Title:

Novel mutations reveal two important regions in *Aspergillus nidulans* transcriptional activator MetR

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Abstract

Expression of the sulfur assimilation pathway in *Aspergillus nidulans* is under control of sulfur metabolite repression, which is composed of *scon* genes encoding subunits of ubiquitin ligase and the *metR* gene coding for a transcriptional activator. In this paper we report three dominant suppressors of methionine requirement isolated from a *metB3* diploid strain. All three mutations lead to the substitution of phenylalanine 48 by serine or leucine in the conserved N-terminal region of the MetR protein. Strains carrying the dominant suppressor mutations exhibit increased activities of homocysteine synthase and sulfur assimilation enzymes as well as elevated levels of the corresponding transcripts. These changes are observed even under conditions of methionine repression, which suggests that the mutated MetR protein may be resistant to inactivation or degradation mediated by sulfur metabolite repression. We also found that a mutant impaired in sulfite reductase activity, known until now as *sG8*, has a frameshift which changes 41 C-terminal amino acids. Therefore, it is now designated *metR18*. This mutant has elevated levels of MetR-regulated transcripts and of activities of sulfur assimilation enzymes (except sulfite reductase), which can be repressed to the wild type level by exogenous methionine. Thus, *metR18* and the three dominant suppressors represent new types of mutations affecting different parts of the *A. nidulans* MetR protein.

Index descriptors:

Aspergillus nidulans, sulfur, *metR*, suppressor, mutant, repression

1. Introduction

Sulfur metabolism in lower eukaryotes has been well studied in the filamentous fungi *Neurospora crassa* (Marzluf, 1997) and *Aspergillus nidulans* (Paszewski et al., 2000), and in the yeast *Saccharomyces cerevisiae* (Thomas and Surdin-Kerjan, 1997). These fungal species can use sulfate as well as cysteine and methionine as a sole sulfur source. The final product of the sulfate assimilation pathway, sulfide, is incorporated into cysteine or homocysteine. Cysteine is synthesized from *O*-acetylserine and sulfide in *A. nidulans*, *N. crassa* and many other fungi. This reaction is catalyzed by cysteine synthase encoded by the *A. nidulans cysB* gene (Figure 1). Cysteine can be converted to methionine by a pathway that begins with cystathionine γ-synthase and cystathionine β-lyase encoded by the *metB* and *metG* genes, respectively. *A. nidulans* also possesses an alternative pathway of cysteine synthesis involving homocysteine synthase, cystathionine β-synthase and cystathionine γ-lyase encoded by the *cysD*, *mecA* and *mecB* genes, respectively (Figure 1). This pathway is activated when the main route of cysteine synthesis is blocked (e.g., in the *cysB* mutant). The main enzyme of the alternative pathway is homocysteine synthase that forms homocysteine. This intermediate metabolite may be further converted to both cysteine and methionine. Therefore, new alleles of *A. nidulans cysA* and *cysB* genes are readily isolated (Natorff et al., 1993) as suppressors of *metA* and *metB* mutations, which affect methionine biosynthesis (Figure 1). Another type of suppressor mutations are those that affect regulatory genes, which in *A. nidulans* and *N. crassa* are designated *scon* (sulfur controller) (Dietrich and Metzenberg, 1973). The *A. nidulans sconB* and *sconC* mutations lead to elevated levels of homocysteine synthase and sulfur assimilation enzymes (Natorff et al., 1993). In this way the alternative pathway of sulfur amino acid synthesis is activated and blocks in the main pathway are suppressed (Figure 1). Mutations affecting orthologs of the *A. nidulans* s*conB* gene - *N. crassa scon2* and

S. cerevisiae MET30 - also lead to derepression of enzymes involved in assimilation of sulfate (Paietta, 1990; Thomas et al., 1995).

The *A. nidulans sconB* and *sconC* genes and their orthologs in filamentous fungi and yeast code for subunits of SCF ubiquitin ligase complex (Skp1 – Cullin – F-box) (for a review see Jackson et al., 2000). The *A. nidulans* SconB protein contains an F-box and WD40 repeats (Natorff et al., 1998), and the latter domain determines the specificity of the whole SCF complex because it is known to bind the substrate for ubiquitination. It has been demonstrated that the *S. cerevisiae* SCF^{Met30} ligase ubiquitinates the Met4 protein, a transcriptional activator specific for sulfur metabolism. Depending on growth conditions, ubiquitinated Met4 becomes inactivated (Kaiser et al., 2000) or is directed to degradation (Rouillon et al., 2000). Degradation takes place in response to an excess of sulfur amino acids in minimal media while in rich media the ubiquitinated protein remains stable (Menant et al., 2006).

The transcriptional activators that regulate the expression of sulfur assimilation genes in fungi - Met4 in *S. cerevisiae* (Thomas et al., 1992), CYS3 in *N. crassa* (Fu et al., 1989) and MetR in *A. nidulans* (Natorff et al., 2003) - belong to the bZIP family of proteins. Loss-of-function mutations affecting these transcriptional activators result in methionine auxotrophy.

S. cerevisiae Met4 possesses an activation domain, but it requires an accessory protein to bind DNA. This latter function may be provided by Cbf1 (Thomas et al., 1992) or by Met31/Met32 proteins (Blaiseau et al., 1997). On the other hand, the *N. crassa* CYS3 protein has an intrinsic ability to bind to DNA and has been demonstrated to recognize the consensus sequence ATGRYRYCAT (Li and Marzluf, 1996; Paietta, 2008). The *A. nidulans* MetR protein apparently recognizes the same sequence, as suggested by complementation of the *metR* deletion by the *A. nidulans metR* gene with the bZIP region replaced by its *N. crassa* counterpart (Natorff et al., 2003).

Both *metR* and *scon* genes in *A. nidulans* constitute a regulatory circuit named sulfur metabolite repression (SMR). Phenotypes of *metR* and *scon* mutants in *A. nidulans* suggest that SconB is a component of the SCF^{SconB} ligase, which controls the MetR transcriptional activator in a way similar to that of the *S. cerevisiae* SCF^{Met30} complex. A low molecular weight effector of SMR is cysteine, which can be readily made from methionine (Figure 1), usually added to the growth medium as a source of sulfur amino acids (Paszewski et al., 1994). It has also been demonstrated that cysteine is essential for regulation of the sulfur assimilation genes in *S. cerevisiae* (Hansen and Johannesen, 2000; Paszewski and Ono, 1992). All sulfur metabolism regulatory mutations isolated so far in *A. nidulans*, affecting either *metR* or *scon* genes, are recessive. The objective of this work was the isolation of suppressors in an *A. nidulans* diploid strain homozygous for the *metB3* mutation (Figure 1). Since dominant regulatory mutations were isolated previously in the *S. cerevisiae MET30* gene (Thomas et al., 1995) and recently in the *S. cerevisiae MET32* gene (Su et al., 2008), it could be expected that such suppressor mutations might affect the regulatory genes involved in SMR in *A. nidulans*. We report here the isolation and characterization of three dominant mutations, which render the *A. nidulans* MetR protein resistant to SMR-mediated inactivation or degradation. We also established that the *sG8* mutation, described originally as affecting sulfite reductase activity (Nadolska-Lutyk and Paszewski, 1988), is in fact an allele of the *metR* gene.

2. Materials and methods

2.1 Aspergillus nidulans strains

A. nidulans strains used in this work are listed in Table 1. Classical genetic methods were used in strain construction and analysis of suppressor mutations (Pontecorvo et al., 1953). The

N0 diploid strain homozygous with respect to the *metB3* mutation was constructed from M5 and M6 haploids. Suppressors of this mutation were allocated to their linkage groups by mitotic haploidization (Forbes, 1959), facilitated by the use of benomyl (Hastie, 1970) at a final concentration of 60 μg per 100 ml of solid medium.

2.2 Media and growth conditions

Complete medium (Cove, 1966) was used for propagation of *A. nidulans* strains, DNA isolation and haploidization of diploids. Minimal medium containing 2 mM sulfate (MM) and minimal medium without sulfate (MM-S), prepared according to Martinelli (1994), were used for growing mycelia for enzymatic assays, RNA isolation and ^{35}S-labeled sulfate incorporation. The MM-S medium was supplemented with the indicated concentrations of sulfate or L-methionine as the sole sulfur source. The minimal media were also supplemented according to the auxotrophic requirements of the strains employed. *A. nidulans* strains were grown on solid media at 37^{o}C and in liquid media in a rotary shaker (200 rpm) for 16 h at 30^{o}C. Mycelia were collected by filtration, washed with distilled water, frozen in liquid nitrogen and kept at –20^{o}C for up to one week.

Tests with selenate, a toxic analog of sulfate, were performed on MM-S medium supplemented with 5 mM methionine as the sole sulfur source. Sulfur assimilation enzymes are repressed by methionine in the wild type strain, but are constitutively derepressed in *scon* mutants, rendering them sensitive to selenate (Natorff et al., 1993). In addition, colonies of some *scon* mutants that possess a very high activity of arylsulfatase can be stained with indoxyl sulfate (Natorff et al., 1993).

2.3 Isolation of mutants

Suppressors of the *metB3* mutation in the N0 diploid strain were isolated as revertants to methionine prototrophy. Conidial suspension ($5x10^7$ per ml) was UV-irradiated to the survival level of 20% and then plated on minimal medium. Ten fastest-growing mutants were selected from among 100 methionine prototrophic colonies. Haploid strains expected to contain a suppressor of the *metB3* mutation were obtained by benomyl-induced chromosome loss.

2.4 Biochemical methods

Frozen mycelia for enzymatic assays were homogenized in a chilled mortar with powdered glass and then suspended in 0.1 M Tris pH 7.5. Protein concentration was estimated by the method of Bradford (1976). All enzymatic reactions were carried out at 37^oC. Homocysteine synthase activity was assayed as described by Paszewski and Grabski (1973), except that the reaction mixture volume was scaled down to 50 μl. Arylsulfatase activity was determined according to Metzenberg and Parson (1966).

ATP sulfurylase activity was determined by the method of de Vito and Dreyfuss (1964) with modifications. The reaction mixture contained: 50 μl of 1 M Tris pH 7.5, 50 μl of 0.1 M $MgCl_2$, 50 μl of 50 mM Na_2MoO_4, 50 μl of 0.1 M neutralized ATP, 200 μl of extract and water up to a total volume of 0.5 ml. The Na_2MoO_4 solution was replaced with water in the blank. The mixture was incubated for 30 min and the reaction was stopped with 0.5 ml of 10% TCA. The protein precipitate was removed by spinning down for 5 min in a microcentrifuge, and 70 μl of supernatant was taken for the colorimetric reaction, to which 175 μl of 2.5% NH_4MoO_4 in 2.5 M H_2SO_4 and 280 μl of water were added, and the mixture was kept for 10 min at room temperature. Then, 800 μl of water and 70 μl of eikonogen

solution (0.25%, prepared in a mortar with hot 14% metabisulfite and filtered) were added. After 10 min at room temperature the extinction at 660 nm was measured.

For estimation of sulfite reductase activity, the colorimetric method of sulfide assay (Siegel, 1965) was modified. The reaction mixture was prepared by combining 100 μl of 1 M Tris pH 7.5, 20 μl of 20 mM NADP, 50 μl of 24 μM FAD, 50 μl of 0.1 M glucose 6-phosphate, extract containing 200 μg of protein, 50 μl of 10 mM sodium bisulfite and adding water up to a total volume of 1 ml. Sodium bisulfite was replaced with water in the blank. The mixture was incubated for 20 min and placed in ice to stop the reaction. After adding 100 μl of 20 mM *N*,*N*-dimethyl-*p*-phenylenediamine in 7.2 M HCl, 50 μl of 10 mM sodium bisulfite in water and 100 μl of 30 mM $FeCl_3$ in 1.2 M HCl, the mixture was incubated for 20 min at 37^{o}C. The solution was clarified by spinning down for 3 min in a microcentrifuge and the extinction at 650 nm was measured. Activities of enzymes were expressed as nmol of product made per min per mg of protein.

Accumulation of ^{35}S-sulfate-labeled sulfur compounds was determined as described by Paszewski and Grabski (1974).

2.5 DNA manipulations and Northern blots

Total DNA from *A. nidulans* was isolated as described by Yelton et al. (1985). DNA for probes and sequencing was amplified by PCR. The DNA was sequenced on both strands with appropriate primers using an ABI 377 (Perkin-Elmer) automatic DNA sequencer. The DNA Sequencing and Oligonucleotide Synthesis Laboratory, Institute of Biochemistry and Biophysics performed the sequencing reactions as well as syntheses of primers listed in Supplementary Table 1. Probes were labeled using Hexanucleotide Kit™ (Fermentas). Total RNA was isolated using TRI Reagent (Molecular Research Center) according to the

manufacturer's protocol (Chomczynski, 1993). Northern blot analysis was carried out as described previously (Natorff et al., 2003).

2.6 Bioinformatics tools

Sequence databases were searched using BLAST programs, release 2.2.18 (Altschul et al., 1990) running on the NCBI server (http://www.ncbi.nlm.nih.gov/). The spliced sequence of the *N. crassa cys3* gene was found on the web site of the *Neurospora crassa* Database, Broad Institute of MIT and Harvard (http://www.broad.mit.edu). The sequences were aligned with Clustal X ver. 1.8 (Thompson et al., 1997) and shaded using GeneDoc ver. 2.6.002 (http://www.nrbsc.org/gfx/genedoc/index.html). Primers were designed with Oligo 4.0 (Molecular Biology Insights, Inc.). The quantities of transcripts detected in Northern blots were estimated by volume integration using Image-Quant 5.2 (Molecular Dynamics).

3. Results

3.1 Isolation, testing and mapping of suppressor mutations

Ten methionine-prototrophic mutants were isolated after UV irradiation of the N0 diploid strain homozygous with respect to the *metB3* mutation. These mutants were haploidized with benomyl as described in Materials and Methods. The obtained methionine-prototrophic haploid strains were tested for reversion of the *metB3* mutation by crossing to the wild type strain. In the progeny of four crosses no methionine auxotrophs were observed suggesting possible reversion of the *metB3* mutation. This was confirmed by sequencing of the PCR-amplified *metB* gene in the *metB3* mutant and the four prototrophic strains. It was found that the *metB3* mutation resulted from a single A to T transversion at position 201 with respect to the ATG codon. This mutation affected the coding sequence of the second exon of the *metB*

gene leading to the E31D substitution. Four out of the ten newly isolated mutants (no. 5, 7, 9, 11) had this aspartate changed to another amino acid (see Table 2 for details).

The remaining six haploid mutants generated methionine auxotrophs in crosses with wild type strains, indicating the presence of non-allelic mutations suppressing *metB3*. These suppressors were tested for dominance by constructing diploids with methionine auxotrophic strains M5 or M6, carrying the *metB3* mutation. Three diploids were able to grow on minimal medium, indicating the presence of a dominant suppressor mutation, and another three diploids required methionine, indicating the presence of a recessive mutation (Table 2).

We found that the recessive mutants 3, 4 and 8 were sensitive to 0.1 mM selenate, and mutants 3 and 4 stained with indoxyl sulfate. Since these characteristics are typical of *scon* mutants, we crossed the suppressor strains 3, 4 and 8, still bearing *metB3,* with a strain carrying the *sconB2* mutation. Very few methionine auxotrophs were obtained in the progeny of these three crosses, which indicated a close linkage of the tested suppressor mutations with the *sconB2* mutation and suggested that these suppressors might occur in the *sconB* gene itself. The three dominant mutants (no. 1, 2 and 10) did not stain with indoxyl sulfate and were resistant to 0.1 mM selenate (sufficient to inhibit growth of *scon* mutants), but were sensitive to 0.5 or 1 mM selenate, which distinguished them from the wild type strain. Thus, these suppressors exhibited a phenotype distinct from all known alleles of *A. nidulans* regulatory genes involved in sulfur metabolism. We started a search for the mutated gene by allocating the suppressor mutations to a chromosome. For this purpose the RM90 strain carrying both the suppressor 10 and the *metB3* mutation was used to construct a diploid with the tester strain FG3 containing *metB3*. This diploid strain was haploidized and the cosegregation of *galA* with suppressor 10 (methionine prototrophy) indicated its localization on chromosome III. Since the *A. nidulans metR* gene is also localized on chromosome III (Natorff et al., 2003), it was possible that the suppressor mutation occurred in the *metR* gene.

To test this hypothesis, the RM90 strain was crossed with the RM107 strain (*metR1, metB3*). The *metR1* mutant requires methionine even when grown on complete medium (Natorff et al., 2003), which discerns it from the *metB3* mutant that is a regular methionine auxotroph requiring methionine only on minimal medium. No segregant bearing only the *metB3* mutation was found in the progeny of this cross, indicating that the suppressor mutation was either closely linked to the *metR* gene or occurred in it.

3.2 Sequencing of the metR *gene in mutants 1, 2 and 10*

Since the dominant suppressor mutations present in strains 1, 2 and 10 might involve the *metR* gene, this gene was PCR-amplified and sequenced in all three strains. It turned out that the mutations took place in the coding sequence of *metR,* indicating that they are alleles of this gene. Hence, the mutations 1, 2 and 10 were denoted *metR21*^{d}, *metR22*^{d} and *metR20*^{d}, respectively.

Interestingly, the three suppressor mutations lead to substitution of F48 with either leucine or serine (Table 2). When the sequence of the *A. nidulans* MetR protein was aligned with sequences of homologous proteins from different fungi (Figure 2), it turned out that F48 lies in a conserved N-terminal region found in all branches of the *Ascomycota* division. This domain is well conserved among fungal species from the *Pezizomycotina* subdivision, and can also be identified in *Schizosaccharomyces pombe* (Sp in Figure 2) and *Schizosaccharomyces japonicus* that belong to the *Thaphrinomycotina* subdivision. Moreover, the N-terminal domain is conserved in *Debaryomyces hansenii* (Dh in Figure 2) and species related to it that belong to the *Saccharomycotina* subdivision. Thus, the newly isolated mutations in the *metR* gene identify a protein domain important for MetR function in *A. nidulans* and many species of *Ascomycota*.

3.3 Specificity of suppressors

Because the three dominant suppressors exhibited a similar phenotype and their mutations affected the same amino acid position, they were used interchangeably in further experiments. Strains possessing the *metR20*^{d} mutation were used in all biochemical analyses. The *metR21*^{d} strains, which set up crosses easier than strains bearing the other alleles of *metR*, were tested for their ability to suppress *A. nidulans* methionine mutations in *loci* other than *metB*. For this purpose, double mutant strains containing *metR21*^{d} and a mutation in the *metA* or *metG* gene, encoding methylenetetrahydrofolate reductase or cystathionine β-lyase, respectively (Figure 1), were constructed and tested for growth on a minimal medium. The *metB3, metR21*^{d} and *metA17, metR21*^{d} double mutants do not require methionine for growth (Figure 3), which means that *metR21*^{d} can completely suppress the *metB3* and *metA17* mutations. But the colony of the *metG55, metR21*^{d} double mutant is smaller on a minimal medium than on a methionine-supplemented medium (Figure 3), which means that *metR21*^{d} only partially suppresses the *metG55* mutation. Similarly, the *metG55, scon* double mutants also grow slower on a minimal medium than on a methionine-supplemented medium (Natorff et al., 1993). Thus, the *metR21*^{d} allele can suppress the *metA17*, *metB3* and *metG55* mutations as effectively as do suppressors assigned to the *scon* genes.

3.4 Characterization of the sG8 *mutation*

Genetic mapping of the *metR* gene (Natorff et al., 2003) and the *sG* gene, the latter affecting the activities of arylsulfatase and sulfite reductase (Nadolska-Lutyk and Paszewski, 1988), suggested their close linkage (less than 5 centimorgans). Taking into consideration some inaccuracy in gene mapping, it seemed possible that the *sG8* mutation occurred in fact within the *metR* gene. This assumption was confirmed by sequencing of the *metR* gene in the *sG8* strain, which revealed a single nucleotide G1251A transition and an adjacent T1252 deletion.

The latter change leads to a frameshift starting from serine 254 and resulting in the substitution of 41 C-terminal amino acids by 46 different residues. Thus, we assigned the *sG8* mutation to the *metR locus* and named it *metR18*. This mutation (marked by a triangle in Figure 2) is located between the leucine zipper and the C-terminal part of MetR, which is less conserved than bZIP and the N-terminal domain. The C-terminal domain is unusually long in *N. crassa* CYS3 and is absent in orthologs of MetR from the *Thaphrinomycotina* subdivision.

3.5 Activities of sulfur metabolism enzymes

It was of interest to establish how the new mutations identified in the *metR* gene affect the activities and regulation of sulfur assimilation enzymes. Under all sulfur source conditions tested, the activities of homocysteine synthase, ATP sulfurylase, sulfite reductase and arylsulfatase were slightly increased in the *metR20*^{d} mutant mycelia compared to the wild type strain (Figure 4).

The regulation of enzymatic activities in the *metR18* mutant exhibits a more complex pattern. The activity of sulfite reductase is lower in the mutant than in the wild type strain when grown on sulfate as the sole sulfur source and is further repressed by exogenous methionine (Figure 4). On the other hand, the activities of homocysteine synthase, arylsulfatase and ATP sulfurylase are highly elevated in the *metR18* strain grown on sulfate, but are repressed to the wild type level when the mutant is grown in the presence of methionine.

The changes in activities of the enzymes of sulfate assimilation pathway in the *metR18* mutant may be an effect of altered levels of the corresponding transcripts. The elevated levels of sulfur assimilation enzymes in the *metR20*^{d} mutant may also result from increased transcription of the corresponding genes. In order to check the above hypotheses we performed a Northern analysis of SMR-regulated genes in *metR20*^{d}, *metR18* and the wild type strain.

3.6 Northern analysis

Transcripts of several sulfur metabolism genes in the *metR20*^{d} and *metR18* mutants were compared to those observed in the wild type strain (Figure 5). The levels of all tested transcripts of sulfate assimilation genes are elevated in the *metR20*^{d} mutant mycelia grown in the presence of 0.1 mM sulfate or 0.25 mM methionine. Particularly increased is the level of the *sB* gene transcript (encoding sulfate permease), being ten times more abundant in the *metR20*^{d} mutant grown on 0.1 mM sulfate than in the wild type strain. The level of the *cysD* gene transcript is 2.7 times higher in the *metR20*^{d} mycelium grown on low sulfate than in the wild type strain (Figure 5, lanes 5 and 9). This result indicates that the twofold increase in homocysteine synthase activity (Figure 4) is a consequence of elevated transcription of the *cysD* gene. The transcript of the *sC* gene encoding ATP-sulfurylase and the transcript coding for the β subunit of sulfite reductase (ORF **AN7600**) are increased, indicating that activities of these enzymes are also elevated due to increased levels of the corresponding transcripts. The elevated levels of MetR-regulated transcripts observed in the *metR20*^{d} mutant suggest that this mutated activator may be partially resistant to inactivation or degradation mediated by SMR. This hypothesis is additionally supported by the fact that the incorporation of ^{35}S-sulfate into amino acids by the *metR20*^{d} mutant is twice that of the wild type strain (results not shown). This increase results from elevated levels of enzymes of the sulfate assimilation pathway in the *metR20*^{d} mutant.

The differences in levels of transcripts between the mutants and the wild type strain are diminished in the presence of exogenous methionine because under these conditions the transcripts are strongly repressed in all of them. Nevertheless, the transcript levels, like the corresponding enzymatic activities, are still higher in the *metR20*^{d} mutant than in the wild type strain.

The level of the transcript encoding the beta subunit of sulfite reductase in the *metR18* mutant grown on 0.1 mM sulfate is lowered to 70% of the wild type level (Figure 5, lanes 1 and 9). It is exactly the same change as the decrease of the sulfite reductase activity in this mutant (Figure 4). In contrast, levels of transcripts encoding other enzymes of sulfur assimilation are elevated. For instance, the *sC* gene transcript is by 60% more abundant in the *metR18* mutant than in the wild type strain (Figure 5), which mirrors exactly the levels of ATP-sulfurylase activity (Figure 4). The *cysD* gene transcript is nearly fivefold more abundant in the *metR18* mutant, which correlates well with the sixfold increase in the homocysteine synthase activity. Similarly to enzymatic activities, transcripts are repressed in the *metR18* mutant by exogenous methionine to the levels observed in the wild type (Figure 5, lanes 3 and 4). Thus, the changes in enzyme activities observed in the tested mutants are accompanied by, and probably result from, similar changes in the levels of the corresponding transcripts.

4. Discussion

We obtained ten prototrophic mutants from the N0 diploid strain homozygous with respect to the *metB3* mutation. Three of these strains, the most interesting ones, carry dominant suppressor mutations. Dominant alleles of regulatory genes specific for sulfur metabolism are rare. Up to date a dominant *met30-1* allele has been reported in *S. cerevisiae* (Thomas et al., 1995), and recently a deletion in the N-terminal domain of Met32 was found to be dominant in the same species (Su et al., 2008). The three dominant mutations described here are the first dominant alleles that affect regulation of sulfur metabolism in *A. nidulans*. Interestingly, they all carry amino acid substitutions in the *metR* gene coding for a transcriptional activator (Natorff et al., 2003).

Activities of sulfate assimilation enzymes are higher in the *metR20*^{d} mutant than in the wild type strain under all growth conditions tested (Figure 4). These results suggest that the MetR protein in the dominant suppressor may be changed in a way that renders it more resistant to inactivation or degradation driven by SMR. The elevated levels of MetR-regulated transcripts, higher assimilation of inorganic sulfur, and increased selenate sensitivity of the *metR20*^{d} mutant in comparison with the wild type strain corroborate this hypothesis.

The *metR* mutations reported here represent a novel, third type of methionine suppressors. The first type comprises c*ysA* and *cysB* mutations (Figure 1), affecting the synthesis of cysteine (Paszewski and Grabski, 1975) – the low molecular weight effector in the SMR system. The second type involves mutations in *scon* genes (Dietrich and Metzenberg, 1973) encoding components of the SCF complex, and in consequence inactivating SMR. The methionine suppressors reported here probably render the MetR protein less susceptible to inactivation by SCF. All three types of methionine suppressor mutations allow effective functioning of the alternative pathway of cysteine and methionine synthesis.

Interestingly, all three dominant suppressor mutations reported here lead to a change of a single amino acid – F48 (Figure 2). This might happen because of structural constraints that limit the possibilities of obtaining suppressor mutations in other positions of the *metR* gene that would result in a properly folded and functional protein. It was found that even small deletions in the N-terminal part of the *N. crassa* Cys3 protein render it non-functional, while amino acid substitutions introduced by alanine saturation mutagenesis result in mutants with either lower or higher activity of arylsulfatase (Kanaan and Marzluf, 1993). However, the results of *in vitro* mutagenesis of the *N. crassa cys3* gene must be treated with caution since the sequence that was then thought to code for the whole protein (**P22697.1** in the Swiss-Prot database) probably lacked the first exon present in the spliced version (**U03536.3**, *Neurospora crassa* Database, Broad Institute).

It is also worth noting that there is only one lysine residue in the N-terminal part of MetR, located 22 amino acids downstream from F48 (Figure 2). Therefore, this K70 is a likely candidate for the ubiquitination site in the *A. nidulans* MetR protein. Thus, the substitution of the nearby F48 in the *metR20*^{d} mutant might result in decreased ubiquitination of such mutated transcriptional activator leading to its accumulation. This hypothesis could be verified experimentally if estimation of the MetR protein content in mycelia were possible. However, MetR could not be detected with polyclonal antibodies in *A. nidulans* extracts from the wild type or the *metR20*^{d} mutant (Natorff R., personal communication).

The *metR18* mutation was originally reported as the *sG* gene, controlling sulfite reductase activity (Nadolska-Lutyk and Paszewski, 1988), and here we assigned this mutation to the *metR locus*. The *metR18* mutation is particularly interesting since it leads to highly elevated activities of several sulfate assimilation enzymes and increased levels of the corresponding transcripts, except for sulfite reductase (Figures 4 and 5). In spite of the high activities of the sulfate assimilation enzymes, *metR18* does not suppress the *metA* or *metG* mutations (Nadolska-Lutyk, 1985). Therefore, we suggest that the high activities of sulfur assimilation enzymes in the *metR18* mutant grown on sulfate might be a secondary effect of cysteine starvation, caused by a low activity of sulfite reductase. This shortage of cysteine may switch off SMR leading to derepression of the sulfate assimilation genes. The supplementation with methionine, which can be readily converted to cysteine (Figure 1), restores repression of genes coding for the sulfur assimilation enzymes, including sulfite reductase (Figure 4 and 5). Therefore, the SMR system in the *metR18* mutant is apparently not affected. It is also worth noting that the function of the C-terminal domains of *A. nidulans* MetR and *N. crassa* CYS3 proteins may be different because of the marked divergence of these two sequences (Figure 2). Besides, the frameshift changing 41 C-terminal amino acids in the *A. nidulans metR18* mutant leads to an increased activity of arylsulfatase (Figure 4) (Nadolska-Lutyk and

Paszewski, 1988), while deletion of the C-terminal part of CYS3 in the *N. crassa* PCYS511 mutant results in a decreased arylsulfatase activity (Kanaan and Marzluf, 1993). It is possible that the C-terminal domain of the *A. nidulans* MetR protein may be specifically required for proper transcription of the gene(s) encoding sulfite reductase. Elucidation of the mode of action of the *metR18* mutation appears an appealing task for future research.

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Table 1

Aspergillus nidulans strains used in this study

Strain	*Genotype*	*Source*
N0	diploid M5+M6 *metB3 nicA2 yA2+ metB3 anA1 biA1*	This study
W1	*pyroA4 yA2*	IBB collection
M1	*metA17 pyroA4 yA2*	IBB collection
M5	*metB3 nicA2 yA2*	IBB collection
M6	*metB3 anA1 biA1*	IBB collection
M25	*metG55 pyroA4 yA2*	IBB collection
R2	*sconB2 metA17 pyroA4 yA2*	IBB collection
RM71	*metR18 pyroA4 yA2*	IBB collection
RM107	*metR1 metB3 anA1 biA1 phenA2 lacA1 facA303*	This study
RM90	*metR20*^{d} *metB3 nicA2 yA2*	This study
RM91	*metR20*^{d} *pyroA4 yA2*	This study
RM92	*metR21*^{d} *metB3 biA1 pyroA4*	This study
RM93	*metR21*^{d} *metA17 anA1 biA1*	This study

RM94	*metR21*[d] *metG55 anA1 biA1*	This study
RM95	*metR22*[d] *nicA2 yA2*	This study
FG3	*metB3 sulA1 AcrA1 galA1 pyroA4 facA303 lacA1 choA1 chaA1*	This study
FGSC465	*sulA1 AcrA1 galA1 pyroA4 facA303 lacA1 choA1 chaA1*	IBB collection, from FGSC

1

Table 2

Summary of properties of newly isolated mutations

Mutant number	**Dominant or recessive**	**Affected gene**	**Nucleotide change**[a]	**Amino acid change**	**Allele name**
1	dominant	*metR*	C144G	Phe48Leu	*metR21*[d]
2	dominant	*metR*	C144A, T120A	Phe48Leu, Asn40Lys	*metR22*[d]
10	dominant	*metR*	T143C	Phe48Ser	*metR20*[d]
3	recessive	*sconB*	nt[b]	nt	na[c]
4	recessive	*sconB*	nt	nt	na
8	recessive	*sconB*	nt	nt	na
5	na	*metB*	A200T	Asp31Val	na
7	na	*metB*	T201A	Asp31Glu	na
9	na	*metB*	T201A	Asp31Glu	na
11	na	*metB*	A200C	Asp31Ala	na

[a] - position of A in the ATG codon was set as +1, please note that the *metB3* mutation is located behind the first 108-bp-long intron,

[b] - not tested,

[c] - not applicable

Figure Legends

Fig. 1

Outline of sulfate assimilation and sulfur amino acid metabolism pathways in *Aspergillus nidulans*. Genes and enzymes: *cysA* - serine acetyltransferase, *cysB* - cysteine synthase, *cysD* - homocysteine synthase, *mecA* - cystathionine β-synthase, *mecB* - cystathionine γ-lyase, *metA* and *metF* – two methylenetetrahydrofolate reductases, *metB* - cystathionine γ-synthase, *metG* - cystathionine β-lyase, *sA* - PAPS reductase, *sB* - sulfate permease, *sC* - ATP sulfurylase, *sD* - APS kinase, *sF* and beta (ORF **AN7600**) – α and β subunits of sulfite reductase, respectively. Metabolites: APS - adenosine-5'-phosphosulfate, PAPS – 3'-phosphoadenosine-5'-phosphosulfate, OAH - *O*-acetylhomoserine, OAS - *O*-acetylserine, SAH - *S*-adenosylhomocysteine, SAM - *S*-adenosylmethionine, THF-tetrahydrofolate, CH_2=THF-methylenetetrahydrofolate, CH_4-THF-methyltetrahydrofolate.

Fig. 2

Comparison of *A. nidulans* MetR protein sequence to its orthologs in several fungal species. An – *Aspergillus nidulans* MetR (**GI:5051964**), Af – *Aspergillus fumigatus* (**GI:238491954**), Ci - *Coccidioides immitis* (**GI:19188249**), Dh - *Debaryomyces hansenii* (**GI:50425459**), Gz - *Gibberella zeae* (**GI:46121585**), Nc – *Neurospora crassa* Cys3 (**GI:164427476**), Pc - *Penicillium chrysogenum* (**GI:255949770**), Sp – *Schizosaccharomyces pombe* Zip1 (**GI:19115435**), Ss - *Sclerotinia sclerotiorum* (**GI:156043879**), cs – consensus sequence. Highly conserved amino acids are in white-on-black, less conserved positions are marked by shading. Phenylalanine 48 is denoted with ↓ and the site of frameshift mutation in the *metR18* allele is marked with ▼ above the alignment. Positions of single lysine in N-terminal domains of *N. crassa* Cys3 and *A. nidulans* MetR proteins are marked with ● and ❍, respectively.

Fig. 3

Growth of *A. nidulans* methionine auxotrophs and their suppression by *metR21*^{d} mutation on minimal medium (MM). Methionine (0.25 mM, +met) was added to support growth of auxotrophs. Selenate sensitivity of strains carrying *metR21*^{d} mutation was tested on sulfate-free minimal medium with 5 mM methionine as a repressing source of sulfur (MM-S+met5). Selenate (1 mM, +sel) inhibits growth of sensitive mutants.

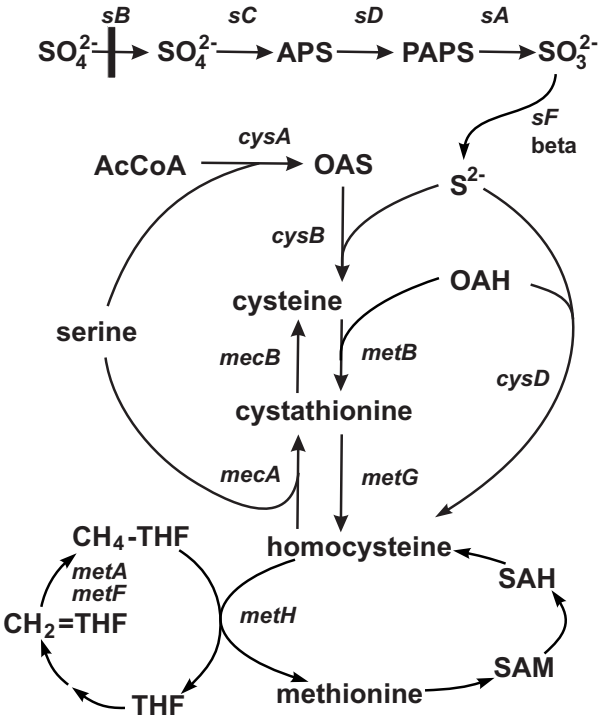
Fig. 4

Activities of sulfate assimilation enzymes in *metR20*^{d} and *metR18* mutants grown with various sulfur supplements. Means and standard deviations calculated from results of six independent experiments are presented as bars and vertical lines.

Fig. 5

Transcriptional regulation of sulfate assimilation genes in *metR20*^{d} and *metR18* mutants. Concentrations of sulfate and methionine added to MM-S medium as a sulfur source are indicated at the top. Probes used for transcript detection are: *sA* – PAPS reductase, *sB* – sulfate permease, *sC* – ATP sulfurylase, beta – β subunit of sulfite reductase (ORF **AN7600**), *cysD* – homocysteine synthase. Actin probe (ORF **AN6542**) was used as a control for mRNA level, and ethidium bromide-stained gel with 28S and 18S rRNA bands marked as a control for total RNA loaded on the gel. Shown is one out of three independent experiments that gave similar results. The quantified transcript levels are normalized to actin and then compared to those in the wild type strain growing on 0.1 mM sulfate, which was set as 1.0.

Figure 1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sD
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APS
PAPS
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sF
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OAS
S^{2-}
cysB
cysteine
OAH
serine
mecB
metB
cysD
cystathionine
mecA
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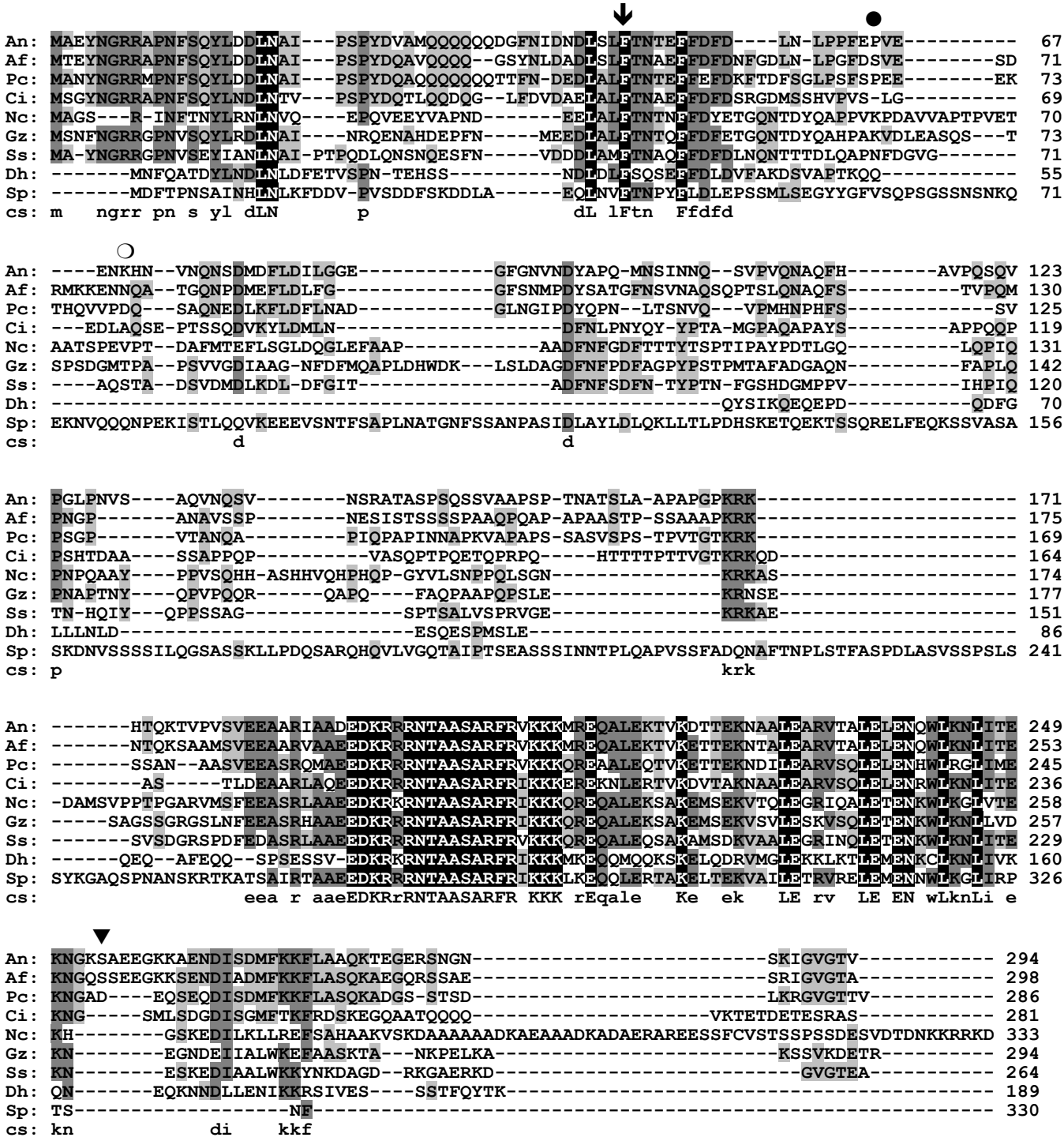
CH_2=THF
metH
SAM
methionine
THF

Figure 2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Af: MTEYNGRRAPNFSQYLDDLNAI---PSPYDQAVQQQQ--GSYNLDADLSLFTNAEFFDFDNFGDLN-LPGFDSVE--------SD 71
Pc: MANYNGRRMPNFSQYLDDLNAI---PSPYDQAQQQQQQQTTFN-DEDLALFTNTEFFEFDKFTDFSGLPSFSPEE--------EK 73
Ci: MSGYNGRRAPNFSQYLNDLNTV---PSPYDQTLQQDQG--LFDVDAELALFTNAEFFDFDSRGDMSSHVPVS-LG---------- 69
Nc: MAGS---R-INFTNYLRNLNVQ----EPQVEEYVAPND-------EELALFTNTNFFDYETGQNTDYQAPPVKPDAVVAPTPVET 70
Gz: MSNFNGRRGPNVSQYLRDLNAI----NRQENAHDEPFN-----MEEDLALFTNTQFFDFETGQNTDYQAHPAKVDLEASQS---T 73
Ss: MA-YNGRRGPNVSEYIANLNAI-PTPQDLQNSNQESFN-----VDDDLAMFTNAQFFDFDLNQNTTTDLQAPNFDGVG------- 71
Dh: -------MNFQATDYLNDLNLDFETVSPN-TEHSS----------NDLDLFSQSEFFDLDVFAKDSVAPTKQQ---------- 55
Sp: -------MDFTPNSAINHLNLKFDDV-PVSDDFSKDDLA------EQLNVFTNPYFLDLEPSSMLSEGYYGFVSQPSGSSNSNKQ 71
cs: m ngrr pn s yl dLN p dL lFtn Ffdfd
An: ----ENKHN--VNQNSDMDFLDILGGE----------GFGNVNDYAPQ-MNSINNQ--SVPVQNAQFH--------AVPQSQV 123
Af: RMKKENNQA--TGQNPDMEFLDLFG----------GFSNMPDYSATGFNSVNAQSQPTSLQNAQFS----------TVPQM 130
Pc: THQVVPDQ---SAQNEDLKFLDFLNAD----------GLNGIPDYQPN--LTSNVQ---VPMHNPHFS----------SV 125
Ci: ---EDLAQSE-PTSSQDVKYLDMLN----------DFNLPNYQY-YPTA-MGPAQAPAYS---------APPQQP 119
Nc: AATSPEVPT--DAFMTEFLSGLDQGLEFAAP----------AADFNFGDFTTTYTSPTIPAYPDTLGQ----------LQPIQ 131
Gz: SPSDGMTPA--PSVVGDIAAG-NFDFMQAPLDHWDK---LSLDAGDFNFPDFAGPYPSTPMTAFADGAQN----------FAPLQ 142
Ss: ----AQSTA--DSVDMDLKDL-DFGIT----------ADFNFSDFN-TYPTN-FGSHDGMPPV----------IHPIQ 120
Dh: ----------QYSIKQEQEPD----------QDFG 70
Sp: EKNVQQQNPEKISTLQQVKEEEVSNTFSAPLNATGNFSSANPASIDLAYLDLQKLLTLPDHSKETQEKTSSQRELFEQKSSVASA 156
cs: d d
An: PGLPNVS----AQVNQSV--------NSRATASPSQSSVAAPSP-TNATSLA-APAPGPKRK---------- 171
Af: PNGP-------ANAVSSP--------NESISTSSSSPAAQPQAP-APAASTP-SSAAAPKRK---------- 175
Pc: PSGP-------VTANQA---------PIQPAPINNAPKVAPAPS-SASVSPS-TPVTGTKRK---------- 169
Ci: PSHTDAA----SSAPPQP----------VASQPTPQETQPRPQ-----HTTTTPTTVGTKRKQD---------- 164
Nc: PNPQAAY----PPVSQHH-ASHHVQHPHQP-GYVLSNPPQLSGN---------------KRKAS---------- 174
Gz: PNAPTNY----QPVPQQR------QAPQ----FAQPAAPQPSLE---------------KRNSE---------- 177
Ss: TN-HQIY---QPPSSAG--------------SPTSALVSPRVGE---------------KRKAE---------- 151
Dh: LLLNLD-------------------------ESQESPMSLE---------- 86
Sp: SKDNVSSSSILQGSASSKLLPDQSARQHQVLVGQTAIPTSEASSSINNTPLQAPVSSFADQNAFTNPLSTFASPDLASVSSPSLS 241
cs: p krk
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Af: -------NTQKSAAMSVEEAARVAAEEDKRRRNTAASARFRVKKKMREQALEKTVKETTEKNTALEARVTALELENQWLKNLITE 253
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Ci: --------AS-----TLDEAARLAQEEDKRRRNTAASARFRIKKKEREKNLERTVKDVTAKNAALEARVSQLELENRWLKNLITE 236
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Ss: -------SVSDGRSPDFEDASRLAAEEDKRRRNTAASARFRVKKKQREQALEQSAKAMSDKVAALEGRINQLETENKWLKNLITE 229
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Af: KNGQSSEEGKKSENDIADMFKKFLASQKAEGQRSSAE----------SRIGVGTA---------- 298
Pc: KNGAD----EQSEQDISDMFKKFLASQKADGS-STSD----------LKRGVGTTV---------- 286
Ci: KNG-----SMLSDGDISGMFTKFRDSKEGQAATQQQQ----------VKTETDETESRAS---------- 281
Nc: KH--------GSKEDILKLLREFSAHAAKVSKDAAAAAADKAEAAADKADAERAREESSFCVSTSSPSSDESVDTDNKKRRKD 333
Gz: KN--------EGNDEIIALWKEFAASKTA---NKPELKA----------KSSVKDETR---------- 294
Ss: KN--------ESKEDIAALWKKYNKDAGD--RKGAERKD----------GVGTEA---------- 264
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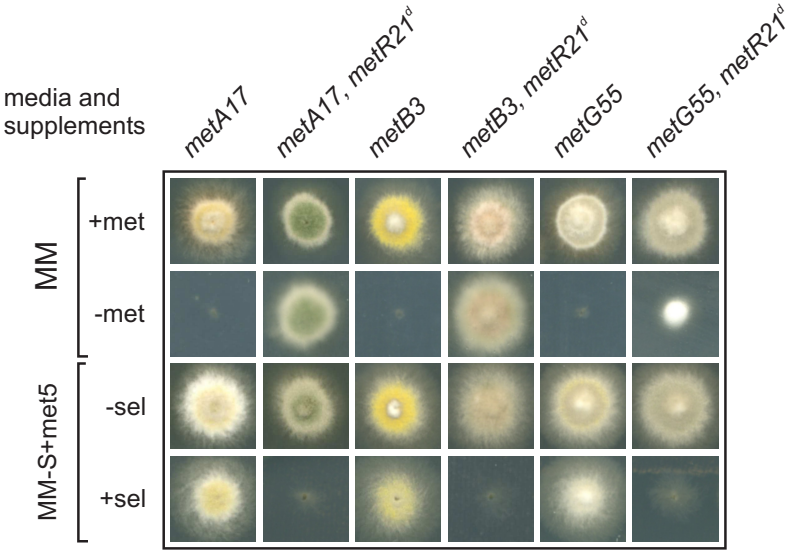
Figure 3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+sel

Figure 4

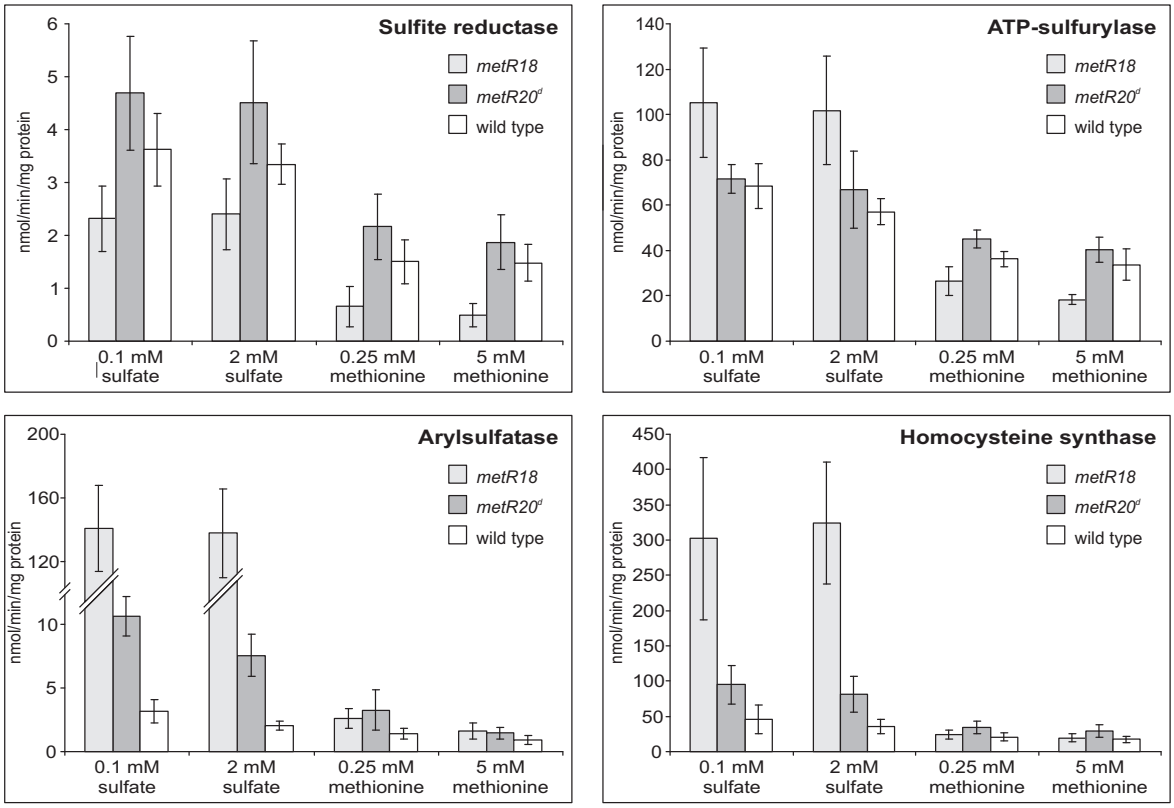
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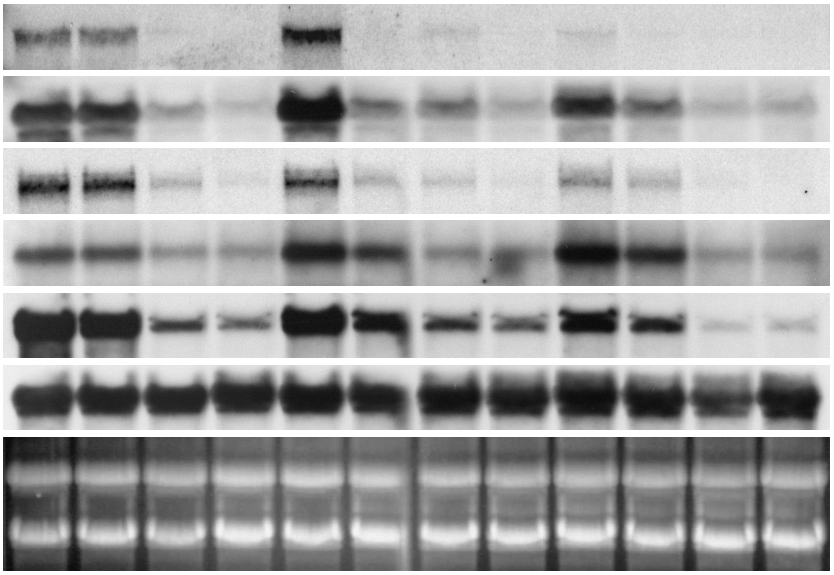
Figure 5



Lane	1	2	3	4	5	6	7	8	9	10	11	12
sB/actin	7.0	4.8	0.4	0.2	10.2	0.2	0.9	0.0	1.0	0.2	0.1	0.0
sC/actin	1.6	1.2	0.4	0.3	2.2	0.6	0.6	0.4	1.0	0.7	0.4	0.3
sA/actin	3.6	3.0	0.5	0.1	2.4	0.6	0.3	0.2	1.0	0.6	0.2	0.1
beta/actin	0.7	0.6	0.3	0.3	1.1	0.7	0.3	0.3	1.0	0.7	0.4	0.3
cysD/actin	4.6	3.2	0.6	0.4	2.7	1.3	0.6	0.5	1.0	0.7	0.2	0.2
actin	0.7	0.8	0.8	0.9	1.0	0.8	0.9	0.9	1.0	0.9	0.7	0.9

Supplementary material

Supplementary Table 1

Primers used for probe amplification

Gene	Primer	Sequence
actin	ANAKT1U	GTGATGAGGCACAGTCCAAG
	ANAKT1L	TACCACCGCTTTCCAGACCA
sA	AnsAup1	ACCACGCCCACCACAACCATA
	AnsAlow1	TGAGAACCAAACGCCAGGAAA
sB	SBU4075	AGGAACTGGTGGCCATTG
	SBL4750	GGTTGCTGTACGTGAAGG
sC	SC1U	GAACGGTGGTTTCAGTCCTCT
	SC1L	GATGATAGCGTGCCAGACAGC
cysD	CYSD2U	TCGGATCCTCGCTACAATGTGCCTGACT
	CYSD2L	ATGGAATTCAAAGTCGGCGATGATGTC
β subunit of sulfite reductase	BU466	TACGGTTTCGGTTCTCTGATT
	BL1454	TCTCCTCGGTGGGCAAGTCCT