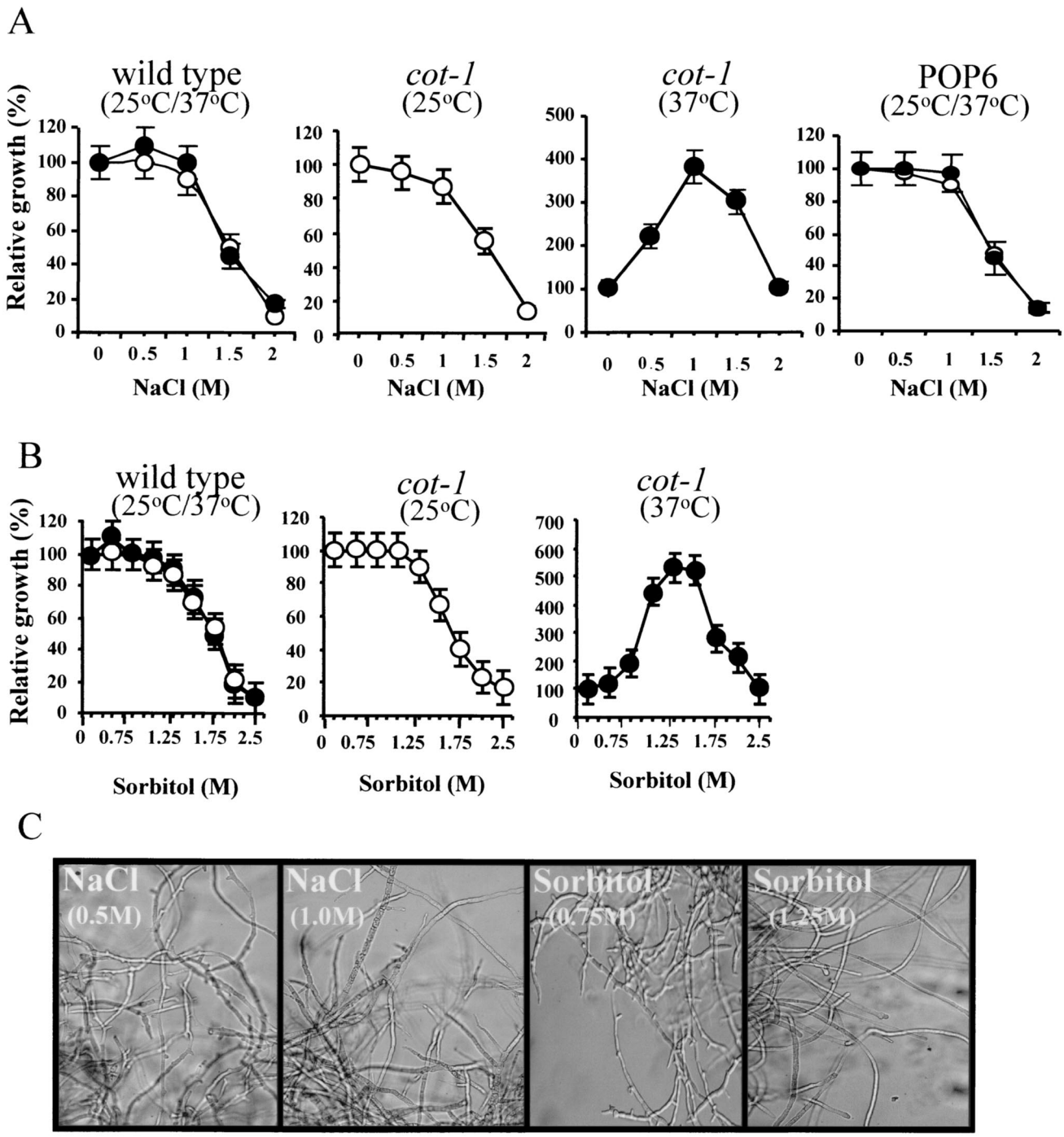

FIG. 2. Relative growth of *N. crassa* strains. (A and B) Relative growth, as a percentage of that of the untreated control, was determined on the basis of radial growth of *N. crassa* strains in the presence of different concentrations of NaCl or sorbitol, respectively. Full and empty circles indicate culture incubation temperatures of 37 and 25°C, respectively. Error bars indicate standard deviations. (C) Effect of NaCl or sorbitol on *cot-1* culture morphology. Pictures were taken 8 h after the cultures were shifted from permissive (25°C) to restrictive (37°C) growth conditions.

to 2 M. The radial growth of the wild type was not significantly affected in media containing NaCl at concentrations of up to 1.2 M (Fig. 2A). However, at the higher concentrations tested (up to 2 M), a clear inhibitory effect was measured, regardless of the growth temperature (25 or 37°C). The sensitivity to NaCl of *cot-1* cultures grown at 25°C was very similar to that of wild-type cultures, but at 37°C, clear suppression of the growth defect was observed in media amended with NaCl at concentrations ranging from 0.5 to 1.5 M (Fig. 2C). Moreover, the radial growth of *cot-1* cultures was more pronounced in media

with NaCl at concentrations of up to 1.5 M, where the hyperbranching phenotype was suppressed (Fig. 2A). A fungal transformant containing both mutant and wild-type alleles of *cot-1* (POP6) (17) exhibited growth characteristics similar to those of the wild type in the presence of increased salt concentrations (Fig. 2A), indicating that the reintroduction of COT1 function results in a resumption of the wild-type growth response to NaCl.

Environmental stresses lead to morphological suppression of the *cot-1* phenotype. Two lines of experimentation were used in order to establish the specificity of the effect of NaCl on the growth of *cot-1* cultures. To determine whether the effect is ion specific, *cot-1* cultures were grown in media amended with other ionic salts at concentrations ranging from 0.5 to 1.5 M. Amending the growth media with KCl and sodium acetate (instead of NaCl) resulted in suppression of the *cot-1* phenotype, similar to that observed in the presence of NaCl (data not shown). Interestingly, adding similar molar concentrations of Ca^{2+} did not alter the growth morphology of *cot-1* cultures.

To determine the specificity of the sodium ion effect, we cultured *cot-1* cells in the presence of LiCl. As Li^+ is apparently transported via Na^+ transporters, it can be used to induce changes in the sodium uptake machinery in a manner similar to that of Na^+ (29). However, LiCl can mimic the physiological effects of NaCl at molar concentrations that are 1 order of magnitude lower than those of the sodium salt. Thus, in this set of experiments, we were able to uncouple the ionic effect from the osmotic effect and determine that LiCl, applied at physiologically active concentrations (50 to 250 mM), does not affect *cot-1* cell morphology at the restrictive temperature (data not shown). Based on these experiments, we concluded that even if increased external Na^+ ion concentrations could restore the ionic balance in *cot-1* cells, the change in the external osmolarity imposed by the salt amendment, rather than the specific effect of sodium, would be the major cause for suppression of the *cot-1* phenotype at the restrictive temperature.

To further confirm this conclusion, we replaced the salt amendment with sorbitol, which does not penetrate cells as readily as salt ions yet, at the appropriate concentrations, will alter the extracellular osmotic pressure. Sorbitol was used at concentrations ranging from 0.25 to 2.5 M. At the higher concentrations tested (1.5 to 2.5 M), wild-type cell growth was inhibited at 25 and 37°C (Fig. 2B), as was *cot-1* cell growth at the permissive temperature (Fig. 2B). When cells were grown in media amended with sorbitol at concentrations of 1.0 to 1.5 M, *cot-1* filament elongation could be observed, even at the restrictive temperature (Fig. 2C). Along with the suppression of the *cot-1* phenotype in the sorbitol-amended medium, we also observed an increase in the relative intracellular sodium content (Table 1) and a reduction in the rate of acidification of the culture medium (to a difference of 0.4 to 0.5 pH unit 6 h after the temperature shift). These results indicate that suppression of the *cot-1* phenotype is not ion specific but is a result of the osmotic changes imposed on growing cells.

Since the *cot-1* hyperbranching phenotype is suppressed by changes in environmental conditions, we complemented the phenotypic analysis of *cot-1* by monitoring changes in the intracellular levels of glycerol (a key fungal osmoregulator). We found that when cells were grown at restrictive temperatures, steady-state levels of glycerol were two- to threefold higher in *cot-1* cultures than in wild-type cultures (1.25 ± 0.25 [mean and standard deviation] and 0.5 ± 0.1 μg/mg of dry weight, respectively). A decrease in glycerol levels (to 1.0 ± 0.15 μg/mg of dry weight) also accompanied the partial phenotypic suppression of *cot-1* obtained by the addition of DES. Whether the changes in the steady-state levels of glycerol in *cot-1* cells were directly imposed by the mutation in *cot-1* (e.g., affecting osmosensing) or were a downstream consequence of the severe phenotype has yet to be determined.

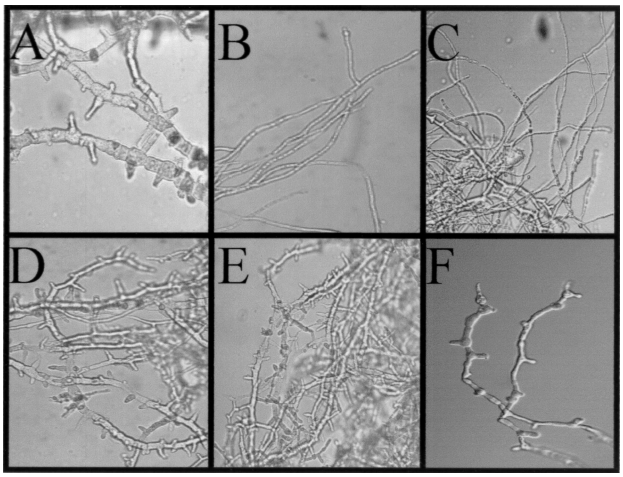

FIG. 3. Effect of medium amendments on the morphology of *cot-1* cultures grown at the restrictive temperature (37°C). Pictures were taken 6 h after the amendments were added and after the cultures were shifted from permissive (25°C) to restrictive (37°C) growth conditions. (A) Nonamended medium. (B) H_2O_2 (7 mM). (C) Ethanol (7.5%). (D) Sorbitol (1.0 M) plus 8-Br-cAMP (1 mM). (E) Glucose (3%). (F) Sorbitol (1.0 M) plus glucose(3%).

In addition to osmotic and salt stresses, we also tested the effects of oxidative stress and the presence of ethanol on the growth of *cot-1* cells. Amending the growth medium with noninhibitory concentrations of either H_2O_2 (5 to 7 mM) or ethanol (7.5%) resulted in the restoration of hyphal elongation and a clear reduction in the branching frequency for *cot-1* cells at the restrictive temperature (Fig. 3B and C). These results suggest that the suppression of *cot-1* is not limited to sensing of osmotic stress but most likely involves a more general stress response mechanism(s).

Impairment of PKA activity leads to suppression of the *cot-1* phenotype. The suppression of the *cot-1* phenotype by various environmental stresses suggests that components of the cellular stress signaling machinery may be linked to COT1 activity. Since PKA has been shown to be involved in fungal stress responses (including salt, ethanol, and oxidative stresses) (26, 27, 38, 53), we monitored PKA levels in extracts prepared from *cot-1* cultures (grown at the restrictive temperature) that had been suppressed by NaCl, H_2O_2, or ethanol. In all cases, a clear reduction in PKA activity levels was observed for protein extracts prepared from the fungal cultures (Fig. 4). While taking into consideration the fact that the assays used were limited to determination of the overall potential of PKA activity within a cell extract, if enhanced PKA activity has a direct effect on the fungal phenotype, then it is expected that the direct inhibition of PKA will also result in the suppression of *cot-1*. This

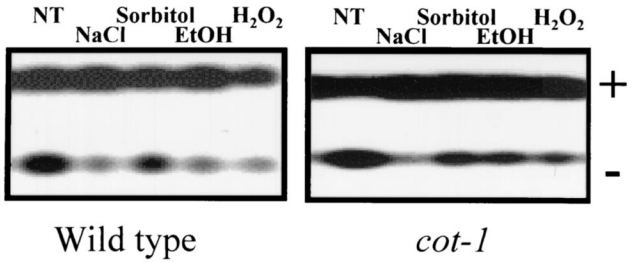

FIG. 4. Relative in vitro PKA activities measured in protein extracts prepared from *N. crassa* wild-type and *cot-1* cultures 5 h after a shift from permissive (25°C) to restrictive (37°C) growth conditions. Cultures were grown in the presence of NaCl (1.0 M), sorbitol (1.25 M), ethanol (EtOH) (7.5%), or H_2O_2 (10 mM). NT, not treated. The nonphosphorylated and phosphorylated (indicating activity) fluorescent Kemptide substrates migrated to the anode and cathode of the agarose gel, respectively.

is, in fact, the case, as amending the growth medium with a PKA inhibitor (KT-5720) resulted in partial suppression of the *cot-1* phenotype (Fig. 5A). This suppression was accompanied by a marked reduction in PKA activity in protein extracts (Fig. 5B). Furthermore, the addition of 1 mM 8-Br-cAMP, an agonist of PKA activity, resulted in increased severity of the *cot-1* phenotype, to the point where cultures could hardly develop (data not shown), along with increased PKA activity (Fig. 5B). In additional experiments, we determined that the presence of the PKA agonist almost completely inhibited the suppressive effect of sorbitol on *cot-1* cell morphology (Fig. 2C and 3D). Nonetheless, when wild-type cultures were grown in the presence of even five times the concentration of the PKA agonist (5 mM), no observable phenotype was evident (data not shown).

A clear linkage between the PKA pathway and carbon source sensing has been established for *S. cerevisiae* (44) as well as for *Aspergillus* (32). To determine whether *cot-1* phenotype suppression is affected by carbon source, we replaced sucrose with either glycerol (3%) or galactose (1.5%). In both cases, the *cot-1* phenotype was suppressed in a manner similar to that observed when *cot-1* cells were cultured in the presence of sorbitol. Furthermore, clear suppression of the *cot-1* phenotype was observed when the mutant was grown in a carbon-poor (0.15% sucrose) medium (data not shown). We also added glucose to sorbitol-amended *cot-1* cell growth medium in order to determine whether the presence of a repressing sugar can alter the suppressive effect of sorbitol. Our results indicated that glucose (also shown to activate PKA [31]) clearly inhibits the suppressive effect of sorbitol (Fig. 3E and F).

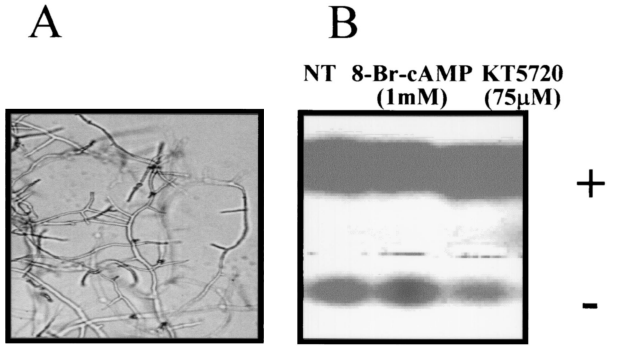

FIG. 5. Suppression of the *cot-1* phenotype by KT-5720. (A) Effect of KT-5720 on *cot-1* culture morphology at the restrictive temperature. (B) Relative in vitro PKA activities in proteins extracted from *cot-1* cultures grown in the presence of KT-5720 or a PKA activator (8-Br-cAMP) 5 min after introduction of the PKA effector. NT, not treated.

These results, along with the effects of the tested stress inducers and PKA agonists and inhibitors, indicate a link among COT1 function, PKA, and the regulation of hyphal elongation.

Correlation between suppression of *cot-1* cell morphology by environmental and genetic factors and the abundance of the 67-kDa COT1 polypeptide. Previously, it was shown that a decrease in the abundance of a 67-kDa polypeptide, which was detected by anti-COT1 antibodies, accompanied the cessation of growth of the *cot-1* mutant at restrictive temperatures. Furthermore, the presence of this polypeptide could be rapidly detected shortly after the cultures were shifted back to permissive temperatures (17). To determine the extent of the correlation between suppression of the morphological defects and the presence or absence of the 67-kDa polypeptide, we performed immunodetection analysis of *cot-1* cultures grown under different suppressive conditions. Western analyses with extracts from *cot-1* cultures that had been exposed to the described environmental stresses showed that suppression of the *cot-1* phenotype, induced by the addition of ion pump inhibitors or defined concentrations of NaCl or sorbitol, was accompanied by only very low, if any, detectable levels of the 67-kDa COT1 polypeptide (Fig. 6A and B). Similarly, the application of additional environmental stresses (H_2O_2 or ethanol) that suppress the *cot-1* phenotype did not result in any significant increase in the abundance of the 67-kDa polypeptide, which was clearly observed when the cultures were grown at permissive temperatures (Fig. 6C). The fact that we could not detect a change in the abundance of the 67-kDa band (which occurs very quickly after *cot-1* cultures are shifted from restrictive to permissive temperatures) suggests that the suppressed phenotype is a result of bypassing the requirement for a functional COT1 protein. This possibility is supported by the results of experiments in which the COT1 polypeptide expression pattern was determined for cultures where PKA activity had been inhibited by the presence of KT-5720 (resulting in suppression of the *cot-1* phenotype). As in experiments with environmental stress suppression, no significant change in the abundance of the 67-kDa band could be observed when PKA activity was inhibited (data not shown). We therefore concluded that the phenotypic response of the mutant to environmental stresses and alterations of PKA activity (which is involved in the cellular responses to a variety of stresses) probably does not involve the regaining of COT1 function (which, to date, has been correlated with the presence of the 67-kDa band).

Genetic suppressors of *cot-1* have been identified on the basis of improved growth at restrictive temperatures (36). Many of these have been determined to belong to the dynein-dynactin cytoskeletal motor complex and have been shown to be allelic to previously isolated *ropy* mutations (35). We used *ro-1* and *ro-3*, two extragenic suppressors of *cot-1* that encode cytoplasmic dynein and dynactin, respectively, to expand our COT1 immunodetection analysis. In contrast to the apparent lack of change in the abundance of the 67-kDa polypeptide

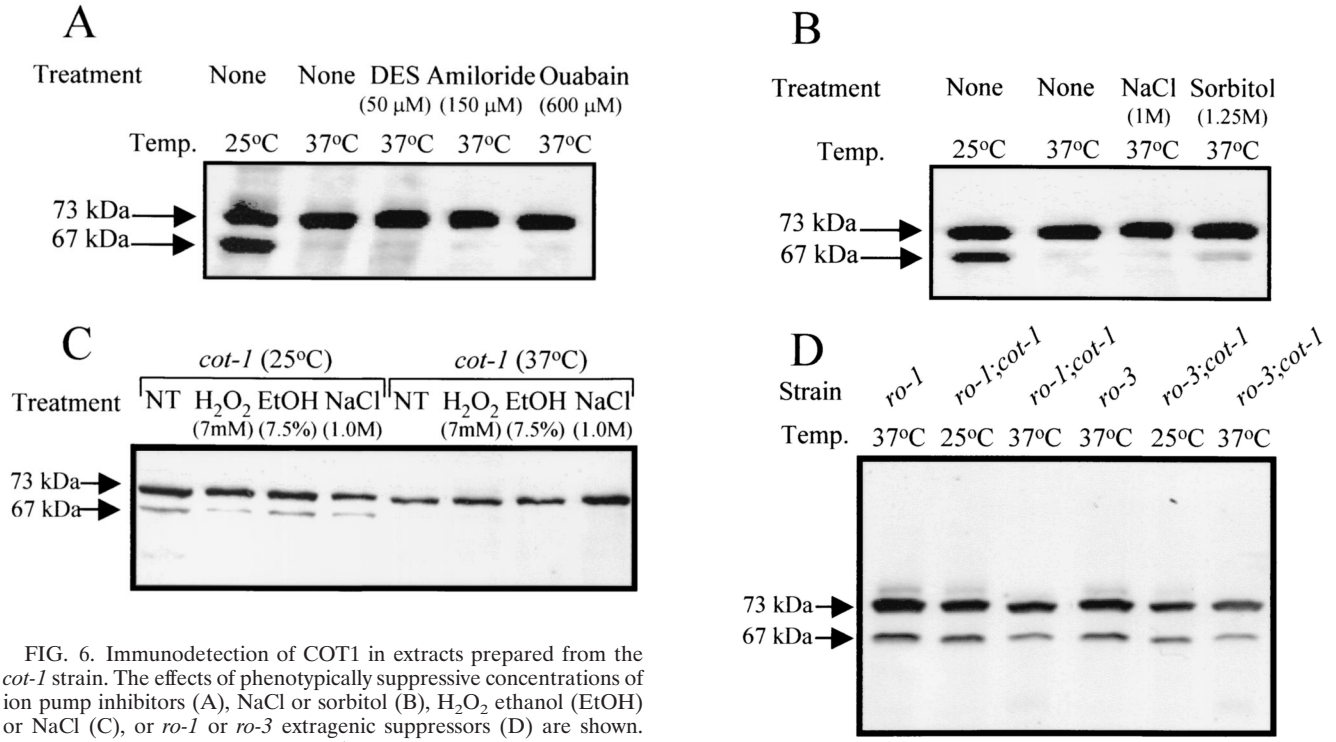

FIG. 6. Immunodetection of COT1 in extracts prepared from the *cot-1* strain. The effects of phenotypically suppressive concentrations of ion pump inhibitors (A), NaCl or sorbitol (B), H_2O_2 ethanol (EtOH) or NaCl (C), or *ro-1* or *ro-3* extragenic suppressors (D) are shown. Temp., temperature; NT, not treated.

band in the environmental stress-suppressed *cot-1* culture extracts, this polypepetide band was clearly seen in the genetically suppressed culture extracts, even though its abundance appeared to be lower than that in the wild-type control (Fig. 6D). Thus, it is conceivable that in these cultures, partial COT1 function was restored.

DISCUSSION

Because hyphal elongation is one of the key complex events in fungal growth, it is highly likely that multiple factors are involved in its regulation. The fact that mutations in a wide variety of genes (39, 50–52) can confer altered hyphal elongation (which, at times, is accompanied by hyperbranching events) is indicative of the fact that polar extension of the fungal cell and maintenance of normal cell shape are dependent upon the proper function of many cellular processes.

Even though COT1 has been identified as a kinase involved in hyphal elongation, the actual role of COT1 kinase in fungal cells has yet to be determined. In this article, we demonstrate that the consequence of impaired *cot-1* not only is represented by the drastic macroscopic or microscopic morphological changes that can be observed following the temperature shift (16) but also is accompanied by changes in ion homeostasis, sensitivity to environmental stresses, and probable alterations in stress sensing machinery. The initial analysis of changes in ion homeostasis were based on evidence of the occurrence of such changes in mammalian myotonic dystrophy cell lines. Results of experiments performed on mammalian cell lines focused mainly on Na^+ or K^+ levels. Our results suggest that changes in Na^+ levels accompany impaired COT1 function but are most likely (along with high proton efflux) a downstream consequence of the genetic defect. This notion is supported by the use of different ion pump inhibitors, which only slightly suppressed the *cot-1* phenotype.

In contrast to the mild suppressive effects of the ion pump inhibitors tested, the effects of various environmental stresses (i.e., osmotic, oxidative, and ethanol) were much more pronounced. The effects of these stresses suggest that a more general response mechanism is involved in mediating the suppressive effect. One of the common denominators of fungal responses to these stresses has been shown to involve cAMP-dependent protein kinase (26, 27, 38, 53). cAMP signaling has already been shown to control a number of developmental events, such as growth polarity in different fungi, including *N. crassa* (6), *Aspergillus niger* (43), and *Candida albicans* (30). For the plant pathogenic fungi *Ustilago maydis* (15), *Magnaporthe grisea* (28), and *Colletotrichum trifolii* (49) and the human pathogen *Cryptococcus neoformans* (24), cAMP signaling has been shown to be directly linked to fungal virulence (reviewed in reference 13).

We suggest that at least some of the altered sensing capabilities found to occur in *cot-1* strains involve PKA function. An increase in PKA activity can confer a defect in growth polarity (which is phenotypically distinct from that of *cot-1*), as occurs in the *N. crassa mcb* strain (6). Inhibiting PKA activity by amending the medium with KT-5720 resulted in a reduction in potential PKA activity (as measured in vitro) and in partial suppression of the *cot-1* phenotype (as is also the case with *mcb*; unpublished data). The link between PKA activity and polar growth is further supported by the fact that adding the PKA activator 8-Br-cAMP to *cot-1* cultures grown at 37°C in

the presence of sorbitol (*cot-1*-suppressing conditions) reverses the suppressive effect. Thus, even though the consequences of impaired COT1 function are pleiotropic, our results suggest that the defect in growth polarity can be attributed to altered PKA activity levels.

Based on our results, the function of COT1 may differ from that of its apparent *S. cerevisiae* homologue—Cbk1p. Screens in *S. cerevisiae* have identified several proteins that could potentially interact with Cbk1p, the yeast homologue of COT1 (21, 22, 40, 47). One of those proteins, Lre1p, shown to affect several stress-related cellular processes (e.g., chitinase activity and heat stress resistance) in yeast cells via inhibition of Cbk1p, does so independently of the cAMP or PKA pathway (47). Even though this finding suggests that at least some Cbk1p-related functions do not involve the PKA pathway, it does not rule out the possibility that Cbk1p and COT1 kinases differ in their function or regulation in yeasts and filamentous fungi. This notion is supported by the fact that based on the full genome sequence of *N. crassa* (14), no apparent structural homologue of Lre1p is present in this organism. Another possibility is that due to the higher morphological complexity of filamentous fungi, COT1 may have multiple functions, with different levels of association with the PKA pathway.

The differences in abundance of the 67-kDa band detected by the anti-COT1 antibodies in the case of genetic suppression versus environmental suppression may be indicative of the actual link between COT1 and the suppressive pathways. Thus, in the case of genetic suppression, the reappearance of the 67-kDa band, concomitant with its phenotypic suppression, suggests the involvement of COT1 abundance in reestablishing polar growth. One possible explanation for this phenomenon is that reducing the efficiency of retrograde transport (by impairing the motors involved in the process) may result in a reduced rate of functional COT1 turnover by maintaining higher levels of the protein in the vicinity of the plasma membrane. In contrast, suppression of the *cot-1* phenotype by environmental stress (which is not accompanied by the reappearance of the 67-kDa polypeptide) is not dependent on increased COT1 function. It is conceivable that COT1 and PKA activity are linked [directly or via another protein(s)] and that altering PKA activity by environmental stimuli or inhibitors bypasses the requirement for the involvement of COT1 in the process. Comparison of the COT1 polypeptide expression patterns indicates that the consequences of impaired COT1 function can be overcome by alternate routes.

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REFERENCES

1. **Ben-Amotz, A. and M. Avron.** 1978. On the mechanisms of osmoregulation in *Dunaliella*, p. 529–541. *In* S. R. Chaplan and M. Ginzburg (ed.), Energetic structure of halophylic microorganisms. Elsevier Press, St. Louis, Mo.
2. **Benders, A., P. Groenen, F. Oerlemans, J. Veerkamp, and B. Wieringa.** 1997. Myotonic dystrophy protein kinase is involved in the modulation of the Ca^{2+} homeostasis in skeletal muscle cells. J. Clin. Investig. **100:**1440–1447.
3. **Benito, B., B. Garciadeblas, and A. Rodriguez-Navarro.** 2000. Molecular cloning of the calcium and sodium ATPases in *Neurospora crassa*. Mol. Microbiol. **35:**1079–1088.
4. **Bidlingmaier, S., E. L. Weiss, C. Seidel, D. Drubin, and M. Snyder.** 2001. The Cbk1p pathway is important for polarized cell growth and cell separation in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **21:**2449–2462.
5. **Blazer, Y. B., and S. I. Helman.** 1997. The amiloride-sensitive epithelial Na+ channel: binding sites and channel densities. Am. J. Physiol. **272:**761–769.
6. **Bruno, K. S., R. Aramayo, P. F. Minke, R. L. Metzenberg, and M. Plamann.** 1996. Loss of growth polarity and mislocalization of septa in a Neurospora mutant altered in the regulatory subunit of cAMP-dependent protein kinase. EMBO J. **15:**5772–5782.
7. **Clausen, T.** 2000. Effects of amylin and other peptide hormones on Na+-K+ transport and contractility in rat skeletal muscle. J. Physiol. **527:**121–130.
8. **Collinge, A. J., M. H. Fletcher, and A. P. J. Trinci.** 1978. Physiology and cytology of septation and branching in a temperature-sensitive colonial mutant (*cot-1*) of *Neurospora crassa*. Trans. Br. Mycol. Soc. **71:**107–120.
9. **Connolly, M. S., N. Williams, C. A. Heckman, and P. F. Morris.** 1999. Soybean isoflavones trigger a calcium influx in *Phytophthora soja*. Fungal Genet. Biol. **28:**6–11.
10. **Coote, P. J., M. B. Cole, and M. V. Jones.** 1991. Induction of increased thermotolerance in *Saccharomyces cerevisiae* may be triggered by a mechanism involving intracellular pH. J. Gen. Microbiol. **137:**1701–1708.
11. **Davis, R. H.** 2000. Neurospora. Contributions of a model organism. Oxford University Press, Oxford, United Kingdom.
12. **Dickman, M. B., and O. Yarden.** 1999. Ser/Thr kinases and phosphatases in filamentous fungi. Fungal Genet. Biol. **26:**99–117.
13. **D'Souza, C. A., and J. Heitman.** 2001. Conserved cAMP signaling cascades regulate fungal development and virulence. FEMS Microbiol. Rev. **25:**349–364.
14. **Galagan, J. E., S. E. Calvo, K. A. Borkovich, E. U. Selker, N. D. Read, W. FitzHugh, L.-J. Ma, S. Smirnov, S. Purcell, B. Rehman, T. Elkins, R. Engels, S. Wang, C. B. Nielsen, J. Butler, D. Jaffe, M. Endrizzi, D. Qui, P. Ianakiev, D. Bell-Pedersen, M. A. Nelson, M. Werner-Washburne, C. P. Selitrennikoff, J. A. Kinsey, E. L. Braun, A. Zelter, U. Schulte, G. O. Kothe, G. Jedd, W. Mewes, C. Staben, E. Marcotte, D. Greenberg, A. Roy, K. Foley, J. Naylor, N. Stange-Thomann, R. Barrett, S. Gnerre, M. Kamal, M. Kamvysselis, C. Bielke, S. Rudd, D. Frishman, S. Krystofova, C. Rasmussen, R. L. Metzenberg, D. D. Perkins, S. Kroken, D. Catcheside, W. Li, R. J. Pratt, S. A. Osmani, C. P. C. DeSouza, L. Glass, M. J. Orbach, J. A. Berglund, R. Voelker, O. Yarden, M. Plamann, S. Seiler, J. Dunlap, A. Radford, R. Aramayo, D. O. Natvig, L. A. Alex, G. Mannhaupt, D. J. Ebbole, M. Freitag, I. Paulsen, M. S. Sachs, E. S. Lander, C. Nusbaum, and B. Birren.** 2003. The genome sequence of the filamentous fungus *Neurospora crassa*. Nature **422:**859–868.
15. **Gold, S., G. Duncan, K. Barrett, and J. Kronstad.** 1994. cAMP regulates morphogenesis in the fungal pathogen *Ustilago maydis*. Genes Dev. **8:**2805–2816.
16. **Gorovits, R., K. A. Sjollema, J. H. Sietsma, and O. Yarden.** 2000. Cellular distribution of COT1 kinase in *Neurospora crassa*. Fungal Genet. Biol. **30:**63–70.
17. **Gorovits, R., O. Propheta, M. Kolot, V. Dombradi, and O. Yarden.** 1999. A mutation within the catalytic domain of COT1 kinase confers changes in the presence of two COT1 isoforms and in Ser/Thr protein kinase and phosphatase activities in *Neurospora crassa*. Fungal Genet. Biol. **27:**264–274.
18. **Gustin, M. C., J. Albertyn, M. Alexander, and K. Davenport.** 1998. MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. **62:**1264–1300.
19. **Han, K. H., and R. A. Prade.** 2002. Osmotic stress-coupled maintenance of polar growth in *Aspergillus nidulans*. Mol. Microbiol. **43:**1065–1078.
20. **Hirata, D., and S. Harada.** 1995. Adaptation to high-salt stress in *Saccharomyces cerevisiae* is regulated by Ca^{2+}/calmodulin-dependent phosphoprotein phosphatase (calcineurin) and cAMP-dependent protein kinase. Mol. Gen. Genet. **249:**257–264.
21. **Ho, Y., A. Gruhler, A. Heilbut, G. D. Bader, L. Moore, S. L. Adams, A. Millar, P. Taylor, K. Bennett, K. Boutilier, L. Yang, C. Wolting, I. Donaldson, S. Schandorff, J. Shewnarane, M. Vo, J. Taggart, M. Goudreault, B. Muskat, C. Alfarano, D. Dewar, Z. Lin, K. Michalickova, A. R. Willems, H. Sassi, P. A. Nielsen, K. J. Rasmussen, J. R. Andersen, L. E. Johansen, L. H. Hansen, H. Jespersen, A. Podtelejnikov, E. Nielsen, J. Crawford, V. Poulsen, B. D. Surensen, J. Matthiesen, R. C. Hendrickson, F. Gleeson, T. Pawson, M. F. Moran, D. Durocher, M. Mann, C. W. V. Hogue, D. Figeys, and M. Tyers.** 2002. Syst. identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. Nature **415:**180–183.
22. **Ito, T., T. Chiba, R. Ozawa, M. Yoshida, M. Hattori, and Y. Sakaki.** 2001. A comprehensive two-hybrid analysis to explore the yeast protein interactome. Proc. Natl. Acad. Sci. USA **98:**4569–4574.
23. **Justice, R. W., O. Zilian, D. F. Woods, M. Noll, and P. J. Bryant.** 1995. The Drosophila tumor suppressor gene warts encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. Genes Dev. **9:**534–546.
24. **Kronstad, J., A. de Maria, D. Funnell, R. D. Laidlaw, N. Lee, M. M. de Sa, and M. Ramesh.** 1998. Signalling via cAMP in fungi: interconnections with mitogen-activated protein kinase pathway. Arch. Microbiol. **170:**395–404.
25. **Mahadevan, M. S., C. Amemiya, G. Jansen, L. Sabourin, S. Baird, C. E. Neville, N. Wormskamp, B. Segers, M. Batzer, J. Lamerdin, P. de Jong, B. Wieringa, and R. G. Korneluk.** 1993. Structure and genomic sequence of the myotonic dystrophy DM kinase gene. Hum. Mol. Genet. **2:**299–304.

26. **Márquez, J. A., and R. Serrano.** 1996. Multiple transduction pathways regulate the sodium-extrusion gene *PMR2/ENA1* during salt stress in yeast. FEBS Lett. **382:**89–92.
27. **Márquez, J. A., A. Pascual-Ahuir, M. Proft, and R. Serrano.** 1998. The Ssn6-Tup1 repressor complex of *Saccharomyces cerevisiae* is involved in the osmotic induction of HOG-dependent and independent genes. EMBO J. **17:**2543–2553.
28. **Mitchell, T. K., and R. A. Dean.** 1995. The cAMP-dependent protein kinase catalytic subunit is required for appressorium formation and pathogenesis by the rice blast pathogen *Magnaporthe grisea*. Plant Cell **7:**1869–1878.
29. **Nakamura, T., Y. Liu, D. Hirata, H. Namda, S. Harada, T. Hirokawa, and T. Miyakawa.** 1993. Protein phosphatase type 2B (calcineurin)-mediated, FK506-sensitive regulation of intracellular ions in yeast is an important determinant for adaptation to high salt stress conditions. EMBO J. **12:**4063–4071.
30. **Niimi, M.** 1996. Dibutyryl cyclic AMP-enhanced germ tube formation in exponentially growing *Candida albicans* cells. Fungal Genet. Biol. **20:**79–83.
31. **Norbeck, J., and A. Blomberg.** 2000. The level of cAMP-dependent protein kinase A activity strongly affects osmotolerance and osmo-instigated gene expression changes in *Saccharomyces cerevisiae*. Yeast **16:**121–137.
32. **Oliver, B. G., J. C. Panepinto, D. S. Askew, and J. C. Rhodes.** 2002. cAMP alteration of growth rate of *Aspergillus fumigatus* and *Aspergillus niger* is carbon-source dependent. Microbiology **148:**2627–2633.
33. **Overgaard, K., O. B. Nielsen, J. A. Flatman, and T. Clausen.** 1999. Relations between excitability and contractility in rat soleus muscle: role of the Na+-K+ pump and Na+/K+ gradients. J. Physiol. **518:**215–225.
34. **Palacek, S. P., A. S. Parikh, and S. J. Kron.** 2002. Sensing, signaling and integrating physical processes during *Saccharomyces cerevisiae* inavasive and filamentous growth. Microbiology **148:**893–907.
35. **Perkins, D. D., A. Radford, and M. S. Sachs.** 2001. The Neurospora compendium. Academic Press, Inc., San Diego, Calif.
36. **Plamann, M., P. F. Minke, J. H. Tinsley, and K. S. Bruno.** 1994. Cytoplasmic dynein and actin-related protein Arp1 are required for normal nuclear distribution in filamentous fungi. J. Cell Biol. **127:**139–149.
37. **Prade, R. A., P. Ayoubi, S. Krishnan, S. Macwana, and H. Russell.** 2001. Accumulation of stress and inducer-dependent plant-cell-wall-degrading enzymes during asexual development in *Aspergillus nidulans*. Genetics **157:**957–967.
38. **Proft, M., and R. Serrano.** 1999. Repressors and upstream repressing sequences of the stress-regulated *ENA1* gene in *Saccharomyces cerevisiae*: bZIP protein Sko1p confers HOG-dependent osmotic regulation. Mol. Cell. Biol. **19:**537–546.
39. **Propheta, O., J. Vierula, P. Toporowski, R. Gorovits, and O. Yarden.** 2001. The *Neurospora crassa* colonial temperature-sensitive 3 (*cot-3*) gene encodes protein elongation factor 2. Mol. Gen. Genet. **6:**894–901.
40. **Racki, W. J., A. M. Becam, F. Nasr, and C. J. Herbert.** 2000. Cbk1p, a protein similar to the human myotonic dystrophy kinase, is essential for normal morphogenesis in *Saccharomyces cerevisiae*. EMBO J. **19:**4524–4532.
41. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
42. **Sarkar, P. S., B. Appukuttan, J. Han, Y. Ito, C. Ai, W. Tsai, Y. Chai, J. T. Stout, and S. Reddy.** 2000. Heterozygous loss of Six5 in mice is sufficient to cause ocular cataracts. Nat. Genet. **25:**110–114.
43. **Saudohar, M., M. Bencina, P. J. I. van de Vondervoort, H. Panneman, M. Legisa, J. Visser, and G. J. G. Ruijter.** 2002. Cyclic AMP-dependent protein kinase is involved in morphogenesis of *Aspergillus niger*. Microbiology **148:**2635–2645.
44. **Thevelein, J. M.** 1994. Signal transduction in yeast. Yeast **10:**1753–1790.
45. **Tutdibi, O., H. Brinkmeier, R. Ruedel, and K. J. Foehr.** 1999. Increased calcium entry into dystrophin-deficient muscle fibres of MDX and ADR-MDX mice is reduced by ion channel blockers. J. Physiol. **515:**859–868.
46. **Verde, F., D. J. Wiley, and P. Nurse.** 1998. Fission yeast orb6, a ser/thr protein kinase related to mammalian rho kinase and myotonic dystrophy kinase, is required for maintenance of cell polarity and coordinates cell morphogenesis with the cell cycle. Proc. Natl. Acad. Sci. USA **95:**7526–7531.
47. **Versele, M., and J. M. Thevelein.** 2001. Lre1 affects chitinase expression, trehalose accumulation and heat resistance through inhibition of the Cbk1 protein kinase in *Saccharomyces cerevisiae*. Mol. Microbiol. **41:**1311–1326.
48. **Xu, W., and A. T. Marshall.** 1999. X-ray microanalysis of the Malpighian tubules of the black field cricket *Teleogryllus oceanicus*: the roles of NaK ATPase and the NaK_2Cl cotransporter. J. Insect Physiol. **45:**885–893.
49. **Yang, Z., and M. B. Dickman.** 1997. Regulation of cAMP and cAMP dependent protein kinase during conidial germination and appressorium formation in *Colletotrichum trifolii*. Physiol. Mol. Plant Pathol. **50:**117–127.
50. **Yarden, O., and C. Yanofsky.** 1991. Chitin synthase 1 plays a major role in cell wall biogenesis in *Neurospora crassa*. Genes Dev. **5:**2420–2430.
51. **Yarden, O., M. Plamann, D. J. Ebbole, and C. Yanofsky.** 1992. *cot-1*, a gene of *Neurospora crassa* required for hyphal elongation, encodes a protein kinase. EMBO J. **11:**2159–2166.
52. **Yatzkan, E., B. Szoor, Z. Feher, V. Dombradi, and O. Yarden.** 1998. Protein phosphatase 2A is involved in hyphal growth of *Neurospora crassa*. Mol. Gen. Genet. **259:**523–531.
53. **Zahringer, H., J. M. Thevelein, and S. Nwaka.** 2000. Induction of neutral trehalase Nth1 by heat and osmotic stress is controlled by STRE elements and Msn2/Msn4 transcription factors: variations of PKA effect during stress and growth. Mol. Microbiol. **35:**397–406.