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Predicting the Identities of su(met-2) and met-3 in Neurospora crassa by Genome Resequencing

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Abstract

A significant number of classical genetic *Neurospora crassa* biochemical mutants remain anonymous, unassociated with a physical genome locus. By utilizing short read next-generation sequencing methods, it is possible to sequence the genomes of mutant strains rapidly and economically for the purpose of identifying genes associated with mutant phenotypes. We have taken this approach to connect genes and mutations to “methioninelessˮ phenotypes in *N. crassa*.

Keywords

Neurospora, genome resequencing, methionine, cysteine, metabolism, mutants

Cover Page Footnote

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Predicting the Identities of *su(met-2)* and *met-3* in *Neurospora crassa* by Genome Resequencing

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Abstract
A significant number of classical genetic *Neurospora crassa* biochemical mutants remain anonymous, unassociated with a physical genome locus. By utilizing short read next-generation sequencing methods, it is possible to sequence the genomes of mutant strains rapidly and economically for the purpose of identifying genes associated with mutant phenotypes. We have taken this approach to connect genes and mutations to "methionineless" phenotypes in *N. crassa*.

Introduction
A systematic understanding of central metabolite biosynthetic pathways and their regulation is critical for metabolic engineering and synthetic biology research in microbes. Many *Neurospora crassa* genes with mutant metabolic phenotypes have been connected to their associated physical locus in the genome (Radford 2004), but many "anonymous" mutants have not yet been fully characterized (Baker 2009). A significant number of *N. crassa* "methionineless" mutants were isolated at the dawn of the biochemical genetics era. Mutants were characterized both genetically and biochemically by testing the auxotrophic mutant strains for growth on a variety of pathway intermediates as well as assaying protein extracts for specific biochemical reactions. For example, *met-3* and *met-7* strains will grow with cystathionine supplementation and their extracts lack cystathionine synthase activity (Kerr and Flavin 1970). Similarly, extracts from *N. crassa* strains with *met-1* and *met-6* mutations lack both cystathionine synthase activity and the ability to transmethylate homocysteine (Selhub *et al.* 1971).

Regulation and synthesis of cysteine and methionine by *Neurospora crassa* has been well described genetically and biochemically over the course of decades (Horowitz 1947; Burton and Metzenberg 1975; Huberman *et al.* 2021). Fungal cysteine and methionine metabolism have largely been explored in ascomycetes and basidiomycetes. Filamentous fungi produce cysteine either via the transsulfuration pathway (homocysteine ↔ cystathionine ↔ cysteine) or the O-acetylserine (OAS) pathway, which produces cysteine from the sulfuration of OAS (Dreyfuss *et al.* 2013). Methionine is generated from homocysteine by a methyltransferase, methionine synthase (methyl tetrahydrofolate homocysteine transmethylase – *met-8* in *N. crassa*). Interestingly, homocysteine is also generated by sulfuration of O-acetylhomoserine by NCU01652 or *cysD* in *A. nidulans* (Sienko *et al.* 1998).

met-2 was mapped to linkage Group IV and mutant strains require methionine or homocysteine for growth and accumulate cystathionine (Horowitz 1947). The gene encoding

met-2, NCU07987, was cloned by complementation and encodes a cystathionine beta-lyase (Reveal and Paietta 2013). It is regulated by sulfur and catalyzes the conversion of L-cystathionine to L-homocysteine and pyruvate (Paietta 2016; Huberman *et al.* 2021).

Quickly following the early publications of methionineless mutants, suppressor mutations were isolated. A mutant that suppresses the nutrition requirements of *met-2*, *su(met-2),* was isolated and mapped to Linkage Group IV (Tokuno *et al.* 1962; Wiebers and Garner 1964). It is clear that *su(met-2)* is not an allele specific suppressor because it is able to suppress both *met-2*, *met-3,* and *met-7* but not *met-5* (Tokuno *et al.* 1962). The suppressors of the methionine phenotype have initially slower growth rates than wild type.

Thus, *N. crassa* sulfur metabolism, which includes biosynthesis of methionine and cysteine, is complex and many genes with these auxotrophic phenotypes remain unassigned to a genomic locus. Next generation sequencing methods enable high throughput resequencing of mutant strains allowing a genomics approach for connecting genes with phenotypes (McCluskey *et al*. 2011). We have utilized this approach to characterize the mutations in a variety of strains with phenotypes impacting methionine biosynthesis including alleles of *met-2*, *met-3*, *su(met-2),* and *T(IV->I)OY333 met*.

Results

Mutant strain genome sequencing

We sequenced the genomes of strains containing *met-2* and *su(met-2)* and used genetic mapping and comparative analysis among strains that are prototrophic for methionine biosynthesis (see Materials and Methods) to identify the underlying mutations (McCluskey *et al.* 2011). We found that the H-98 allele of *met-2* has a missense mutation which would lead to the replacement of a glutamic acid (E) residue with a lysine (K) at amino acid 181 of the protein encoded by NCU07987. In the H-98-Su allele of *su(met-2)* we identified a missense mutation leading to replacement of glycine (G) with cysteine (C) at amino acid 296 of NCU00536, which encodes the serine O-acetyl transferase that functions in cysteine biosynthesis pathway (Table 1 and Figure 1).

In addition, we sequenced the genomes of FGSC strains #3666 and #3667. The strains both of have the translocation, *T(IV->I)OY333 met*, which is associated with a methionine auxotrophy. We traced the genetic change likely causing the auxotrophy in *T(IV->I)OY333 met* to a chromosomal breakpoint in *met-2* (NCU07987). The other chromosomal breakpoint is in NCU04386 encoding a member of the Nur1/Mug15 protein family. The chromosomal insertion location in Chromosome 1 lies in a region devoid of genes and high in repetitive sequence (Figure 2).

Finally, we sequenced the genome of *Neurospora crassa* strain FGSC#502, which has a mutation in *met-3*. Interestingly, we identified a nucleotide difference that would replace amino acid 21, a glutamic acid (E) with a glycine (G) in NCU09545. NCU09545 encodes a predicted methylenetetrahydrofolate reductase (Table 1).

Phylogenetic analysis of OAS

BLASTP analysis of NCU00536 against filamentous fungal and yeast genomes located in the National Center for Biotechnology Information-Non Redundant (NCBI-NR) database indicated that most fungi contain a single serine O-acetyltransferase (E value of 0.0) and a single, functionally related homoserine *O*-acetyltransferase (E value range 1×10^{-31} to 1×10^{-37}) as essentially expected (Table 2; Bastard *et al.* 2017). We performed phylogenetic analysis of 26

putative fungal and eight yeast serine O-acetyltransferases and homoserine *O*-acetyltransferases. Our analyses agree with prior analysis showing that these proteins share homology and cluster into two distinct clades with 100% bootstrap support (Bastard *et al.* 2017) (Figure 3). In all predicted proteins in both clades, the G at 296 of NCU00536, which is mutated to a cysteine in FGSC#689 and FGSC#690 *su(met-2)*, is perfectly conserved indicating a critical role for this amino acid in serine *O*-acetyltransferase and homoserine O-acyltransferase activity (Figure 4). Notably, the fungus *Fusarium verticillioides* contained two additional putative homoserine acyltransferases of which one, FVEG 12519, encoding FUB5, has been shown to be involved in the synthesis of the secondary metabolite fusaric acid in *F. verticillioides* (Brown *et al.* 2012) and *F. fujikuroi* (Niehaus *et al.* 2014).

Discussion

Regulation of metabolic pathways is complex, integrating feedback at levels of transcription, translation, and enzyme activity. Considerable biochemical and genetic research into fungal cysteine and methionine amino acid biosynthesis has been performed in the model genetic systems, *N. crassa* and *Aspergillus nidulans*. In this study we used genome resequencing to identify previously anonymous methionine biosynthetic pathway genes and characterized a translocation leading to a methionine auxotrophy phenotype. This research adds an additional layer of information regarding *N. crassa* methionine and cysteine metabolism to prior genetic and biochemical studies.

We have determined the molecular basis for the methionine auxotrophy phenotypes associated with *met-2*, *T(IV->I)OY333 met* and *met-3.* Previous work had shown that *met-2* is associated with NCU07987 and encodes cystathionine gamma-synthase. The *met-2* allele we sequenced, H-98, has a mutation that changes NCU07987 amino acid 181 from glutamic acid (E) to lysine (K). Glutamic acid in this position is well-conserved and based on structural studies, shown to be in the cofactor binding pocket (Clausen *et al.* 1996). Thus, mutation would be expected to cause a loss of function. Genome resequencing and assembly of two *N. crassa* strains with the translocation *T(IV->I)OY333 met* show that the mutation associated with methionine auxotrophy is the result of one of the chromosome IV breakpoints occurring in the middle of NCU07987, close to the encoded pyridoxal-5'-phosphate (PLP)-cofactor binding pocket. The N-terminal domain in this enzyme family also contributes to the formation of the quaternary tetrameric structure, including substrate and cofactor binding (Clausen *et al.* 1996). Given the importance of the *met-2* protein N-terminus to its structure and activity, the truncation in *T(IV->I)OY333* is consistent with a loss of function. The *met-2* gene disruption in this mutant is accompanied by disruption of NCU04386, a hypothetical protein with 4 transmembrane domains, in the Nur1/Mug15 family. In fission yeasts, Nur1 is part of the perinuclear network that controls recombination at multiple loci to maintain genome stability (Banday *et al.* 2016).

The *met-3* gene in *N. crassa* had been hypothesized to encode the beta subunit of cystathionine synthase (Nagai and Flavin 1966). However, our genome resequencing indicates that the underlying mutation occurs in NCU09545, predicted to encode methylenetetrahydrofolate reductase. Within the conserved methylenetetrahydrofolate reductase domain, the glutamic acid residue at amino acid 21 is mutated to a glycine. While most ascomycete fungi encode two methylenetetrahydrofolate reductases, in some cases, mutation of each, individually, results in methionine auxotrophy indicating complexity in regulation of these two proteins (Raymond *et al.* 1999; Naula *et al.* 2002; Sienko *et al.* 2007; Frandsen *et al.* 2010; Yan *et al.* 2013). Previous studies indicated that cystathionine synthase is activated by

methyltetrahydrofolate explaining why mutations in *met-3* result in a lack of cystathionine synthase activity (Selhub *et al.* 1971). Future studies should include complementation experiments to definitively confirm that the nucleotide differences identified are responsible for the mutant phenotypes.

Genetic interaction-based suppression of auxotrophy is an indication of this complexity, inferring that multiple biosynthetic routes to metabolites exist. Because the enzymes encoded by *met-2*, *met-3,* and *met-7* are part of the transsulfuration pathway to methionine, our results indicate that activation of the O-acetylserine (OAS) pathway to methionine occurs as a consequence of inactivation of serine O-acetyl transferase by mutation of *su(met-2)*/NCU00536. The *cysA* gene, AN8565, is the *A. nidulans* ortholog of *su(met-2)*, NCU00536 (Grynberg *et al.* 2000). Studies of sulfur metabolism and regulation from *A. nidulans* showed that *cysA* mutations suppress *A. nidulans metD*/*metH* similar to suppression of *met-2* by *su(met-2)* (Paszewski and Grabski 1975). By comparison, we conclude that suppression of *met-2* is due to derepression of the O-acetylhomoserine pathway caused by mutation of NCU00536 that leads to dysregulation in the OAS and transsulfuration pathways.

Conclusion

In summary, we used next generation sequencing to identify the mutations underlying both the *met-2, met-3, T(IV->I)OY333 met,* and *su(met-2)* phenotypes. This research fully supports work in filamentous ascomycetes and furthers our understanding of fungal cysteine and methionine amino acid biosynthesis.

Materials and Methods

Resequencing and genome analysis of FGSC strains. Strains were maintained as dried spores on anhydrous silica gel and revived according to standard practices (Ogata 1962). DNA was prepared from 10 ml cultures in Vogels medium as described previously using the ZR Fungal/Bacterial DNA MiniPrep, Zymo Research, Irvine, CA) (Bredeweg *et al*. 2022). DNA was characterized with a nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) prior to freezing at -80 °C and shipment to the JGI. Library construction, sequencing and analysis were performed as detailed previously (McCluskey *et al.* 2011; Bredeweg *et al.* 2022). The genomes of *su(met-2)* strains, FGSC 689 and FGSC 690, were compared against the following FGSC strain genome sequences (NCBI BioProject number in parentheses): FGSC 10, (PRJNA249716), FGSC 137 (PRJNA249735), FGSC 340 (PRJNA249856), FGSC 380 (PRJNA249870), FGSC 632 (PRJNA249952), FGSC 633 (PRJNA249953), FGSC 939 (PRJNA250034), FGSC 1113 (PRJNA249727), FGSC 1630 (PRJNA249740), FGSC 2197 (PRJNA249791), FGSC 2319 (PRJNA249801), FGSC 2320 (PRJNA249795), FGSC 2384 (PRJNA249797). The genome of the *met-3* strain, FGSC 502, was compared against the following FGSC strain genome sequences (NCBI BioProject number in parentheses): FGSC 12 (PRJNA251075), FGSC 13 (PRJNA249809), FGSC 14 (PRJNA251073), FGSC 49 (PRJNA251072), FGSC 57 (PRJNA249813), FGSC 64 (PRJNA251069), FGSC 101 (PRJNA251067), FGSC 104 (PRJNA251082), FGSC 185 (PRJNA251081), FGSC 239 (PRJNA251250), FGSC 255 (PRJNA251085), FGSC 317 (PRJNA251068), FGSC 336 (PRJNA251074), FGSC 419 (PRJNA251131), FGSC 492 (PRJNA251130), FGSC 506 (PRJNA251125), FGSC 507 (PRJNA251124), FGSC 746 (PRJNA251140), FGSC 793 (PRJNA251137), FGSC 943 (PRJNA251139), FGSC 981 (PRJNA251143), FGSC 993 (PRJNA251132), FGSC 575 (PRJNA251127), FGSC 1400 (PRJNA251191)), FGSC 6946 (PRJNA251261).

Evolutionary analysis of NCU00536. Proteins related to NCU00536 and AN8565 were identified by Basic Local Alignment Search Tool (BLASTP) analysis (ZHANG *et al.* 1998) of a diverse group of filamentous fungal and yeast genomes located in the NCBI-NR database (Table 2) using NCU00536 as a query. Protein sequences with E values of 0.0 to the query were considered likely orthologs of NCU00536 (Koonin 2005). Protein sequences with E values less than 1 x 10^{-30} to the query were considered likely L-homoserine-*O*-succinyltransferases (HSTs) or homoserine acyltransferases (HATs). Amino acid sequence of 32 HATs and HSTs were aligned by CLUSTALW, as implemented in MEGA X (Build#: 10210118) with default parameters (Kumar *et al.* 2018). The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987) and evolutionary analysis was conducted in MEGA X. Branch support was determined with 500 bootstrap replicates. The *Bacillus cereus* IIW_03477 served as the outgroup.

Genome assembly. Reads stored at the Short Read Archive (SRR25764243 and SRR10513761) for strains 3666 and 3667 respectively were uploaded to Galaxy (https://usegalaxy.eu/) for processing by the following tools: Faster Download and Extract Reads in FASTQ format from NCBI SRA was used to retrieve the sequence data, and separate the forward and reverse strands with --split-3 option; FastQC was run on the paired-end imported data to check quality. 'SPAdes' (Bankevich *et al.* 2012) was operated in 'Assembly and error correction mode', paired-end short reads, with the additional options of –isolate (on a single strain), auto k-mer detection, auto Phred quality offset. The SPAdes assemblies (contigs) were made into a nucleotide blast database using 'NCBI BLAST+ makeblastdb' for nucleotide input. After upload of the NCU07987 coding sequence to Galaxy, matching contigs were found using megablast within 'NCBI BLAST+ blastn' with default options. The resulting contigs (NODE 101 and 130 for 3666, NODE 101 and 122 for 3667) were analyzed through the NCBI BLAST web interface, which matched CDS regions around translocation junctions. This process was repeated for NCU04386 (NODE 101 and 15 for 3666, and NODE 101 and 68 for 3667).

Data and Resource Availability

Strains FGSC 689, FGSC 690, FGSC 502, FGSC 3666 and FGSC 3667 can be obtained from the Fungal Genetics Stock Center (FGSC; McCluskey *et al.* 2010). Datasets can be obtained from NCBI via BioProject identifiers from Table 1 and Material and Methods.

Competing Interests

The authors declare no competing interests.

Author Contributions

K.M. and S.E.B. conceived the overall mutant resequencing project. K.M grew mutants and generated genomic DNA for sequencing. D.W.B. performed phylogenetic analysis and S.E.B. and K.M. performed genome analyses to identify mutations. E.L.B. generated genome assemblies for strains 3666 and 3667 and identified translocation breakpoints. S.E.B. wrote the first draft of the manuscript. All authors contributed to manuscript review and editing. All authors discussed the data and approved the manuscript.

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Table 1 Summary of identified mutations from strain resequencing

Strain[1]	Gene	Allele / isolate	Acc[2]	NCU# (Mutation)	Mutation Position[3]
689	*su(met-2)* *met-2*	H-98-Su; H-98	256750	00536 (G296C) 07987 (E181K)	Sc_1:8097517, G->T Sc_4:3819325, C->T
690	*su(met-2)*	H-98-Su	249967	00536 (G296C)	Sc_1:8097517, G->T
502	*met-3*	36104	251133	09545 (E21G)	Sc_5:4544750; T->C
3666	*T(IV>I) OY333 met*	OY333	346014	07987 and 04386 (Breakpoints)	See Figure 2
3667	*T(IV>I) OY333 met*	OY333	346015	07987 and 04386 (Breakpoints)	See Figure 2

[1]FGSC number. [2]NCBI BioProject accession number (PRJNA######). [3]Abbreviation: Sc, Supercontig.

Table 2 Identification of HATs and HSTs by BLASTP with NCU00536

Locus Tag (Gene)	Species[1]	Class[2]	Score (bits), Expect	Function, Gene Name[3]
NCU00536	*N. crassa*	Sor	n.a.[4]	HAT, metA
NCU07001	*N. crassa*	Sor	134, 4e-31	HST, metX
FVEG_00204	*F. verticillioides*	Sor	740, 0.0	HAT
FVEG_06826	*F. verticillioides*	Sor	135, 9e-35	HST
FVEG_12519	*F. verticillioides*	Sor	128, 4e-32	FA, FUB5
FVEG_03150	*F. verticillioides*	Sor	144, 3e-38	unknown
FGSG_00186	*F. graminearum*	Sor	727, 0.0	HAT
FGSG_05658	*F. graminearum*	Sor	134, 2e-34	HST
NECHADRAFT_41345	*F. vanettenii*	Sor	755, 0.0	HAT
NECHADRAFT_83647	*F. vanettenii*	Sor	154, 3e-41	HST
NECHADRAFT_82780	*F. vanettenii*	Sor	156, 2e-42	unknown
MGG_14202	*P. oryzae*	Sor	751, 0.0	HAT
MGG_01469	*P. oryzae*	Sor	124, 2e-30	HST
G4B84_008494	*A. flavus*	Eur	739, 0.0	HAT
G4B84_008026	*A. flavus*	Eur	139, 5e-36	HST
M747DRAFT_69036	*A. niger*	Eur	745, 0.0	HAT
M747DRAFT_305479	*A. niger*	Eur	133, 4e-34	HST
ANIA_08565	*A. nidulans*	Eur	731, 0.0	HAT, cysA
ANIA_2229	*A. nidulans*	Eur	144, 1e-37	HST, metE
COCC4DRAFT_139598	*C. heterostrophus*	Dot	720, 0.0	HAT
COCC4DRAFT_170484	*C. heterostrophus*	Dot	129, 3e-32	HST
YALI0_C24233g	*Y. lipolytica*	Sac	598, 0.0	HAT
YALI0_E00836g	*Y. lipolytica*	Sac	137, 4e-35	HST
LIPSTDRAFT_73260	*L. starkeyi*	Sac	616, 0.0	HAT
LIPSTDRAFT_69933	*L. starkeyi*	Sac	178, 1e-50	HST
SPBC106.17c	*Sc. pombe*	Sch	555, 0.0	HAT
SPBC56F2.11	*Sc. pombe*	Sch	132, 5e-34	HST
UMAG_06047	*U. maydis*	Bas	511, 0.0	HAT
UMAG_03425	*U. maydis*	Bas	115, 1e-27	HST
BD309DRAFT_970577	*D. squalens*	Bas	510, 1e-177	HAT
BD309DRAFT_850810	*D. squalens*	Bas	151, 3e-40	HST
YNL277W	*Sa. cerevisiae*	Sac	144, 2e-36	HAT
IIW_03477	*B. cereus*	Bac	155, 9e-41	HAT

[1]Abbreviations: *A, Aspergillus*; *B, Bacillus; C, Cochliobolus; D, Dichomitus; F, Fusarium; L, Lipomyces; N, Neurospora; P, Pyricularia, Sa, Saccharomyces; Sc, Schizosaccharomyces; U, Ustilago; Y, Yarrowia.* [2]Abbreviations: Bac, Bacteria; Bas, Basidiomycetes; Dot, Dothideomycete; Eur, Eurotiomycete; Sac, Saccharomycetes; Sch, Schizosaccharomyces; Sor, Sordariomycete. [3]Abbreviations: HAT, homoserine O-acetyltransferases; HST, serine O-acetyltransferases; FA, fusaric acid synthesis. [4]n.a., not applicable

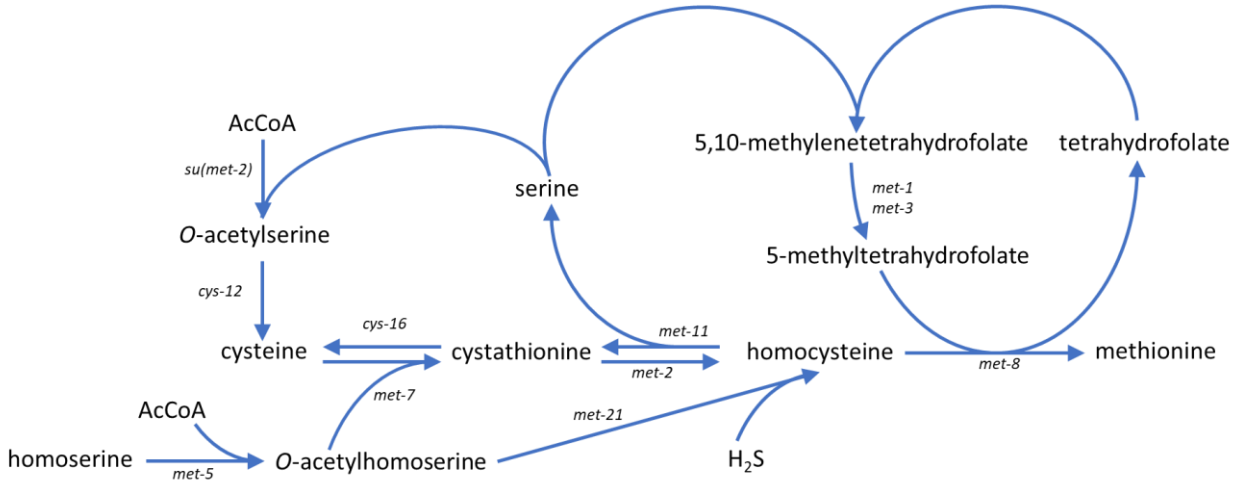
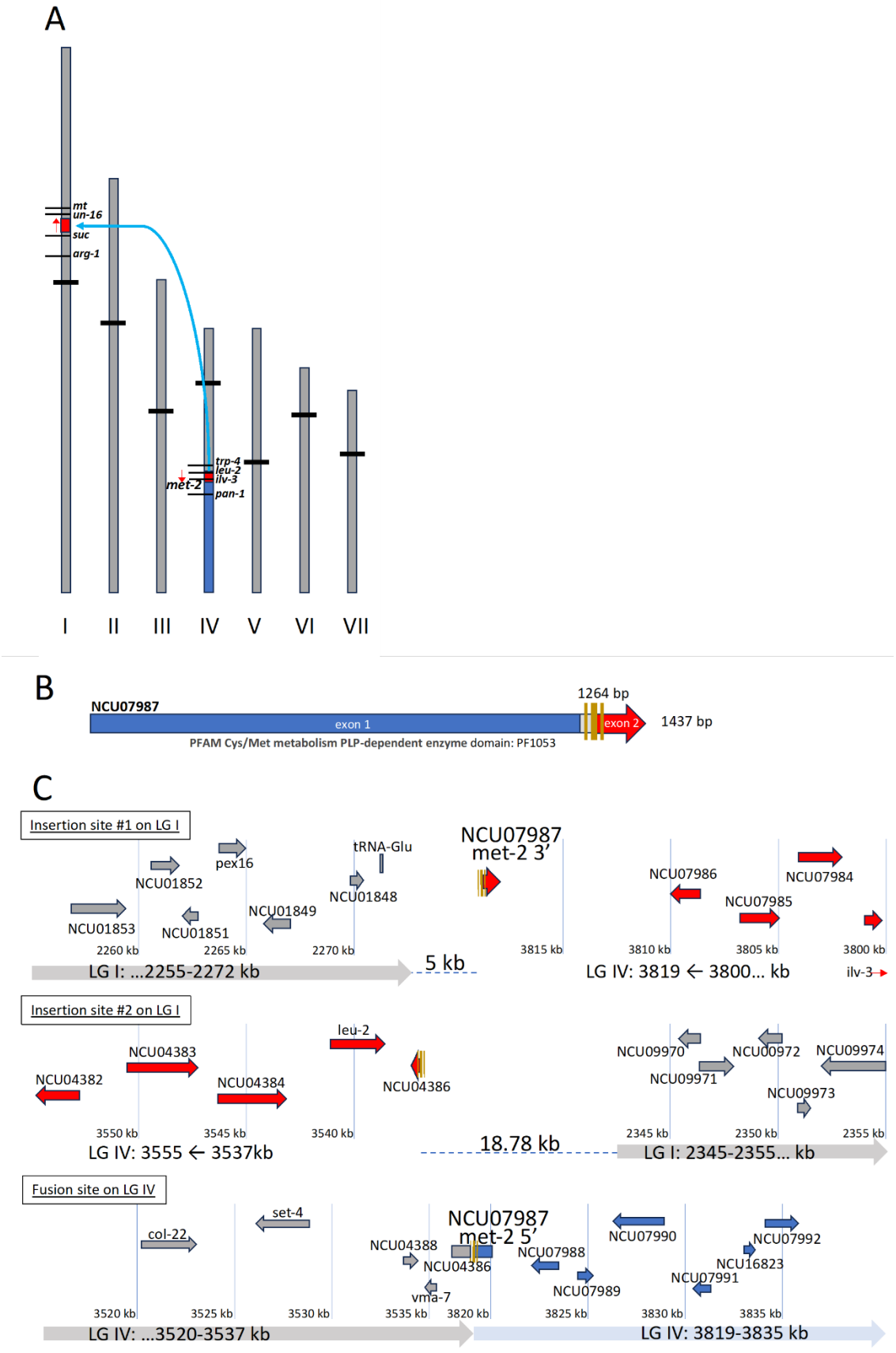


Figure 1. Sulfur amino acid synthesis in *Neurospora crassa*.


A
mt
un-16
suc
arg-1
trp-4
leu-2
ilv-3
met-2
pan-1
I
II
III
IV
V
VI
VII
B
NCU07987
1264 bp
exon 1
exon 2
1437 bp
PFAM Cys/Met metabolism PLP-dependent enzyme domain: PF1053
C
Insertion site #1 on LG I
NCU01852
pex16
tRNA-Glu
NCU07987
met-2 3'
NCU07986
NCU07984
NCU01853
NCU01851
NCU01849
NCU01848
NCU07985
2260 kb
2265 kb
2270 kb
3815 kb
3810 kb
3805 kb
3800 kb
LG I: ...2255-2272 kb
5 kb
LG IV: 3819 ← 3800... kb
ilv-3
Insertion site #2 on LG I
leu-2
NCU04383
NCU09970
NCU09972
NCU09974
NCU04382
NCU04384
NCU04386
NCU09971
NCU09973
3550 kb
3545 kb
3540 kb
2345 kb
2350 kb
2355 kb
LG IV: 3555 ← 3537kb
18.78 kb
LG I: 2345-2355... kb
Fusion site on LG IV
set-4
col-22
NCU07987
met-2 5'
NCU07990
NCU07992
NCU04388
NCU07988
NCU16823
NCU04386
NCU07991
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3520 kb
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Figure 2. The transposition resulting in *met-2* disruption in *T(IV> I) OY333 met* is depicted on breakpoints from assembly by SPAdes (version 3.15.4) (Bankevich *et al.* 2012). A) A segment of chromosome 4 was transpos*ed into chromosome 1 in* reverse orientation. (Galagan *et al.* 2003; Galazka *et al.* 2016). Nearby mapping markers are included (Perkins 1997; Perkins *et al*. 2000). B) The breakpoint in *met-2* is after amino acid position 415, or 1264 from the start codon, which falls within an intron; remaining intron sequence and the beginning of the second exon are lost. Homology resumes for the remaining DNA sequence on a separate SPAdes node. PFAM domain 1053 spans amino acids 46–417, indicating transposition removes the final domain amino acids. Breakpoints are indicated by vertical yellow lines (B and C). C) The Chromosome I integration location is within a 74 kb 'gene desert' in the NC12 assembly. Genome context and gene fragments were identified by BLAST of contigs containing breakpoints against NC12 (GCA_000182925.2) (Galagan *et al.* 2003; Galazka *et al.* 2016). The *met-2* gene disruption in this mutant is accompanied by disruption of NCU04386, a hypothetical protein with four transmembrane domains, in the Nur1/Mug15 family.

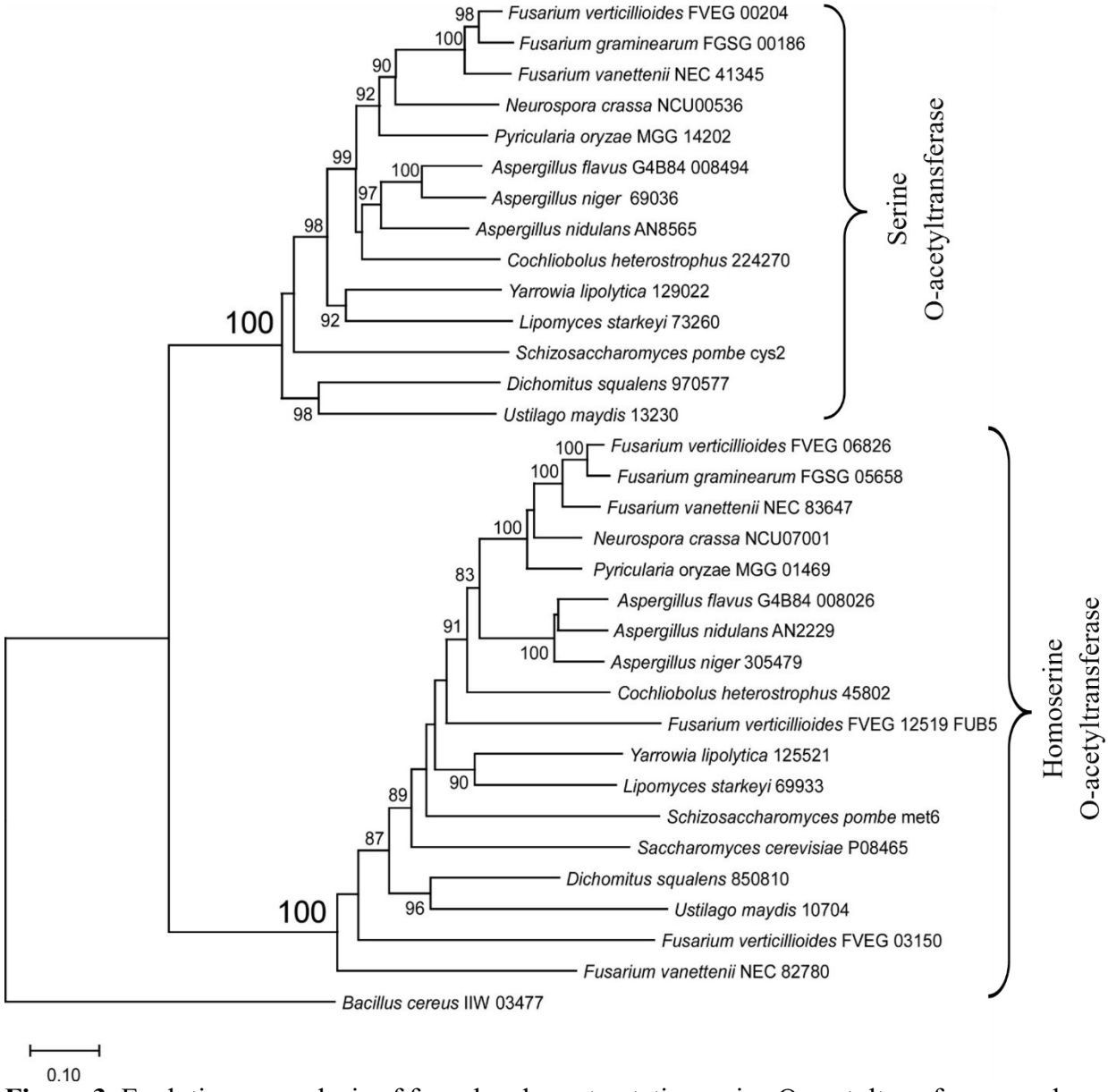

Figure 3. Evolutionary analysis of fungal and yeast putative serine O-acetyltransferases and homoserine O-acetyltransferases.

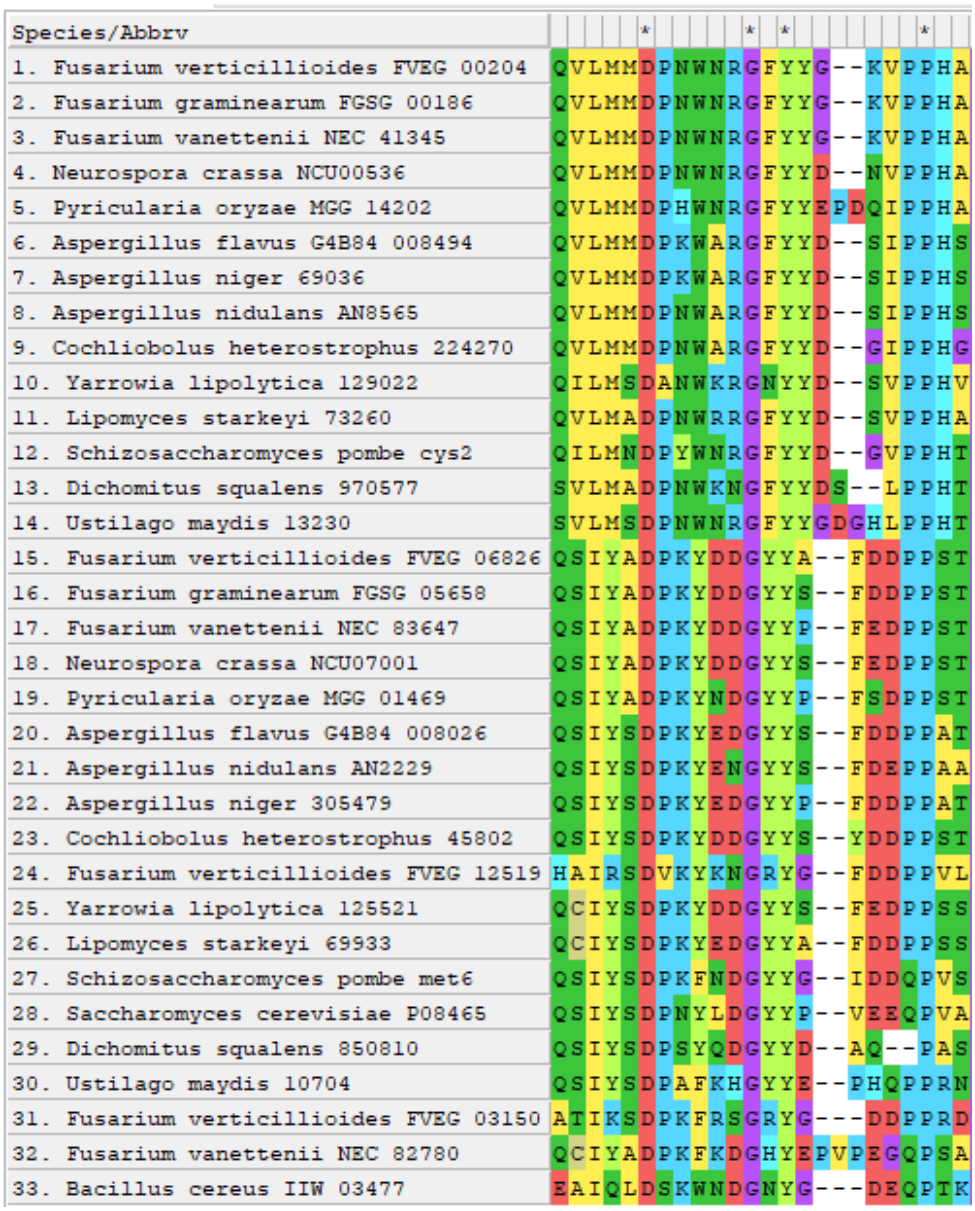

Figure 4. Alignment of region around glycine (residue 296 in NCU00536, 4th row) which is mutated in *su(met-2).* The glycine (in purple) is conserved across fungal and yeast putative serine O-acetyltransferases and homoserine O-acetyltransferases. Conserved amino acids are noted by * at the top of the alignment.