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Recommendations for assigning symbols and names to Neurospora crassa genes now that its genome has been sequenced.

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Recommendations for assigning symbols and names to Neurospora crassa genes now that its genome has been sequenced.

Abstract

Originally, *Neurospora crassa* genes were named for their mutant phenotypes or natural variant properties. Genes are now increasingly named on the basis of cross-species sequence similarity. These names may also be supported by predicted or experimentally identified molecular function. As a consequence, *N. crassa* gene nomenclature in practice is frequently no longer adequately covered by the established conventions (Perkins *et al.* 2001). Here we provide additional nomenclature guidelines relevant to these new circumstances, and some general guidelines for providing information on the identity of *N. crassa* genes in scientific communications.

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The *Neurospora crassa* colonial temperature sensitive 2, 4 and 5 (*cot-2*, *cot-4* and *cot-5*) genes encode regulatory and structural proteins required for hyphal elongation and branching

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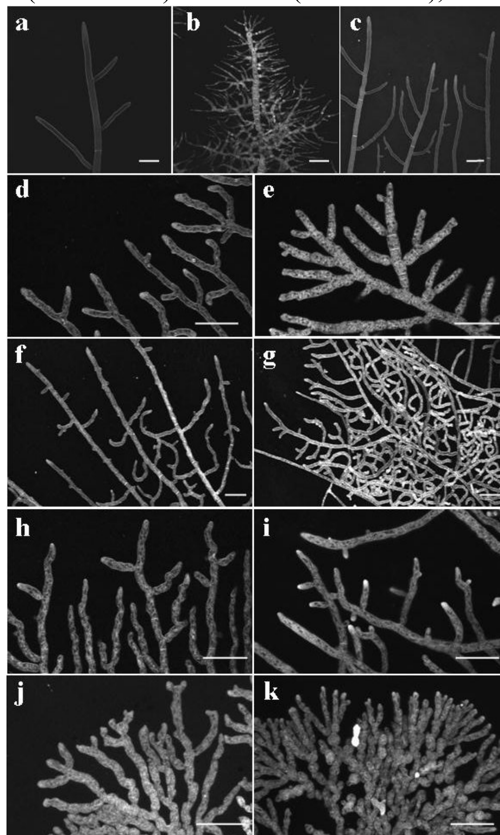
The morphology and the genetic defects of the *Neurospora crassa* colonial temperature-sensitive-*2*, *-4* and *-5* mutants were analyzed. *cot-2* is allelic to *gs-1* and encodes a component of the glucan synthesis process. *cot-4* encodes the catalytic subunit of a type 2B phosphatase and is allelic to calcineurin (*cna-1*). *cot-5* encodes a homologue of the *S. cerevisiae* ALG2 manosyltransferase-encoding gene, a component of the dolichol pathway.

A group of five non-allelic *Neurospora crassa* colonial temperature sensitive (*cot*) mutants was described by Garnjobst and Tatum (1967). The *cot-1* gene was found to encode a Ser/Thr protein kinase (Yarden *et al.* 1992) which is the founding member of the NDR kinase family. The nature of the *cot-3* defect has also been analyzed and the *cot-3* gene was found to encode protein elongation factor 2 (Propheta *et al*. 2001). In order to expand our understanding of the genetic defects that can confer abnormal hyphal elongation/branching patterns, we have performed morphological and genetic analyses of the three remaining *cot* mutants isolated by Garnjobst and Tatum. We found that even though they all exhibit compact temperature-sensitive macroscopic colonial features, their microscopic hyphal morphology and branching patterns differ. Furthermore, the genetic defects involved in conferring their phenotypes include both regulatory as well as structural factors, all of which are required for maintaining proper hyphal elongation and branching patterns.
Confocal microscopic examination, using the membrane-selective dye FM4-64 (as described by Hickey *et al.* 2005) of *Neurospora crassa* wild-type (74-OR23-1A; FGSC987), *cot-1* (FGSC 4065), *cot-2* (FGSC 1512), *cot-3* (FGSC 1517), *cot-4* (FGSC 3600) and *cot-5* (FGSC 1362), revealed significant morphological differences between the different strains (Fig. 1).



As the morphological features of *cot-1* and *cot-3* have been studied in depth (Collinge and Trinci, 1974; Collinge *et al*. 1978; Propheta *et al*. 2001), we focused on the quantification of the observed differences on *cot-2*, *cot-4* and *cot-5*. Hyphal extension rates of *cot-2* *cot-4* and *cot-5* were measured on a standard solid medium at permissive and restrictive conditions. All of the mutants exhibited a significant reduction (75 to 99%) in elongation rates (to 0.15±0.03 mm/h, 0.27±0.05 mm/h and negligible elongation for *cot-2*, *cot-4* and *cot-5*, respectively) and an increase in branching rates when cultured at the restrictive temperature. Even though the mutant's phenotypes are clearly temperature sensitive, we found that their branching rates were significantly higher (60 to 160%) even at the permissive temperatures (Table 1). For the most part, the hyperbranching patterns observed are lateral, rather than apical (Watters *et al*. 2000). Nonetheless, some apical/dichotomous branching was evident in the *cot-5* strain (regardless of temperature; Fig. 1j-k).

Figure 1. Morphology of wild type and colonial temperature sensitive strains of *N. crassa* at permissive (24 °C) and restrictive (37 °C) temperatures. Fungi were stained with FM4-64 and imaged using a confocal microscope. (a) *cot-1* grown at 24 °C; (b) *cot-1*, 37 °C; (c) wild type, 37 °C; (d) *cot-2*, 24 °C; (e) *cot-2*, 37 °C; (f) *cot-3*, 24 °C; (g) *cot-3*, 37 °C; (h) *cot-4*, 24 °C; (i) *cot-4*, 37 °C; (j) *cot-5*, 24 °C; (k) *cot-5*, 37 °C. Bars are 50 um.

Strain	Average distance (um) between branches at 25 ^{o} C	Average distance (um) between branches at 34 ^{o} C	Increase in branching frequency (%) at 34 ^{o} C
wt	180±4.0	176±14.1	≈0
cot-2	110±7.0	30±1.8	360%
cot-4	70±1.6	30±0.8	230%
cot-5	90±7.0	20±0.4	450%

Table 1. Average length between branches of *cot-2, cot-4, cot-5* and the *wt* strain grown at permissive (25 ^{o}C) or restrictive (34 ^{o}C) temperatures.

We cloned the three genes by complementation (Davis 2000), utilizing the Orbach/Sachs *N. crassa* genomic DNA cosmid library (Orbach and Sachs 1991). The *cot-2* and *cot-4* genes are unlinked. However, as both genes reside on linkage group V (Perkins *et al.* 2001) the same complementation strategy was employed for identifying both genes. A single cosmid (G23:G5) capable of complementing the *cot-2* phenotype was isolated from a cosmid library that had been assigned to linkage group V. A 6kb *Hind*III fragment isolated from the cosmid was sufficient to complement the mutant. Sequence analysis of this fragment revealed the presence of a single predicted gene encoding a protein designated GS-1. GS-1 has been shown to be required for beta (1,3) glucan synthase activity in *N. crassa* (Enderlin and Selitrennikoff 1994). During the course of the complementation experiments, we determined that a DNA fragment encoding a C-terminal-truncated version of the gene (corresponding to a gene product 188aa shorter than the original protein) was sufficient for complementation of *cot-2*.

The mutant *cot-2* allele was amplified (using primers ACCTGTAGAAAGCGGAAAGT and AGCCGTGAGAGAGGTTGT) and sequenced. A single C to T substitution in codon 167 resulted in a Ser to Phe substitution of a residue found to be identical (and within a conserved region) in 6 other fungal glucan synthases. This substitution is likely to result in impaired glucan synthase activity. This is supported by the observation that in addition to the hyperbranching phenotype, *cot-2* also exhibited significantly higher sensitivity, with respect to the wild type, to the echinocandin glucan synthase inhibitor caspofungin (Kurtz *et al*. 1996), as extensive hyperbranching of the *cot-2* mutant was evident in the presence of 0.01ug/ml caspofungin, at permissive temperature, whereas a ten-fold higher concentration of the inhibitor was required to induce a comparable phenotype in the wild type. The fact that caspofungin-induced hyperbranching was observed in the *cot-2* mutant even at 25 ^{o}C strongly suggested that glucan synthase activity is lower in this strain, even at the permissive temperature. GS-1 has been shown to be involved in beta (1,3) glucan synthesis of the fungal cell wall. Based on our analyses, a truncated version of the gene was sufficient for complementation of the elongation/branching defect of *cot-2*. When first describing *gs-1*, Enderlin and Selitrennikoff (1994) stated that a truncated (≈140 amino acids shorter) wild type *gs-1* allele was sufficient for complementation. In this study we found that even a shorter version of the gene is sufficient. The apparently unnecessary C-terminal region of the protein may be involved in additional cellular functions not examined in this study. Support for this possibility can be found in reports suggesting that the role of the *S. cerevisiae* SM1/KNR4 homologue of COT2 is, most likely, regulatory (Martin *et al*. 1999), and that on the basis of additional analyses in yeasts (Dagkessamanskaia *et al*. 2001), it is conceivable that COT2 participates, via interactions with several proteins, in additional cellular processes. Other alleles of *cot-2*/ *gs-1* have been identified in an additional screen (Seiler and Plamann 2003). However, the genetic nature of the defect in these additional mutants has yet to be elucidated.

A 4.2kb *Bam*HI/ *Apa*I subclone, containing NCU03804.3, isolated from cosmid X15:E10 was sufficient to fully complement the *cot-4* mutant. NCU03804.3 is the *N. crassa* type 2B protein phosphatase (PP2B/calcineurin) which, when impaired, was shown to induce hyperbranching in this fungus (Prokisch *et al*. 1997). Further experiments revealed that *cot-4* was more sensitive than the wild type to the specific calcineurin inhibitors, Cyclosporin A and FK-506, but not to hygromycin B, a general inhibitor of protein synthesis (Fig 2.)

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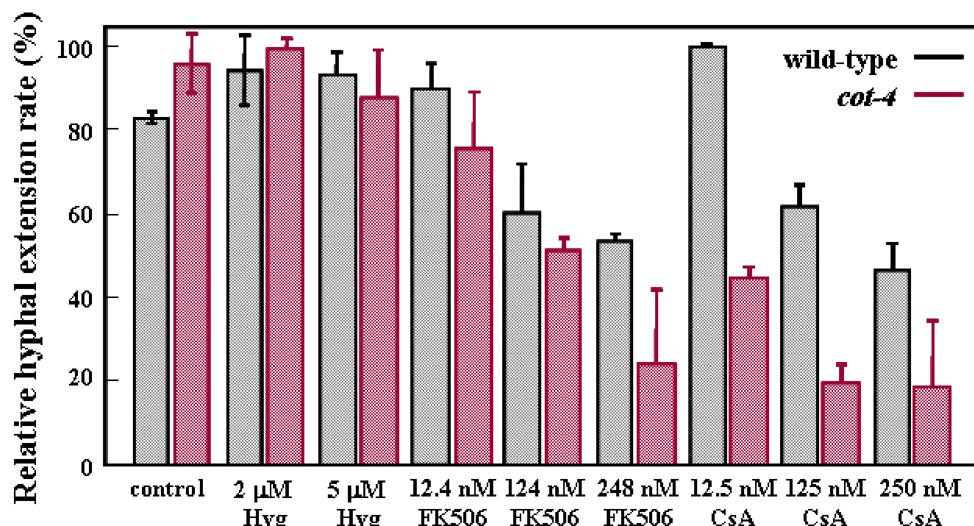

Figure 2. The effect of two calcineurin inhibitors, FK506 and Cyclosporin A (CsA), on the relative hyphal extension rates of wild type and *cot-4* colonies grown on a solid medium at 24 °C. Hyphal extension rates were determined by the change in colony diameter over time. Hygromycin B (hyg), a general protein synthesis inhibitor, was used as a control. Bars indicate standard deviation based on a 6 replicate experiment.

The *cot-4* mutant allele was amplified (using primers ACCCGGTGACTTTTATGCAG and TTGGCGAGCTATTCGATCTT) and sequenced. A single C to T substitution in codon 25 was found in the mutant allele. This mutation results in a Pro to Arg change in a residue that is highly conserved within fungal calcineurins, but variable in many higher eukaryotes.
Complementation of the *N. crassa cot-5* mutant was assisted by the fact that a chromosome walk had been performed in the vicinity of *cot-5* (Saupe *et al*. 1996). A 3kb *Sac*I/ *Hind*III fragment, encoding NCU03503.3, a homologue of the *S. cerevisiae* ALG2 gene encoding a mannosyl transferase (Jackson *et al*. 1993; Borkovich *et al*. 2004), isolated from cosmid G20:11 was sufficient to complement *cot-5*.

Based on a sequence comparison between the mutant and wild type alleles of *cot-5* (amplified with primers GGTACCATGATGATGATGAGGC and GGAGACGCAAGCGGCTATG), the mutation responsible for the *cot-5* phenotype resides in codon 37 of *cot-5*. The C to T transition in the mutant changes the wild type CAG codon (encoding glutamine) to an amber stop codon (TAG). This stop codon resides in a highly conserved part of the protein. Additional support linking the morphological defect with impaired N-glycosylation was obtained by applying Tunicamycin, an inhibitor of the dolichol pathway (Kukuruzinska *et al.* 1987) to wild type and *cot-5* cultures. At concentrations of 4 to 10ug/ml, Tunicamycin was able to induce hyperbranching in a manner that phenocopies the morphology of *cot-5*. Thus, it is quite possible that the *cot-5* mutation results in reduced glycosylation efficiency. Our results suggest a connection between a normally functioning *N*-glycosylation pathway and proper hyphal elongation. The fact that a defect in the dolichol pathway can affect polarity is also supported by the identification of a mutant in a gene encoding an additional mannosyltransferase, NCU07261.3 (a homologue of the *S. cerevisiae* Alg1), which, when mutated, confers a temperature sensitive colonial growth phenotype (Seiler and Plamann 2003). We suggest two possible explanations for the link between the genotypic defect found in *cot-5* (amber codon at amino acid position 37) and the temperature sensitive phenotype: (i) it is possible that premature translational termination occurs, indicating that COT5 is partially redundant. If that is the case, perhaps an additional manosyltransferase within the dolichol pathway can compensate, in a limited and temperature-sensitive manner, for the lack of COT5 function (two such candidates are the structurally-related NCU03317.3 and NCU00163.3, which are similar to the *S. cerevisiae* Alg6 protein); (ii) as amber suppressors can be obtained in *N. crassa* (Fang *et al.* 2002), it is conceivable that such a mutation accompanies the *cot-5* strain, resulting in a full or partial readthrough despite the amber codon.

The *cot* mutants were originally isolated by the fact that they showed colonial growth at 34 °C (the optimal temperature for *N. crassa* growth), but improved growth at a lower temperature (24 °C). Despite the improvement at permissive temperatures, the hyphal elongation/branching rates exhibited by some of these mutants at the permissive temperature are significantly different from the wild type under the same conditions. Nonetheless, the assumption that the phenotypic severity of such mutants positively correlates with the increase in temperature may not necessarily be accurate. Though not tested in this study, it is highly possible that phenotypic changes with temperature are incremental (rather than directly proportional). It is also possible that lowering the temperature to sub-optimal levels will, in some cases, also result in increased hyperbranching as it

occurs, for example, when *cot-5* is grown at 18 °C (Resheat-Eini and Yarden, unpublished).
The comparative functional analysis of factors involved in hyphal elongation and branching within the fungal kingdom is in its infancy. On the one hand, inactivation of *cot-1* homologues in several fungi (including *Aspergillus nidulans*, *Colletotrichum trifolii*, *Calviceps purpurea* and *Ustilago maydis*) results in similar defects in polarity (Buhr *et al.* 1996; Durrenberger and Kronstad 1999; Scheffer *et al*. 2005; Johns *et al*. 2006), suggesting that the mutations identified in the genes analyzed during this study may confer polarity/cell shape defects in additional fungi.

On the other hand, even though reduced calcineurin activity has already been shown to affect hyphal polarity in *N. crassa* and *A. nidulans*, no significant effect on hyphal growth of *C. albicans* was observed (Bader et al. 2003), despite the protein having a clear effect on pathogenicity. Calcineurin was also shown to be required for growth and virulence of *Cryptococcus neoformans* at mammalian body temperature (Fox et al. 2001). In addition to affecting human pathogens, impaired calcineurin expression also affected development and pathogenicity in the polyphagous plant pathogen *Sclerotinia sclerotiorum* (Harel *et al*. 2006).

Although the *cot* mutants were originally identified and grouped together based on their phenotypic responses to temperature, these responses are clearly the result of mutations in different genes whose functions vary immensely. Though eventual functional links between the different gene products may become evident in the future, this observation is not unexpected, given the complexity of hyphal elongation and branching and the fact that it requires the proper function of a wide array of cellular processes.

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