Analysis of the DNA sequence of the putative ABC transporter NCU09975 in *Neurospora crassa* strains carrying acriflavin resistance markers.

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Genomic DNA sequence was determined for the putative *Neurospora crassa* ABC transporter NCU09975 from several different classical mutant strains including several acriflavin resistant mutants. The sensitivity of these strains to acriflavin was tested. While the open reading frame NCU09975 has multiple polymorphisms in strains sequenced for other purposes, none of the acriflavin resistant classical mutants tested had polymorphisms in the NCU09975 coding region or in the 195 bases upstream of the translation start site.

Introduction

While years of mutagenesis and subsequent characterization in Neurospora crassa produced one of the most densely saturated genetic maps (Perkins et al. 2001), many of the classical mutants are not yet associated with open reading frames from the genome sequence (Galagan et al. 2003). Among these are acriflavin resistant mutants which have been assigned to seven different loci. A subset of these genes also confer resistance to multiple related drugs, including acridine orange, malachite green, and aminotriazole (Akiyama and Nakashima 1996). Moreover some acriflavin resistance genes are dominant while others are recessive. Because we wanted to find a dominant selectable marker for transformation of Neurospora and because the abc-3-like ORF NCU09975 had a large number of polymorphisms in its primary sequence among a group strains subject to whole genome sequence (McCluskey et al. 2011) we undertook to characterize this locus in strains carrying the acriflavin resistance markers acr-1 and Acr-3. We additionally validated the acriflavin sensitivity in the classical mutant strains carrying polymorphisms in NCU09975.

Materials and methods

Strains were cultured on Vogel's minimal medium (Vogel 1956) using standard practices (Davis and De Serres 1970) (Table 1).

Table 1. Strains and their characteristics

FGSC number	Genotype	Marker Location	Acriflavin sensitivity
2489	*mat-A*	IL	Sensitive
1215	*Acr-3 mat-a*	IL IL	Resistant
1209	*Acr-3 mat-A*	IL IL	Resistant
875	*acr-1 mat-a*	IL IL	Sensitive
305	*amyc mat-A*	IL IL	Sensitive
1363	*smco-1 mat-A*	I IL	Sensitive
3921	*tng mat-A*	IIL; IL	Sensitive
7022	*fld*	IVR; IL	Sensitive

Acriflavin was dissolved in water and filter sterilized prior to addition to sterile culture medium. It was stored in the dark and fresh stocks were prepared regularly. Acriflavin sensitivity testing was carried out by pipetting 10 ul of a freshly prepared suspension of conidia and hyphal fragments in water (approximately 10^3cfu/ ml) onto the surface of agar solidified medium in 10 x 75 mm glass culture tubes. Results were logged after two, four and ten days. Genomic DNA was extracted from 2 - 3 day old liquid shake cultures using the ZR Fungal DNA kit (Zymo Research, Irvine, CA). NCU09975 DNA was amplified as shown in Figure 1, using primers specific for this ORF (Table 2). Primers 1F and 5R were used together to amplify a 2848 base fragment including the start codon and 195 upstream bases. Primers 7F and 11R were used to amplify a 2878 base fragment including the stop codon and 667 bases downstream (Figure 1). These large fragments which overlap by 370 bases were directly sequenced at the UMKC School of Biological Sciences core facility using an Applied Biosystems 3100 Genetic Analyzer (Foster City, USA). Sequences were aligned and analyzed using Sequencher (Gene Codes Corporation, Ann Arbor, USA).

Table 2. Primers

Primer Designation and Orientation	Position	Sequence
1F	-195	ATTCGTCTCGACTTGCGACT
2F	24	TTCGTTGTCACTCGTCTTGG
3F	306	CGTTTGAGTTGGCGATCA
4F	784	AGCAGCCCTGACTTGCAT
5F	1303	CCTTCTTCGCTGCCTTTG
6F	1813	TCATCGACCGCAAGTCAA
7F	2283	AGCGGCTCGTTGAAGATG
8F	2784	AAATTGGAGGAGCTGCGATA
9F	3277	TCCGCTTCTATCGGTACACA
10F	3778	TCGATGGCATTGGGTTTT
11F	4295	CCACCGGGTCAGTTTGTC
12F	4786	CGAAGGTGGAGCAGATGG
1R	641	TGAGGGGTCTCGTCTTCCT
2R	1135	ATCCTGATGGCGTTGGTG
3R	1634	TCCAGCGTACACGCAAAA
4R	2149	CCTGCATGCTTACCTGCTG
5R	2653	CGACCGTTGGACATGACA
6R	2750	CTTTTGGGGTTCGTCATCAT
7R	3163	AGTTGCCAAGGGCTACGA
8R	3635	TGGCCATGATAGCCTCAGA
9R	4136	CGCCGTTGCTACGTTTTT
10R	4628	GAGATCCGCAGGGGGTAG
11R	5161	GACGGAGATGACCCGAAA

Figure 1. Primer map for NCU09975. Primers (Table 2) were used for amplification or sequencing of NCU09975.

Results and Discussion

The primary sequence of NCU09975 was identical to wild type in strains FGSC 1215 and FGSC 1209 (Acr-3) and also in strain FGSC 875 (acr-1, Table 1). Strain FGSC 1215 and FGSC 1209 were both resistant to acriflavin at the highest concentration tested (50 ug/ml). Strain FGSC 875 which caries the acr-1 mutation was sensitive to acriflavin at all concentrations above 2.5 ug/ ml, as was the reference genome strain FGSC 2489 (74-OR23-1VA, Table 1 and Figure 2).

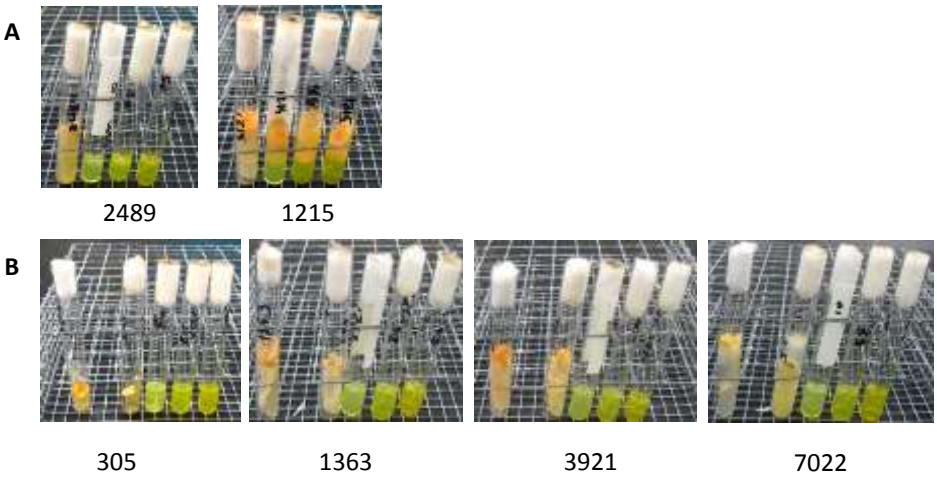

Figure 2. Acriflavin sensitivity tests A) Wild type (2489) and *Acr-3* mutant strains (1215) of *Neurospora crassa*. Left to right (in each test tube): 2.5 ug/ml acriflavin, 10 ug/ml, 25 ug/ml, 50 ug/ml. B) Acriflavin sensitivity of classical mutant strains of *N. crassa*. Left to right (in each test tube): 0 ug/ml acriflavin, 2.5 ug/ml, 10 ug/ml, 25 ug/ml, 50 ug/ml.

Single Nucleotide Polymorphisms (SNPs) in NCU09975 were detected in four strains subject to whole genome sequence as part of another study (McCluskey et al. 2011). These strains, FGSC 305, 1363, 3921 and 7022, all have mutations unrelated to their potential response to acriflavin (Table 1). While most of the polymorphisms at NCU09975 in these strains are shared among all four strains, strain 3921 has ten unique SNPs and strain 7022 has two unique SNPs. Both of the unique SNPs in FGSC 7022 are in the ninth intron. The unique SNPs in strain 3921 include four synonymous SNPs, four non-synonymous SNPs and one SNP each in intron one and two, respectively. Among the shared SNPs in these strains are fourteen that are shared among multiple strains. One SNP is present in two strains, ten SNPs are found in three strains and three SNPs are found in all four strains. Eight of these SNPs are synonymous while three are non-synonymous and three are found in introns. None of these strains had any insertions or deletions in NCU09975. All four of these strains were sensitive to acriflavin at 10 ug/ ml, as was the wild type strain FGSC 2489 (Figure 2). Strain FGSC 305 contains the morphological mutation *amyc* which causes it to grow as a small dot-like colony (Perkins 1959) and while this makes comparison to wild-type difficult, the comparison between growth of this strain on Vogels medium without acriflavin and with acriflavin was straightforward (Figure 2B). Strains FGSC 1363, 3921 and 7022 each carry morphological

mutations (Table 1), but these did not impact the ability to score their growth on acriflavin containing medium.

The acriflavin resistance gene *Acr-2* (NCU05733) was previously characterized as a Zn(II)Cys6 binuclear domain containing protein and disruption of this gene rendered progeny acriflavin sensitive (Akiyama and Nakashima 1996). Like *Acr-3*, *acr-4* is also on linkage group IL and it confers resistance to 50 ug/ml acriflavin. Genetic mapping places *acr-4* 5 map units away from *Acr-3* (Hsu 1965) and while *Acr-3* is a dominant mutation, *acr-4* is recessive. *acr-5* is on linkage group II L and unlike other acriflavin resistance traits does not confer resistance to other drugs such as malachite green or acridine orange (Akiyama and Nakashima 1996). Some alleles of *acr-5* are only manifest in strains also carrying the morphological marker *mo*(KH161). *acr-6*, on linkage group III R, also confers resistance to acridine orange, but not to malachite green (Akiyama and Nakashima 1996).

While this work did not identify the ORF responsible for acriflavin resistance conferred by mutations at the *Acr-3* locus in *N. crassa*, analysis of additional ORFs in the region near NCU09975 is ongoing.

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