Title:

The *Aspergillus nidulans metZ* gene encodes a transcription factor involved in regulation of sulfur metabolism in this fungus and other *Eurotiales*

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Abbreviation:

OE::*metZ* - overexpression of the *metZ* gene

SMR - sulfur metabolite repression system

Summary

In *Aspergillus nidulans*, expression of sulfur metabolism genes is activated by the MetR transcription factor containing a basic region and leucine zipper domain (bZIP). Here we identified and characterized MetZ, a new transcriptional regulator in *Aspergillus nidulans* and other *Eurotiales*. It contains a bZIP domain similar to the corresponding region in MetR and this similarity suggests that MetZ could potentially complement the MetR deficiency. The *metR* and *metZ* genes are interrupted by unusually long introns. Transcription of *metZ*, unlike that of *metR*, is controlled by the sulfur metabolite repression system (SMR) dependent on the MetR protein. Overexpression of *metZ* from a MetR-independent promoter in a *ΔmetR* background, activates transcription of genes encoding sulfate permease, homocysteine synthase and methionine permease, partially complementing the phenotype of the *ΔmetR* mutation. Thus, MetZ appears to be a second transcription factor involved in regulation of sulfur metabolism genes.

Introduction

Transcription factors containing bZIP domains (basic region, leucine zipper) are engaged in the regulation of diverse metabolic pathways in eukaryotes. They form homo- and heterodimers through the leucine-zipper domains. Different combinations of monomers recognize different sequence elements in the promoters of target genes (Glover and Harrison 1995; Grigoryan and Keating 2008).

Expression of sulfur metabolism genes is controlled by bZIP transcription factors in numerous fungi: CYS3 in *Neurospora crassa* (Marzluf 1997), Zip1 in *Schizosaccharomyces pombe* (Harrison et al. 2005), Met4 and Met28 in *Saccharomyces cerevisiae* (Thomas and Surdin-Kerjan 1997). CYS3 from *N. crassa* is one of the best characterized bZIP family proteins in filamentous fungi. It is involved in coordinated expression of genes encoding enzymes responsible for acquisition and utilization of sulfur (Marzluf and Metzenberg 1968). CYS3 functions as a homodimer (Kanaan et al. 1992) and recognizes the palindromic sequence 5-ATGRYRYCAT-3 (Li and Marzluf 1996) present in promoters of sulfur assimilation genes (Fu and Marzluf 1990; Fu et al. 1989). In *Aspergillus nidulans*, an ortholog of CYS3 is MetR activating transcription of sulfur metabolism genes, in particular those involved in sulfate assimilation (Natorff et al. 2003). The *Aspergillus fumigatus* MetR protein is a bZIP transcription factor important for sulfur assimilation and pathogenicity (Amich et al. 2013).

Transcription factors specific to sulfur metabolism in fungi are controlled by activity of ubiquitin ligases from the SCF family. The role of the SCF^{Met30} complex in ubiquitination of the Met4 bZIP transcription factor was extensively studied in *S. cerevisiae*. 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 *A. nidulans* ubiquitin ligase controlling activity of MetR is encoded by the *scon* genes (Natorff et al. 1998; Piotrowska et al. 2000). The level of the active MetR protein is apparently dependent on the sulfur status. When cysteine or methionine (which is readily metabolized to cysteine) are abundant, MetR is probably inactivated and/or degraded by the SCF^{SconB} ubiquitin ligase complex, analogously to the *S. cerevisiae* Met4 protein. In consequence, expression of MetR-regulated genes is repressed to its minimal constitutive level. This regulatory system is known as the sulfur metabolite repression system (SMR) (Paszewski et al. 1994). In contrast to *cys-3* in *N. crassa*,

transcription of the *metR* gene itself is not controlled by SMR. Regulation of bZIP transcription factor stability may be more complex as an ortholog of MetR in *S. pombe*, Zip1, which is phosphorylated and this phosphorylation leads to an interaction with Pof1 and subsequent degradation (Harrison et al. 2005). Moreover, recent transcriptomic analyses found interaction of sulfur metabolism with metabolism of iron (Amich et al. 2013) and stress responses (Sieńko et al. 2014).

The *A. nidulans* genome contains 22 proteins, which are annotated in the Broad Institute database (http://www.broadinstitute.org), as containing bZIP-domain. Using the MetR amino acid sequence as a query in a BLAST similarity search of the *A. nidulans* genome reveals that one of these proteins, encoded by the AN5218 open reading frame (ORF), contains a bZIP-domain very similar to that of MetR. This finding prompted us to search for a function of the novel gene, which we named *metZ*. Orthologs of *metZ* are present in *Eurotiales* only, indicating specific regulation of sulfur metabolism in this taxon, which distinguishes it from other orders of *Ascomycota*. The high similarity of the MetR and MetZ bZIP domains suggested that overexpression of *metZ* could complement some deficiencies of the *ΔmetR* mutant. To verify this assumption, the *metZ* promoter was replaced with a MetR-independent one, which led to transcriptional activation of some genes, including those for transporters of sulfate and sulfur amino acids.

Materials and methods

Strains and plasmids

Aspergillus nidulans strains from our collection carrying standard markers (Martinelli 1994), used in the study, are listed in Table 1 along with plasmids and an *Escherichia coli* strain. The W1 wild-type (WT) strain of *A. nidulans* (Glasgow) was used as a reference for growth and qPCR experiments. *A. nidulans* chromosome-specific gene libraries constructed on cosmids pWE15 and pLORIST2 (Brody et al. 1991) were obtained from the Fungal Genetics Stock Center, Kansas, USA.

Growth conditions and genetic analysis

For protoplast or DNA isolation, mycelia were grown in complete (C) medium (Cove 1966). For RNA isolation, minimal sulfur free (MM-S) medium (Lukaszkiewicz and Paszewski 1976) supplemented with either 0.1 mM sulfate (low inorganic sulfur), 2 mM sulfate (high inorganic sulfur), 0.3 mM L-methionine (low organic sulfur), 1 or 5 mM

L-methionine (repressing conditions) as a sole sulfur source was used. The MM-S medium was also supplemented according to the auxotrophic requirements of the strain employed. Liquid cultures were grown in 100 ml of MM-S medium at 37°C for 16 h in a rotary shaker (200 rpm). *Escherichia coli* was grown in standard LB medium supplemented with antibiotics as required (Sambrook et al. 1989).

Nucleic acids manipulations

Standard procedures for plasmid propagation and isolation were according to (Sambrook et al. 1989). Sequences of primers used are listed in Supplementary Table S1. DNA from *A. nidulans* was isolated by the salting out method by grinding frozen mycelia under liquid nitrogen, followed by immediate suspension in warm STEN buffer (1% SDS, 100 mM Tris pH 7.5, 50 mM EDTA pH 8, 100 mM NaCl) (Sambrook et al. 1989).

Total RNA was isolated from powdered mycelia using TRI Reagent (Molecular Research Center) according to the manufacturer's protocol (Chomczyński 1993) and subsequently precipitated with lithium chloride added to a final concentration of 3.42 M as described by (Barlow et al. 1963). RNA for Northern blot analysis (30 μg of total RNA in each lane) was fractionated in 1% agarose gels containing 1% formaldehyde, and 1×NBC buffer (0.5 M boric acid, 10 mM sodium citrate, 50 mM NaOH) and transferred to nylon membranes (BrightStar®-Plus, Ambion) using a Turbo blotter (Schleicher & Schuell, Inc.). ^{32}P-labeling was done with DecaLabel DNA Labeling Kit according to the manufacturer (Thermo Scientific, Fermentas). Hybridization signals were visualized in a Fuji FLA-7000 PhosphorImager. Reverse transcription was performed using the RevertAid™ H Minus First Strand cDNA Synthesis Kit according to the manufacturer's protocol (Thermo Scientific, Fermentas).

Plasmids bearing the *metZ* gene under the *A. nidulans alcA* or *trpC* promoter were constructed in the pAL3 vector bearing the *N. crassa pyr-4* selection cassette (Waring et al. 1989). The entire *metZ* ORF with 3'UTR (from -12 bp upstream of the ATG start codon through 786 bp after the stop codon) was amplified by PCR, cut and ligated into KpnI-BamHI restriction sites of the vector yielding the PALMZ8 plasmid (Table 1). Next, the SpeI-KpnI fragment of the *alcA* promoter was replaced with PCR-amplified 377-bp fragment of the *trpC* promoter (Hamer and Timberlake 1987) yielding the kTRMZPG plasmid (Table 1).

The TOPO5'3'UTRMZPG plasmid (Table 1) for deletion of the entire *metZ* gene, including its promoter and 3'UTR, comprised the *N. crassa pyr-4* selection marker

surrounded by the 5'- and 3'-flanking regions of *metZ* (positions -2735 through -1070 relative to the *metZ* ATG codon and 365 through 2017 relative to the *metZ* stop codon, respectively). Both flanks of *metZ* along with the *pyr-4* marker were PCR-amplified (sequences of primers are shown in Supplementary Table S1) and cloned initially in the PCR2-TOPOII vector (LifeTechnologies™). Then, the 5'- and 3'-flanks were cut and cloned respectively in the XbaI-NotI and BglII-KpnI restriction sites of the TOPOpyr4 plasmid (Table 1) yielding TOPO5'3'UTRMZPG, which was used for *A. nidulans* transformation. Deletion of *metZ* was attempted in the W12 wild type strain and in strains bearing *ΔnkuA* (TN02A25) or *ΔmetR* mutation (RM131), all bearing the *pyrG89* mutation.

Transformation of A. nidulans

Mycelia for transformation were collected by filtration, washed with 0.6 M KCl and suspended in 0.6 M KCl buffered with 10 mM potassium phosphate pH 6.5 and containing lytic enzymes: 15-20 mg/ml of Glucanex® 200G (Novozymes), 2 mg/ml of Driselase® (Sigma-Aldrich) and 1 mg/ml of snail acetone powder (Sigma-Aldrich). Protoplasts were prepared and transformed by the PEG method (Kuwano et al. 2008). Transformants were selected for uracil prototrophy on MM-S medium supplemented with 2 mM sulfate and 1.2 M sorbitol.

Microarray analysis

Transcriptomes of two congenic strains, K1 (*ΔmetR,pyr-4+*) and TZ12 (*ΔmetR,trpC*^{pr}*::metZ,pyr-4+*), both grown in MM-S supplemented with 0.3 mM methionine, were compared in three biological replicates, each in two technical replicates with dye swap. cRNA probes fluorescently labeled with Cy3 or Cy5 were synthesized using Quick Amp Labeling Kit, two-color (Agilent Technologies) according to manufacturer's protocol, using 5 μg of total RNA as a template. Labeled probes were hybridized concurrently to *A. nidulans* custom designed microarray slides (purchased from Agilent) in an 8×15k format containing oligonucleotides representing all known *A. nidulans* genes identified in the Aspergillus Genome Database (AspGD) version s06-m01-r07. Following hybridization the microarrays were scanned with an Axon GenePix 4000B microarray scanner (Molecular Devices, LLC). Feature extraction was done with GenePix Pro 6.1. Raw LogRatio results from all biological and technical replicates were Lowess normalized, the resulting data for each gene were averaged and the statistical significance (p-values) were calculated with Acuity 4.0 software.

Additional data manipulations were done in Microsoft Excel. A gene was considered to be differentially expressed between the *ΔmetR* and *ΔmetR,trpC*^{pr}*::metZ* strains if its transcript level differed between the two strains at least twofold ($|\log_2 Ratio|>1$) and the probability of such a difference by chance was less than 0.05 ($p<0.05$). The resulting lists of differentially expressed genes (Supplementary Table S2) were subjected to further bioinformatics analysis. ORF descriptions were retrieved from the Aspergillus Genome Database (http://www.aspgd.org/). The data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al. 2002) and are accessible through GEO Series accession number GSE62548 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62548).

Quantitative real-time RT-PCR

Real-time RT-PCR was performed using the LightCycler®480 System (Roche Laboratories) with SYBR Green detection, according to the manufacturer's instructions. The primers used to quantify expression of target genes (Supplementary Table S1) were designed using Primer3 software (Untergasser et al. 2012). Primer specificity was verified by melting curve analysis. qRT-PCRs were performed in triplicate in 96-well plates with each 10-µl reaction mixture containing 5 µl of LightCycler® 480 SYBR Green I Master mix (Roche Laboratories), two primers (3 pmol of each) and 1µl of diluted template cDNA. cDNA was synthesized from 5 µg of total RNA treated with DNaseI (Roche) using RevertAid™ H Minus M-MuLV Reverse Transcriptase kit (Fermentas) according to supplier's protocol. The *actA* actin gene (AN6542) was used as the normalization reference (internal control) for target gene expression ratios. Average cycle thresholds were calculated and the Pfaffl method (Pfaffl 2001) was applied to calculate relative expression with respect to that of actin.

Bioinformatics tools

Homology searches were carried out against the GenBank (release 95.0) database with BLAST algorithm (Altschul and Lipman 1990). DNA and protein sequences were aligned with ClustalX (Thompson et al. 1997). Evolutionary tree based on protein distance matrix was built with the Kitsch program, which is part of the PHYLIP package version 3.6. (Felsenstein 1989). Primers were designed with Clone Manager Suite 9 (Scientific & Educational Software, Cary NC). Predicted dimers formed by bZIP proteins were drawn using the DrawCoil program (http://www.grigoryanlab.org/drawcoil/). Functional categories enriched among genes up- or down-regulated in the *ΔmetR* strain overexpressing *metZ* were identified

using the FungiFun web server (https://sbi.hki-jena.de/FungiFun/FungiFun.cgi) (Priebe et al. 2011).

Results

The *metZ* gene was found by a BLAST search of the *A. nidulans* genome using the MetR protein sequence as a query, which yielded only one additional sequence with e-value amounting 7.0e-20. This gene, encoded by the AN5218 ORF, we named *metZ*. The newly identified gene is located on chromosome V and codes for a putative protein of 279 amino acids showing 26% identity with MetR. The putative MetZ protein contains a bZIP domain showing 77% identity and 91% similarity to the bZIP domain of MetR (Fig. 1A). Such a high similarity suggests that MetZ could be a bZIP transcription factor potentially recognizing sequences in DNA similar to the MetR targets.

The UmetZ and LmetZ primers (Supplementary Table S1) designed to the central part of the *metZ* gene allowed for identification of the SL10G06 cosmid in the LORIST/pWE library. A 3470-bp SalI-KpnI fragment bearing the *metZ* gene was excised and cloned into the pBluescript KS(–) vector yielding the kKSMZ plasmid, then the insert was verified by DNA sequencing. The sequence of the *metZ* gene was submitted to the EMBL/GenBank database under accession number KJ195521 and it is identical to the sequence of the AN5218 ORF in the *A. nidulans* FGSC A4 strain. Both *metR* (Natorff et al. 2003) and *metZ* genes are interrupted by a single unusually long intron, located in similar position of both genes. The intron in the *metZ* gene is 570 bp long (Fig. 1B), as determined by comparison of the RT-PCR-generated cDNA obtained on the template of mRNA with the genomic sequence.

Orthologs of MetZ are present in the *Eurotiales* order only, in contrast to MetR having orthologs in the entire *Pezizomycotina* subphylum (Fig. 1A). The MetZ protein form a single branch of evolutionary tree (highlighted by a shaded box on tree in Fig. 1A), which suggests duplication of the ancestral MetR gene during evolution of *Eurotiales*. Analysis of promoter regions of *metZ* and its orthologs shows that all of them contain conserved palindromic 5'-ATGRYRYCAT-3' elements located in the same position (Fig. 1C). Moreover, similar sequence motifs are also found within the long introns of *metZ* and its orthologs (Fig. 1D). This sequence motif is recognized by the *N. crassa* CYS3 protein (Li and Marzluf 1996), an ortholog of the MetR protein. Such motifs are also present in the promoters of the *A. nidulans* genes regulated by MetR. It has been shown earlier that the bZIP domains of MetR and CYS3

are functionally interchangeable (Natorff et al. 2003), thus the two proteins are likely to bind the same sequences.

In order to identify the function of the *metZ* gene we tried deleting it (see Materials and Methods), but failed despite having repeated the procedure several times: all the transformants obtained contained an intact *metZ* gene. Thus, to identify the physiological function of *metZ* an alternative approach had to be applied. To study the effect of MetZ on expression of other genes the *metZ* ORF was fused with strong promoters *alcA* or *trpC* and these constructs were introduced into the W12 wild type strain and the *metR* deletion mutant (RM131). Each construct partially complemented the defect of the *ΔmetR* strain because transformants overexpressing *metZ*, in contrast to the parental *ΔmetR* strain, did not need methionine for growth on complete medium though they still required it on minimal medium (Fig. 2). Moreover, the *ΔmetR* mutant overexpressing the *metZ* gene could be rescued with cysteine when grown on minimal medium, in contrast to the *ΔmetR* strain requiring supplementation with methionine (Fig. 2). These results suggest that MetZ could induce transcription of genes encoding sulfur amino acids permeases. This assumption is supported by the fact that overexpression of *metZ* in the wild-type strain resulted in an elevated sensitivity to ethionine (Fig. 2), a toxic analog of methionine. Moreover, overexpression of *metZ* in the *ΔmetR* mutant rendered this strain sensitive to chromate and selenate (toxic analogs of sulfate) even under repressive conditions. This result suggests that MetZ can activate transcription of the *sB* gene encoding sulfate permease.

To identify potential targets of the MetZ transcription factor we compared the transcriptome of the *ΔmetR* strain overexpressing *metZ* from the *trpC* promoter with that of the *ΔmetR* strain transformed with an empty vector, showing very low constitutive level of the *metZ* transcript. Overexpression of the *metZ* gene up-regulated 750 genes and down-regulated 552 genes (Supplementary Table S2). In order to find biological significance of the observed transcriptomic changes we analyzed KEGG pathways, GO terms and the Functional Catalogue categories assigned to all the genes whose transcript levels were changed at least twofold in *ΔmetR* overexpressing *metZ*. Those genes belonged to diverse main categories of the Functional Catalogue (Fig. 3). The most frequently represented among the differentially expressed genes was the main category Metabolism containing 238 up-regulated and 180 down-regulated genes. Some of the up-regulated genes were those involved in sulfur metabolism, including methionine permeases (AN1631, AN8538, and AN12483), arylsulfatase (AN8341), taurine dioxygenases (AN2960, AN4111, and AN4108) and

homocysteine synthase (*cysD*) (Fig. 4, see Supplementary Table S2 for details). However, some genes encoding enzymes of the sulfate assimilation pathway were unaffected by overexpression of *metZ* (*e.g.*, *sA* coding for phosphoadenosine phosphosulfate reductase or the AN1752 encoding α-subunit of sulfite reductase) or were even down-regulated (*e.g.*, *sC* encoding ATP sulfurylase or AN7600 encoding β-subunit of sulfite reductase). Thus, overexpression of *metZ* leads to elevated transcription of permeases and enzymes incorporating sulfide into sulfur amino acids while transcription of sulfate assimilation genes is decreased (Fig. 4). The simultaneous activation of two genes encoding oxidoreductases transferring electrons from sulfide to quinone (AN1825 and AN8346) could additionally contribute to controlling intracellular sulfide concentration. Interestingly, analysis of KEGG pathways revealed that the *metZ* overexpressing strain accumulates transcripts encoding three consecutive enzymes of the ubiquinone biosynthesis pathway (AN1743, AN3586 and AN4569). Among other up-regulated genes many are involved in biosynthesis of lipids, which might be components of biological membranes: ergosterol (AN0451, AN1901, AN4094, AN6506, AN8907), fatty acids (AN9407, AN9408) and unsaturated fatty acids (AN1037, AN4135, AN6731, AN7204). GO term analysis of genes up-regulated by overexpression of *metZ* showed that 58 of them are involved in transmembrane transport, including methionine permeases mentioned above. However, the *sB* transcript encoding sulfate permease was not detected in the microarray analysis while the results of growth tests suggested that it could be elevated (Fig. 2). Hence, we performed quantitative real-time PCR analysis for the *sB* transcript and additionally for some other relevant genes to complement the microarray data (Table 2). Levels of the *sB* and *cysD* transcripts were almost threefold induced and that of AN1631 (encoding an ortholog of the *Saccharomyces cerevisiae* Mup1p high-affinity methionine permease) even sevenfold in the strain overexpressing *metZ*. As expected, overexpression of *metZ* resulted in at least 70-fold induction of its own transcript. Using two pair of primers we found five-fold stronger up-regulation of the second exon than the first one under starvation conditions (Table 2) suggesting independent expression of the second exon, presumably starting from a putative second promoter located in the intron. This seems likely since the intron does contain conserved sequences that could activate transcription (Fig. 1D).

Expression of the *metZ* gene was studied in more detail using Northern blotting to reveal two bands in the wild type strain under derepressing conditions (up to 4 h of sulfur starvation) (Fig. 5), supporting the above assumption that there is a second transcription start

site located in the *metZ* intron. Transcription of *metZ* was strongly regulated by the sulfur metabolite repression system (SMR), being repressed in *A. nidulans* grown in the presence of methionine or high sulfate and derepressed after a shift to sulfur starvation conditions (Fig. 5). However, under the sulfur starvation conditions expression of only the short *metZ* transcript was up-regulated as shown by a real-time PCR analysis (Table 2). One should note here that in the overexpression experiment where the full *metZ* ORF was fused to a strong MetR-independent promoter, only the long (spliced) transcript was overproduced (Table 2, Fig. 5). Transcription of *metZ* depends on the MetR transcription factor because only a very low level of the *metZ* transcript was observed in the *ΔmetR* mutant even under sulfur starvation conditions (Fig. 5). The latter result confirms that the expression of the *metZ* gene is controlled by SMR and the MetZ protein is a second, beside MetR, transcription factor affecting expression of sulfur metabolism genes.

Discussion

In this study we describe a novel paralog of the MetR bZIP transcription activator of *A. nidulans*, encoded by the *metZ* gene. The deduced amino acid sequences of both MetR and MetZ proteins contain almost identical bZIP domains so they could in principle recognize similar target sequences in DNA and consequently, affect transcription of the same genes. Since bZIP proteins usually form dimers (Pu and Struhl 1993), it seems plausible that MetZ could form the MetZ-MetZ homodimer or heterodimers with other transcription factors. Analysis of putative interactions between leucine zipper domains shows that MetZ and MetR proteins have very similar leucine zippers so they could potentially form homodimers and the MetZ-MetR heterodimer, each of them stabilized by six ionic bonds (Fig. 1E). In *S. cerevisiae*, two proteins involved in regulation of sulfur metabolism genes (Met4p and Met28p) form a heterodimer through their leucine zipper domains (Lee et al. 2010). However, Met4p and Met28p show low similarity to other fungal bZIP transcription factors involved in regulation of sulfur metabolism (Fig. 1A).

In order to study the role of MetZ as a putative transcription factor we analyzed transcriptomic effects of the *metZ* gene overexpression in the *ΔmetR* background. Microarray analysis revealed elevated expression of 58 genes involved in transmembrane transport including three genes encoding methionine permeases. Elevated expression of these genes could lead to complementation of the methionine requirement of the *metR* mutant on complete medium. One can speculate that genes encoding methionine permeases are normally activated

by the MetR protein. If so, in the *ΔmetR* strain methionine could only be taken up by a general amino acid permease for which it would compete with other amino acids present in complete medium. Thus, to allow *ΔmetR* to grow, complete medium must be additionally supplemented with methionine. The induction of high-affinity methionine permeases by the overexpressed MetZ protein could allow methionine to enter the cell from complete medium even in the absence of methionine excess. This assumption is supported by the sensitivity of transformants overexpressing *metZ* to ethionine, a toxic analog of methionine. Overexpression of *metZ* induces also expression of the *sB* gene encoding sulfate permease, which results in elevated sensitivity of *metZ* transformants to structural analogues of sulfate – chromate and selenate.

On the other hand, decreased expression of the *sC* gene and the AN7600 ORF encoding β-subunit of sulfite reductase could explain why the *metR* mutant can not be complemented by overexpression of *metZ* on minimal medium. Thus, MetZ seems to be involved in the regulation of sulfur metabolism-related genes by fine tuning their expression (Fig. 4).

Microarray analysis of *metZ* overexpression revealed also up-regulated genes taking part in synthesis of ergosterol, fatty acids and unsaturated fatty acids which might be components of biological membranes. However, it is possible that some changes in the transcript levels could in fact be side effects of the MetZ protein excess resulting in the formation of non-physiological heterodimers with other bZIP transcription factors. Therefore, we have not attempted a more detailed analysis of possibly non-specific effects of gene overexpression.

Of interest are also the unusually long introns and the common exon-intron organization of the *metR* (Natorff et al. 2003) and *metZ* genes (Fig. 1B). The large intron of *metZ* contain several sequence motifs identical with the recognition sequence of the *N. crassa* CYS3 transcription factor, which could activate transcription from a second start point. Since MetR and MetZ proteins share similar bZIP domains with the *N. crassa* CYS3 protein (Fig. 1A), it seems likely that they could also recognize similar target sequences in DNA. Indeed, qPCR results (Table 2) have confirmed that expression of the second exon of *metZ* can be independently driven from an intronic promoter. We can speculate that the *N. crassa cys-3* gene has similar expression pattern since two transcripts and two protein bands were detected under derepressing conditions (Fu et al. 1989; Tao and Marzluf 1998). The presence of two types of the *metZ* transcript, the spliced one and the short one containing only the second exon

of the *metZ* gene suggests complex regulation of sulfur metabolism by MetR and two variants of MetZ. This type of gene organization, with an alternative promoter localized within an intron, has also been observed earlier in other genes, *e.g.*, the human TP53 gene (Marcel et al. 2011).

We found that expression of *metZ* is MetR-dependent. Since high activity of MetR leads to accumulation of sulfide and activation of stress responses (Sieńko et al. 2014) then simultaneously elevated expression of *metZ* could decrease stress by lowering production of sulfide. The *A. nidulans metZ* gene, but not the *metR* gene, is regulated by the SMR system similarly to the *cys-3* gene encoding the single sulfur-specific transcription factor in *N. crassa*. It appears therefore that the existence of two transcription factors activating sulfur metabolism genes only in *Eurotiales* discerns their regulation of sulfur metabolism from that of other fungi, and makes it potentially more sophisticated.

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Figure legends

Fig. 1

In silico analysis of *Aspergillus nidulans metZ* gene and its orthologs. A) The evolutionary tree and alignment of the MetZ and MetR bZIP domains from *A. nidulans* and other fungi: ***A****spergillus,* ***G****ibberella,* ***M****agnaporthe,* ***N****eurospora,* ***P****enicillium,* ***S****accharomyces,* ***T****alaromyces*. B) Exon-intron structure of the *metZ* gene and its orthologs. Positions of conserved regions in promoters and introns are marked by dark boxes. Arrows indicate primers used in qPCR (see Materials and Methods). C) Alignment of sequences conserved in promoters of *metZ* and its orthologs in *Eurotiales*. Conserved nucleotides are shaded and putative CYS3 (MetR)-binding sites are underlined. D) Alignment of conserved regions located in introns (at *c.a.* 2/3 of their length) of *metZ* orthologs in different species of *Eurotiales*. Conserved sequences and putative CYS3 (MetR)-binding sites are marked as above. E) Putative dimers formed by MetR and MetZ proteins. Broken lines indicate ionic interactions stabilizing each dimer.

Fig. 2

Growth of *A. nidulans* strains on solid media supplemented with various sulfur sources. Wild-type strain W1 and the K1 Δ*metR* mutant are compared with congenic transformants overexpressing the *metZ* gene.

Fig. 3

Categories of Functional Catalogue enriched among genes affected by overexpression of *metZ*. The number of up- (top) or down-regulated genes (bottom) is also shown.

Fig. 4

Effect of the *metZ* gene overexpression on sulfur metabolism. Up-regulated genes are indicated by thick arrows, whereas down-regulated ones by empty arrows.

Fig. 5

metZ gene expression in wild type and *ΔmetR* strains. Transcript levels under sulfur starvation conditions in wild type and *ΔmetR* strains is shown on the three leftmost lanes and the three central lanes, respectively. Overexpression of the *metZ* gene under the *trpC* promoter

is compared to its endogenous expression in the recipient *ΔmetR* strain (two rightmost lanes). The *actA* transcript was used for normalization of RNA loading.

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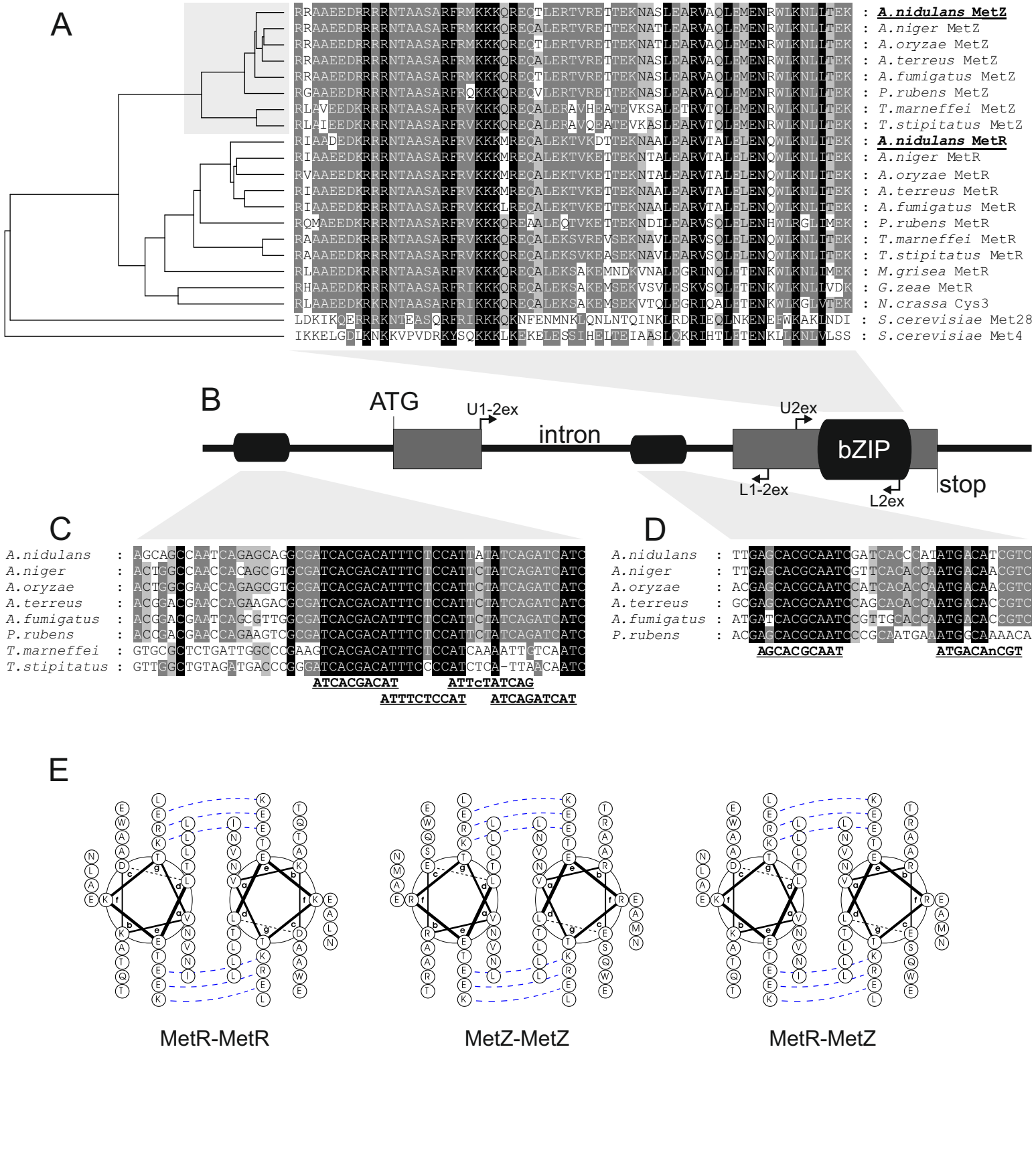
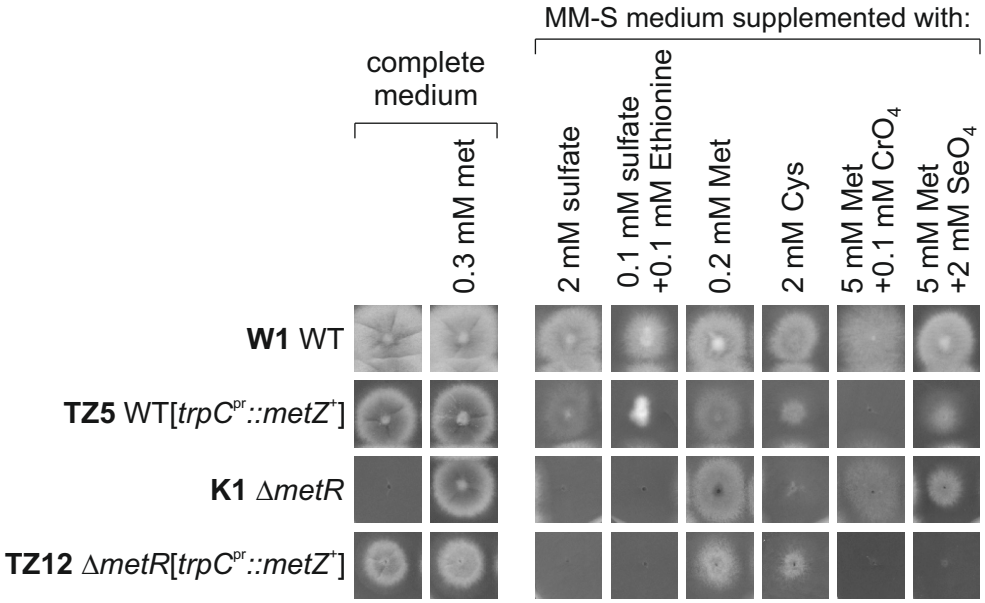
Table 1. List of strains and plasmids

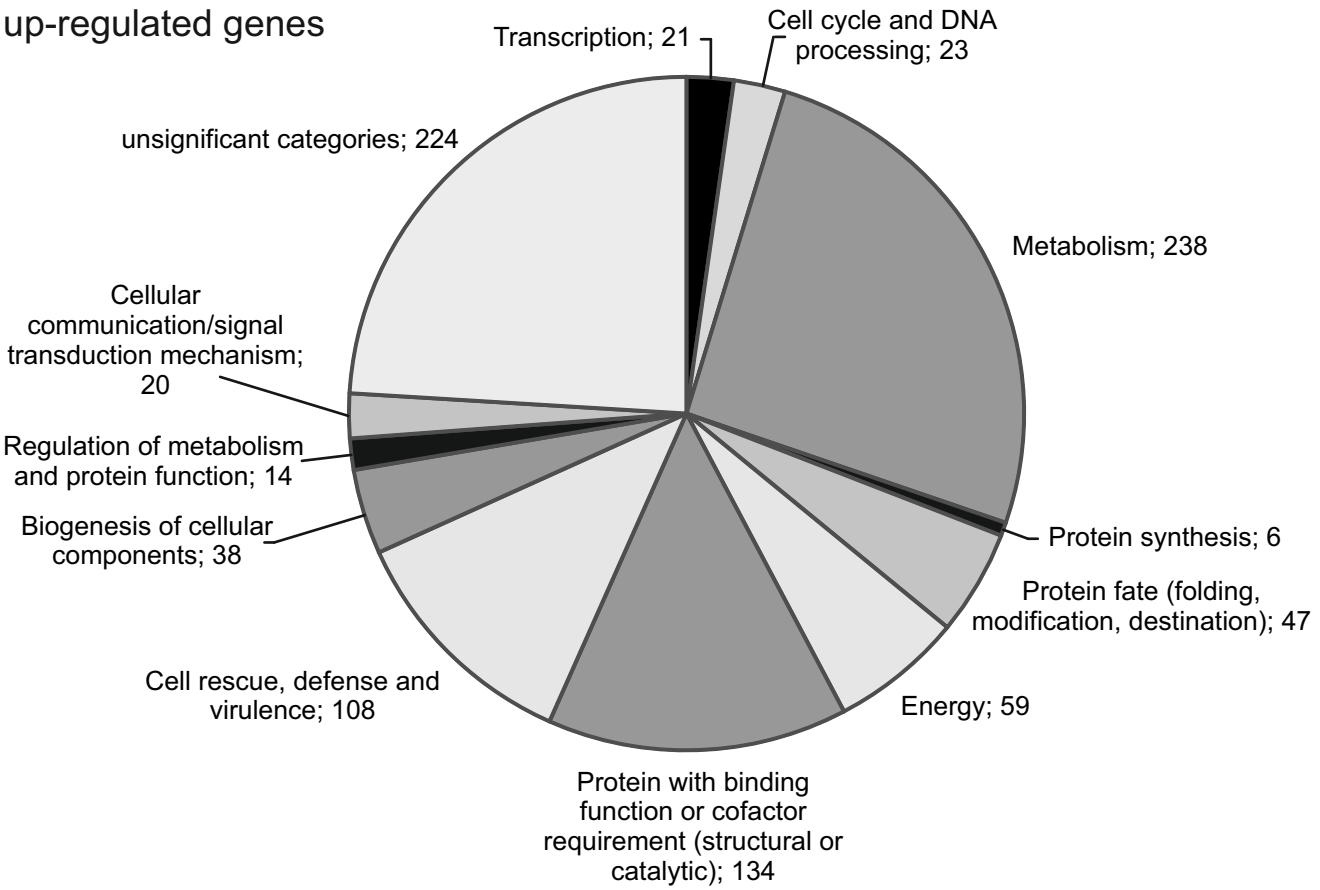
Stains	Genotype or relevant features	Reference or source
E.coli		
XL1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI*^{q} *ZΔM15* Tn10 (Tetr)]^{c}	Stratagene
A. nidulans		
W1	*pyroA4 yA2*	Laboratory collection
W12	*pabaA1 pyrG89*	Laboratory collection
TN02A25	*argB2 nkuA::argB pabaB22 pyrG89 riboB2*	FGSC
RM117	*ΔmetR biA1 nicA2*	Laboratory collection
RM131	*ΔmetR pyrG89 yA2*	Laboratory collection
K1 RM131 [*pyr-4*$^{+}$]	*ΔmetR pyrG89 yA2 pyr-4*$^{+}$	this study
TZ12 RM131 [*trpC*^{pr}*::metZ*$^{+}$*::pyr-4*]	*ΔmetR pyrG89 yA2 trpC*^{pr}*::metZ*$^{+}$*::Ncpyr-4*	this study
TZ5 W12 [*trpC*^{pr}*::metZ*$^{+}$*::pyr-4*]	*pabaA1 pyrG89 trpC*^{pr}*::metZ*$^{+}$*::Ncpyr-4*	this study
Plasmids	Description	
pBluescript KS(–)	cloning vector, Amp^{r}	Stratagene
PCR2-TOPOII	cloning vector, Amp^{r} Km^{r}	LifeTechnologies™
ARp1	increases *A. nidulans* transformation efficiency about 200-fold	Gems and Clutterbuck, 1993
HELp1	increases *A. nidulans* transformation efficiency about 200-fold	Gems and Clutterbuck, 1993
pSF20	carries γ-actin gene	Prof.G. Turner kind gift
pAL3	expression vector containing *A. nidulans alcA* promoter and *N. crassa pyr-4* gene as selectable marker	Waring et al. 1989
L10G06	cosmid bearing *metZ* gene	this study
kKSMZ1	KpnI-SalI fragment bearing *metZ* gene ligated into KS KpnI-SalI site	this study
PALMZ8	KpnI-BamHI fragment bearing *metZ* gene ligated into pAL3 KpnI-BamHI site	this study
kTRMZPG	*metZ* entire ORF cloned under *trpC* promoter, with selectable *pyr-4* cassette	this study
TOPOpyr4	*N. crassa pyr-4* gene PCR-cloned into PCR2-TOPOII	this study
TOPO5'3'UTRMZPG	PCR-cloned 5' and 3' *metZ* UTRs, ligated into TOPOpyr4	this study

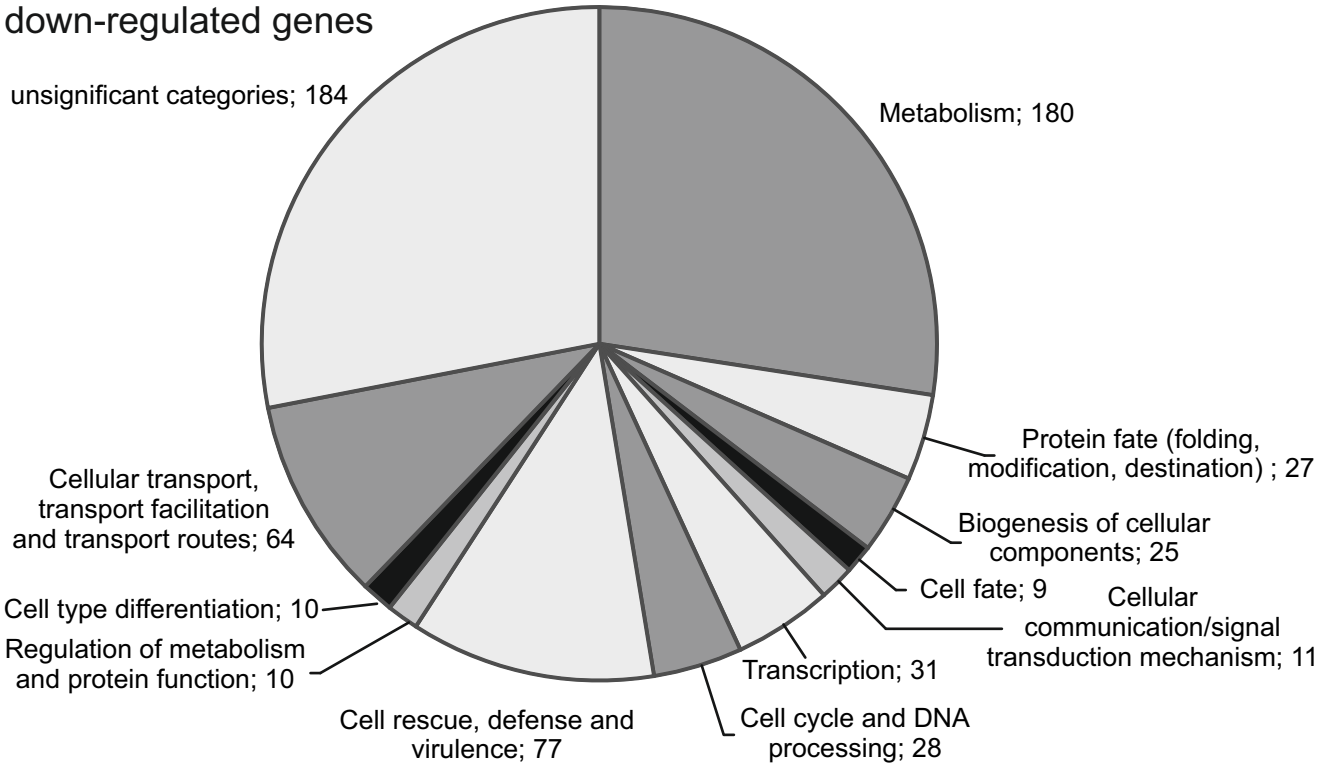
Table 2. Expression of selected genes in *Aspergillus nidulans* strains. Transcript levels in indicated strains were compared by qPCR to those in Δ*metR* strain grown on 0.3 mM methionine. Strains used: Δ*metR*,OE::metZ - *ΔmetR* transformant overexpressing the *metZ* gene from the *trpC* promoter, WT repressed with 1 mM methionine and WT derepressed by 4 h of sulfur starvation after shift from 1 mM methionine.

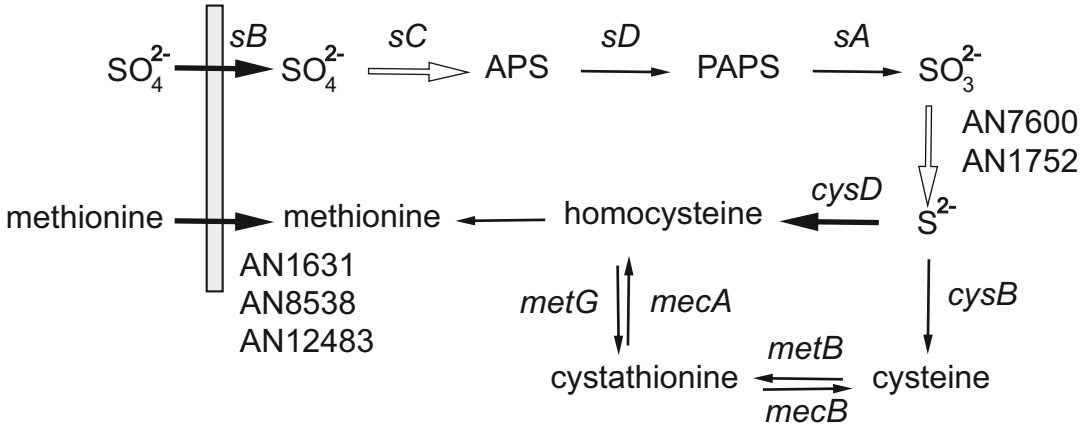
gene	relative expression level			primers used
	Δ*metR*, OE::metZ	WT repressed	WT derepressed	
metZ (spliced)	70	9	5	$U_{1\text{-}2ex}$, $L_{1\text{-}2ex}$
metZ (2nd exon)	88	9	26	U_{2ex}, L_{2ex}
sB	3	13	60	U_{4139}, L_{4258}
AN1631	7	5	100	U_{mupA}, L_{mupA}
cysD	2.5	5	12	$_{cysDq\text{-}}$U1, $_{cysDq\text{-}}$L1

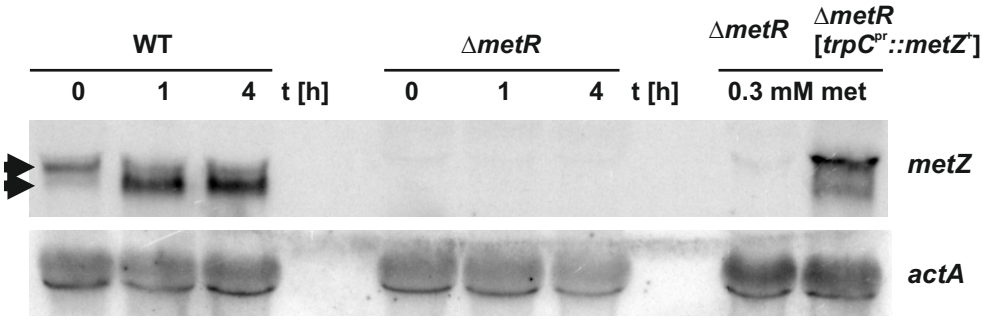

A
A.nidulans MetZ
A.niger MetZ
A.oryzae MetZ
A.terreus MetZ
A.fumigatus MetZ
P.rubens MetZ
T.marneffei MetZ
T.stipitatus MetZ
A.nidulans MetR
A.niger MetR
A.oryzae MetR
A.terreus MetR
A.fumigatus MetR
P.rubens MetR
T.marneffei MetR
T.stipitatus MetR
M.grisea MetR
G.zeae MetR
N.crassa Cys3
S.cerevisiae Met28
S.cerevisiae Met4
B
ATG
U1-2ex
intron
U2ex
bZIP
L1-2ex
L2ex
stop
C
A.nidulans
A.niger
A.oryzae
A.terreus
A.fumigatus
P.rubens
T.marneffei
T.stipitatus
ATCACGACAT
ATTcTATCAG
ATTTCTCCAT
ATCAGATCAT
D
A.nidulans
A.niger
A.oryzae
A.terreus
A.fumigatus
P.rubens
AGCACGCAAT
ATGACAnCGT
E
MetR-MetR
MetZ-MetZ
MetR-MetZ


MM-S medium supplemented with:
complete medium
0.3 mM met
2 mM sulfate
0.1 mM sulfate +0.1 mM Ethionine
0.2 mM Met
2 mM Cys
5 mM Met +0.1 mM CrO_4
5 mM Met +2 mM SeO_4
W1 WT
TZ5 WT[*trpC*^{pr}::*metZ*$^{+}$]
K1 Δ*metR*
TZ12 Δ*metR*[*trpC*^{pr}::*metZ*$^{+}$]


up-regulated genes
Transcription; 21
Cell cycle and DNA processing; 23
unsignificant categories; 224
Metabolism; 238
Cellular communication/signal transduction mechanism; 20
Regulation of metabolism and protein function; 14
Biogenesis of cellular components; 38
Protein synthesis; 6
Protein fate (folding, modification, destination); 47
Cell rescue, defense and virulence; 108
Energy; 59
Protein with binding function or cofactor requirement (structural or catalytic); 134


down-regulated genes
unsignificant categories; 184
Metabolism; 180
Protein fate (folding, modification, destination) ; 27
Cellular transport, transport facilitation and transport routes; 64
Biogenesis of cellular components; 25
Cell fate; 9
Cellular communication/signal transduction mechanism; 11
Cell type differentiation; 10
Regulation of metabolism and protein function; 10
Transcription; 31
Cell rescue, defense and virulence; 77
Cell cycle and DNA processing; 28


SO_4^{2-}
sB
SO_4^{2-}
sC
APS
sD
PAPS
sA
SO_3^{2-}
AN7600
AN1752
methionine
methionine
homocysteine
cysD
S^{2-}
AN1631
AN8538
AN12483
metG
mecA
cysB
metB
cystathionine
cysteine
mecB


WT
ΔmetR
ΔmetR
ΔmetR [trpC^pr::metZ^+]
0 1 4 t [h]
0 1 4 t [h]
0.3 mM met
metZ
actA