RESEARCH ARTICLE

Molecular analysis of mutants of the *Neurospora* adenylosuccinate synthetase locus

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Abstract

The *ad-8* gene of *Neurospora crassa*, in addition to being used for the study of purine biology, has been extensively studied as a model for gene structure, mutagenesis and intralocus recombination. Because of this there is an extensive collection of well-characterized *N. crassa ad-8* mutants in the Fungal Genetics Stock Center collection. Among these are spontaneous mutants and mutants induced with X-ray, UV or chemical mutagens. The specific lesions in these mutants have been genetically mapped at high resolution. We have sequenced the *ad-8* locus from 13 of these mutants and identified the molecular nature of the mutation in each strain. We compare the historical fine-structure map to the DNA and amino acid sequence of each allele. The placement of the individual lesions in the fine-structure map was more accurate at the 5′ end of the gene and no mutants were identified in the 3′ untranslated region of this gene. We additionally analysed *ad-8*$^{+}$ alleles in 18 *N. crassa* strains subjected to whole-genome sequence analysis and describe the variability among *Neurospora* strains and among fungi and other organisms.

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Introduction

The *Neurospora crassa* adenosine biosynthetic pathway was one of the first where genetics and biochemistry were integrated, leading to a deep understanding of the biology of nucleotide synthesis and also providing a valuable tool for the study of gene structure and function, gene conversion, mutation, and DNA repair (Westergaard 1957). One area where the *Neurospora* genetic system was exploited was in the characterization of intragenic recombination, and *ad-8* locus was particularly illustrative (Ishikawa 1962a).

The *ad-8* genes encode an adenylosuccinate synthetase (EC 6.3.4.4). Mutation in *ad-8* causes auxotrophy in haploid *Neurospora* cells (Johnson and Giles 1956). The genetic order of the specific lesions in *ad-8* were mapped by intraallelic recombination (Ishikawa 1962a,b), and many of these strains are available in the collection of the Fungal Genetics Stock Center (FGSC), University of Missouri, although the molecular nature of these mutations has never been determined. Notwithstanding this, the *Neurospora* purine biosynthetic pathway continues to serve as a valuable model for regulation and integration of general metabolism into processes such as DNA repair and even human circadian rhythms (Baggott *et al*. 2011) and response to chemotherapeutic agents (Miller *et al*. 2009). The branch point in purine biosynthesis occurs after the production of inosine-5′-P (IMP). Adenylosuccinate synthetase, using GTP as an energy source, catalyses the Mg^{2+}-dependent transfer of aspartate to IMP, yielding adenylosuccinate. The subsequent enzymatic step is catalysed by adenylosuccinate lyase (EC 4.3.2.2), leading to the production of adenosine monophosphate, and the release of fumarate. Thirty per cent of T-cell acute lymphocytic cancers are deficient in purine salvage pathway and must rely solely on *de novo* synthesis of purines (Batova *et al*. 1999). The adenylosuccinate synthetase 1 gene is also frequently deleted in carcinogen-induced mouse lung adenocarcinomas (LAC) both *in vitro* and *in vivo* (Miller *et al*. 2009). A similar frequency of deletion of the human adenylosuccinate synthetase gene was also observed in the human LAC cell lines (Miller 2010). Because survival of cancer cells bearing a deletion of adenylosuccinate synthetase requires an active purine salvage pathway, deletion is never seen in cells that have a deletion in the 5′-methylthioadenosine phosphorylase gene (Miller 2010). As it becomes practical to elucidate markers of drug sensitivity for cancer cells in

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human patients (Garnett *et al*. 2012), manipulation of purine pools may be a practical and less lethal approach to selectively starving cancer cells. Turning these observations into applications depends on the robust history of research into purine biosynthesis. Much of this early research was conducted in microbial model systems (Levine and Taylor 1982). Building on earlier genetic work with auxotrophic mutants in *Neurospora* (Gross and Tatum 1956), Ishikawa found that the location of *ad-8* on linkage group 6 was 26 crossover units from the centromere and 20 crossover units distal to *lys* (*lys-5*) based on the frequency of second-division segregation in 158 ascospores. The genome sequence reveals the distances to be ~1.64 Mb and ~0.49 Mb, respectively (Galagan *et al*. 2003), reinforcing the observation of nonlinearity of genetic distances. Because the *ad-8* locus was stable and known to correspond to one reaction in a well-characterized biochemical pathway, rare prototrophic recombinants were used to demonstrate that these recombinants could be used to map the original unique mutations within one locus. This pioneering work also investigated the frequency of mutations at the same site as well as the inability of some mutants to revert. Following up on this work using molecular genetic analysis provides unique insight into the nature of the genetic events underlying gene conversion and mutation.

Materials and methods

All strains listed in table 1 were obtained from FGSC (Kansas City, USA). Two of these are in the St Lawrence genetic background (the reference genome) and 11 are in a 'mixed' genetic background. Genomic DNA was extracted using the ZR Fungal/Bacterial DNA kit (Zymo Research, Irvine, USA). The *ad-8* gene was amplified via PCR from genomic DNA using primers F_ad8_102 (CCG TTC CGT ACC TTG AGG GCG) and sR6_ad8_2563 (TGG AAT CAT GTG AAA GAA GGA TG). The resulting bands were purified using Qiaquik columns (Qiagen, Valencia, USA) according to the manufacturer's instructions. Sequencing reactions were performed by the University of Missouri Kansas City Genomics Facility using an Applied Biosystems 3100 Genetic Analyzer (Foster City, USA). Sequences were aligned and analysed using Sequencher (Gene Codes Corporation, Ann Arbor, USA).

Results

Thirteen alleles of *ad-8* were characterized to the level of the DNA sequence. Eight of the strains contained mutations at *ad-8* that were nonsynonymous nucleotide replacements (table 2). Two of the *ad-8* alleles characterized had insertions of four nucleotides each, and three had deletions of various numbers of nucleotides. Of the nonsynonymous substitutions, all were transitions and none were transversions. The transition, in strain FGSC 3496, causes a stop codon at amino acid 294. Similarly, the indels all cause stop codons, although some stop codons are significantly distal to the actual mutation. Of the five mutations induced by irradiation (UV or X-ray), one had an insertion, one had a deletion, and three had single-nucleotide substitutions (SNPs). Of the spontaneous mutations, two had deletions and two had SNPs. Other than these specific changes, all of the *ad-8* sequence was identical to the sequence of the reference genome and there was no association with the genetic background of a strain (mixed versus St Lawrence). Finally, no changes were detected in the 3′ untranslated region (UTR) of this gene (figure 1).

This locus was also characterized in 18 lab isolates subjected to whole-genome sequencing (McCluskey *et al*. 2011). These strains were all mutant at characterized loci unrelated to adenine biosynthesis and all were able to grow on minimal medium. Among the 18 strains, 11 have an *ad-8* allele identical to that of the reference genome strain and seven have

Table 1. Strains used in the current study.

FGSC #	*ad-8* allele	Mutagen*	Genetic background**	Reference
3489	E2	X	M	Ishikawa (1962b)
3490	E10	X	M	Ishikawa (1962b)
3494	E35	S	M	Ishikawa (1962b)
3495	E36	S	M	Ishikawa (1962b)
3496	E42	S	M	Ishikawa (1962b)
3500	E96	S	M	Ishikawa (1962b)
3501	E110	UV	M	Ishikawa (1962b)
3502	E115	UV	M	Ishikawa (1962b)
3504	E121	UV	M	Ishikawa (1962b)
5428	E226	EMS	SL	Ishikawa (1962b)
5429	E227	EMS	SL	Ishikawa (1962b)
5430	E255	BUDR	SL	Ishikawa (1962b)
5434	E326	NA	SL	Ishikawa (1965)

*Mutagen: X, X-ray; S, spontaneous; UV, ultraviolet light; EMS, ethyl methanesulphonate; BUDR, 5-bromodeoxyuridine; NA, not available; **genetic background: M, mixed; SL, St Lawrence.

Table 2. Mutations detected in *ad-8* alleles.

Strain	*ad-8* allele	Mutation	gDNA base*	Amino acid change
3489	E2	Deletion of 8 nucl.	139–146	Stop
3490	E10	C→T	1106	S237L
3494	E35	Deletion of 18 nucl.	1431–1448	AAs 318–323 deleted
3495	E36	Deletion of A	885	Stop
3496	E42	G→A	1367	W294Stop
3500	E96	T→C	573	L82P
3501	E110	A→G	142	K15E
3502	E115	Insertion of 4 As	1313–1316	Stop
3504	E121	C→T	495	S56P
5428	E226	T→C	1462	L326P
5429	E227	G→A	1084	G230D
5430	E255	Insertion of 4	467–470	Stop
5434	E326	G→A	1127	G244D

*gDNA base is the position in the genomic DNA sequence.

alleles that differ from that of the reference genome. The polymorphisms among these 18 strains are grouped into four different alleles (table 3). Strains FGSC 322, FGSC 3562, and FGSC 3921 have allele 1 with 27 SNPs and three indels. Strains FGSC 2261 and FGSC 3564 have allele 3, which has four indels and 27 SNPs, while strains FGSC 1363 and FGSC 3114 have unique alleles, the former (allele 2) with four indels and 25 SNPs and the latter (allele 4) with one indel and no SNPs. After the wildtype allele (reference genome), allele 1 is the most common, being found in three strains. Allele 3 is the second most common, occurring in two strains, and alleles 2 and 4 each occur in one strain. Among these polymorphisms, only two SNPs were nonsynonymous and none of the indels were in protein-coding sequence.

Discussion

The *ad-8* mutant strains in the FGSC collection allow a unique perspective on the accuracy of genetic mapping by intragenic recombination and also provide insight into strain lineages among classical mutant strains with defects in other pathways.

Among the *ad-8* mutant strains analysed in the present study are strains treated with a variety of chemical and ionizing radiation mutagens. Because of the relatively small sample size, it is not possible to draw conclusions about the type of mutation associated with each mutagen. The placement of lesions on the genetic map was generally accurate, with markers closer to the 5′ end of the gene being more accurately placed than those at the 3′ end of the gene (figure 1). While it may be attractive to speculate that this is due to differences in recombination frequencies at the 5′ versus the 3′ end of the gene, the current data do not address this issue. Also, the fact that none of the lesions occurred in the 3′ UTR suggests that this region may be dispensable for protein function. Moreover, the 3′ UTR is not present in the *Sordaria ad-8* orthologue (Nowrousian *et al*. 2010). Finally, while the original study oriented the mutations at *ad-8* relative to the centromere using the frequency of second-division segregation among ordered asci from independent crosses, the orientation of the *ad-8* gene (NCU09789) in the genome sequence (Galagan *et al*. 2003) is reversed relative to the maps in the original studies (Ishikawa 1962a).

Because of the diversity of the FGSC collection, similar studies might be carried out with a number of different loci

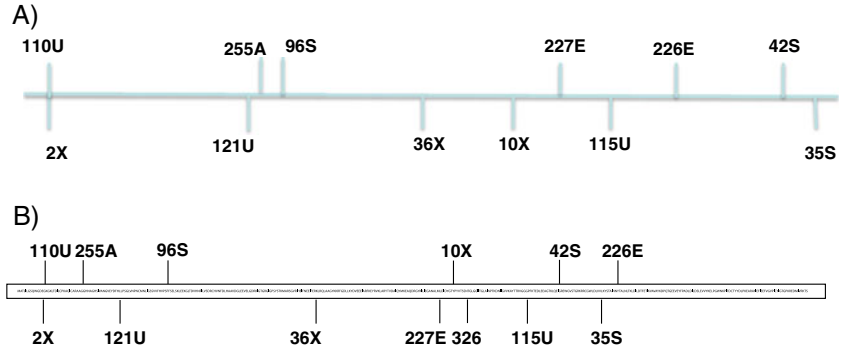

Figure 1. Distribution of mutations in *ad-8* alleles. (A) Mutations mapped by intra-allelic recombination from Ishikawa (1962b). (B) Mutations identified by targeted gene sequencing.

Table 3. *ad-8* alleles in whole-genome-sequenced strains.

Strain	Allele
106	WT
305	WT
309	WT
322	1
821	WT
1211	WT
1303	WT
1363	2
2261	3
3114	4
3246	WT
3562	1
3564	3
3566	WT
3831	WT
3921	1
7022	WT
7035	WT

for which different strains are available. A similar analysis of mutations at the *al-2* locus in *N. crassa* was successful in associating protein domains with function in nine mutants, some of which retained partial activity (Diaz-Sanchez *et al*. 2011).

The *ad-8* gene is highly conserved among fungi at the level of the amino acid sequence. Orthologues of *ad-8* in *N. tetrasperma* (Ellison *et al*. 2011) and *Sordaria macrospora* (Nowrousian *et al*. 2010) show over 97% conservation of the amino acid sequence while conservation among other fungi drops off with taxonomic distance (figure 2). Nevertheless, even fungi as distant as the basidiomycete smut *Ustilago maydis* (Kamper *et al*. 2006) or the saprophyte *Coprinopsis cinerea* (Stajich *et al*. 2010) show over 50% amino acid sequence identity, demonstrating the high conservation of this function. The *ad-8* orthologue in *Saccharomyces cerevisiae* (Dorfman 1969) is 54% identical and 69% similar to the *Neurospora* gene with regard to the amino acid sequence. Moreover, while there are several additional open reading frames (ORFs) in Saccharomyces which have shorter regions of homology to the *Neurospora ad-8* amino acid sequence, in most fungi this gene is present in a single copy. Direct searches of the genomes of several different fungal genomes using the BLAST algorithm (Altschul *et al*. 1997) revealed that *N. tetrasperma* (Ellison *et al*. 2011), *Magnaporthe grisea* (Dean *et al*. 2005), most *Aspergillus* species (Galagan *et al*. 2005), *Podospora anserina* (Espagne *et al*. 2008) and *S. macrospora* (Nowrousian *et al*. 2010) each have only one copy of the *ad-8* orthologue. Conversely, several *Fusarium* species appear to have multiple *ad-8* orthologues, although *F. graminearum* (*Giberella zeae*) has only one *ad-8* orthologue. *F. verticillioides* has two high-scoring sequences (FVEG_04286 on supercontig 4 and FVEG_05923 on supercontig 7) and *F. oxysporum* f. sp. *lycopersici* has five high-scoring ORFs including four hypothetical proteins (Ma *et al*. 2010). The *Trichoderma reesei ad-8* orthologue (Martinez *et al*. 2008) differs markedly by the presence of 18 additional amino acid residues after position 280.

Adenylosuccinate synthetase has been well studied in a number of systems. The *E. coli* mutants S240A exhibited modest changes in their initial rate kinetics relative to the wildtype enzyme, suggesting that Ser240 does not play an essential role in substrate binding or catalysis (Wang *et al*. 1997). However, FGSC 3490 is mutated at the homologous serine (S237L) and is auxotrophic for adenine. Further demonstration that the *Neurospora* adenylosuccinate synthetase is unique is found in studies of the *Aspergillus nidulans* orthologue, variously known as *adB*, *oxpA* and AN0893. The *A. nidulans adB57* mutant is an adenine auxotroph and is also oxypurinol resistant. The *A. nidulans oxpA5* mutation confers resistance to oxypurinol and is the result of a C-to-T transition at nucleotide 297 of the AN0893 ORF resulting in replacement of the proline at position 56 with a

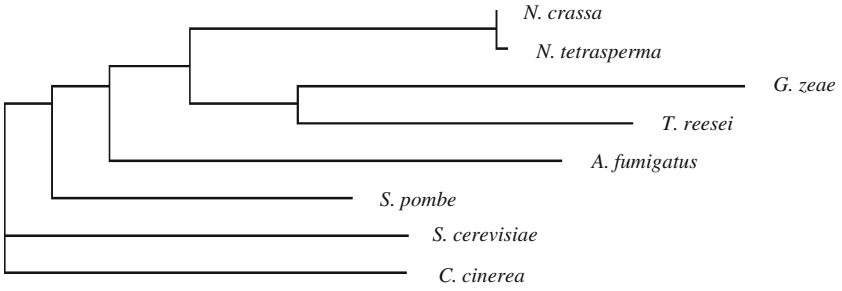

Figure 2. Comparative analysis of adenylosuccinate synthetase genes from a variety of fungi. *N. crassa*, XP_960187.1; *N. tetrasperma*, EGZ68389.1; *G. zeae*, XP_385363.1; *T. reesei*, EGR50168.1; *A. fumigatus*, XP_752913.1; *S. pombe*, NP_594664.1; *S. cerevisiae*, EGA85175.1; *C. cinerea*, EAU88944.1. This cladogram was generated using the ClustalW2 phylogeny platform at http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/ using the default settings for a neighbour-joining tree.

serine. P56S occurs in a highly conserved region of the adenylosuccinate synthetase protein (Ribard *et al*. 2001) and does not result in adenine auxotrophy. The *ad-8* mutation in *N. crassa* strain FGSC 3504 is in the corresponding orthologous residue resulting in S56P and this strain is auxotrophic for adenine.

The analysis of the *ad-8* locus among 18 strains subject to whole-genome analysis shows the impact of different lineages among laboratory stocks. Because none of the polymorphisms in these 18 strains are unique (all are shared among more than one strain), and the polymorphisms occur in different combinations, it is possible that intragenic recombination is occurring in lab strains in the lineage of these 18 classical mutant strains. For example, alleles 2 and 3 (table 3) differ only by two SNPs at positions 1,127,015 and 1,128,567. Alternatively, since the *ad-8* allele in strain FGSC 1363 differs from allele 3 only in the absence of the SNPs at positions 1,127,015 and 1,128,567, it is possible that allele 2 represents a progenitor state with allele 3 being derived from allele 2.

Neurospora mutants at *ad-8* and in other genes encoding proteins required for the synthesis of adenine have been important both in elucidating the fundamental nature of a gene (Ishikawa 1965), as well as in the effect of ionizing radiation (de Serres and Webber 1997) and chemical mutagens (de Serres *et al*. 1997). This retrospective analysis of materials used in foundational research into intragenic recombination provides unprecedented insight into the accuracy and efficacy of classical genetic analysis.

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