

**Title:**

Regulatory Mutations Affecting Sulfur Metabolism Induce Environmental Stress Response in *Aspergillus nidulans*

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**Keywords:**

*Aspergillus nidulans*, sulfur metabolism, microarray, stress response, carbohydrate

**Abbreviations:**

FunCat – (a category of) the Functional Catalogue

## Abstract

Mutations in the *cysB*, *sconB* and *sconC* genes affect sulfur metabolism in *Aspergillus nidulans* in different ways. The *cysB* mutation blocks synthesis of cysteine by the main pathway and leads to a shortage of this amino acid. The *sconB* and *sconC* mutations affect subunits of the SCF ubiquitin ligase complex, which inactivates the MetR transcription factor in the presence of an excess of cysteine. In effect, both *cysB* and *scon* mutations lead to permanent derepression of MetR-dependent genes. We compared transcriptomes of these three mutants with that of a wild type strain finding altered expression of a few hundred genes belonging to various functional categories. Besides those involved in sulfur metabolism, many up-regulated genes are related to stress responses including heat shock and osmotic stress. However, only the *scon* strains are more resistant to exogenous stress agents than the wild type strain while *cysB* is more sensitive. The two-component signal transduction system is a functional category, which is most enriched among genes up-regulated in the *cysB*, *sconB* and *sconC* mutants. A large group of up-regulated genes are involved in carbohydrate and energy metabolism, including genes coding for enzymes of trehalose and glycerol synthesis. The altered expression of these genes is accompanied by changes in sugar and polyol accumulation in conidia of the mutants. Genes encoding enzymes of the glyoxylate bypass and the GABA shunt are also up-regulated along with genes coding for enzymes of alcohol fermentation. Among the down-regulated genes the most numerous are those encoding membrane proteins and enzymes involved in secondary metabolism, including the penicillin biosynthesis cluster.

## 1. Introduction

*Aspergillus nidulans* is a fungal model organism for studying various metabolic processes, including sulfur metabolism (Paszewski et al., 2000). This fungus can use sulfate as well as cysteine or methionine as a sole sulfur source. The final product of the sulfate assimilation pathway is sulfide, which can be further incorporated into cysteine or homocysteine in reactions catalyzed by cysteine synthase and homocysteine synthase, encoded by the *cysB* and *cysD* genes, respectively (Fig. 1A).

Cysteine is synthesized by cysteine synthase in wild type strains of *A. nidulans* and many other fungi (Paszewski et al., 1994) and serves as a substrate for methionine synthesis (Fig. 1A). Strains carrying mutations in the *cysB* gene are prototrophic because *A. nidulans* possesses an alternative pathway of sulfur amino acid biosynthesis, involving homocysteine

synthase, cystathionine  $\beta$ -synthase and cystathionine  $\gamma$ -lyase, which is activated when the main route of cysteine synthesis is blocked (Fig. 1A).

The expression of the *cysD* gene encoding the first enzyme of the alternative pathway (homocysteine synthase) and genes coding for enzymes of the sulfate assimilation pathway is controlled by the sulfur metabolite repression system (Natorff et al., 1993). This control involves the positive-acting MetR protein and negative-acting (Scon) regulatory proteins (Natorff et al., 1998; Piotrowska et al., 2000) (Fig. 1B). The MetR protein is a bZIP transcription factor indispensable for transcriptional activation of sulfur assimilation genes and *cysD* (Natorff et al., 2003).

Scon proteins are components of the SCF complex, which are the largest family of ubiquitin-protein ligases, comprising four constant and one variable subunit (for a review see Willems et al., 2004). The latter determines specificity of the complex to the protein to be degraded or inactivated and in *A. nidulans* one of such variable subunits is encoded by the *sconB* gene. The SCF<sup>SconB</sup> complex negatively regulates the level or activity of the MetR protein when cysteine, a low molecular weight effector of sulfur metabolite repression, is in excess (Fig. 1B) so that the MetR-controlled genes remain repressed (Natorff et al., 2003). SconC is one of the four constant subunits of the SCF complex (Piotrowska et al., 2000), which binds variable subunits, including SconB (Natorff et al., 1998).

Mutations in either *cysB* or *scon* genes abolish sulfur metabolite repression, resulting in activation of MetR-regulated genes. All three mutations lead to activation of the alternative pathway of cysteine synthesis (Natorff et al., 1993; Paszewski and Grabski, 1975) and thereby suppress mutations in the *metB* and *metG* genes coding for cystathionine  $\gamma$ -synthase and cystathionine  $\beta$ -lyase (Fig. 1A). In addition, the *sconB* strain accumulates two and a half times more of both cysteine and glutathione compared to the wild type strain (Natorff et al., 1993). In the case of *sconC* this increase is by about 50% (Natorff et al., 1993). An opposite situation takes place in the *cysB* mutant, which can synthesize cysteine only by the less efficient alternative pathway (Fig. 1A) and produces four times less cysteine and glutathione (Paszewski and Grabski, 1975; Wrobel et al., 2008) despite having the sulfate assimilation pathway derepressed. The derepression of this pathway in all three mutants in question leads to overproduction of sulfide causing dissipation of ATP and NADPH (Fig. 1A).

While the effects of the regulatory mutations on sulfur metabolism *per se* are fairly well understood, including the molecular mechanisms, little is known on how they affect other aspects of cell functioning. Since the *cysB*, *sconB* and *sconC* mutants are sulfur prototrophic,

they seem particularly suitable for such a study because gene expression can be investigated without any interference caused by supplementation of media with organic sulfur sources, which could influence the metabolism of the strains studied.

We report here global effects of the *cysB*, *sconB* and *sconC* mutations on gene expression in *A. nidulans*. This study reveals that dysregulation of sulfur metabolism stimulates transcription of genes implicated in multiple stress responses as well as those involved in sugar and energy metabolism - an effect similar to that caused by adverse environmental agents.

## 2. Materials and methods

### 2.1 Strains and media

The *Aspergillus nidulans* wild type strain W1 from our collection, carrying standard markers *pyroA4* and *yA2* (Clutterbuck, 1994), was used as a reference in all experiments. The three mutant strains - C8 (*cysB102*, *pyroA4*, *yA2*), R17 (*sconB2*, *pyroA4*, *yA2*) and R22 (*sconC3*, *pyroA4*, *yA2*) – were compared to the reference strain.

Mycelia were grown for 18-21 h at 30°C and 200 rpm in 300-ml Erlenmeyer flasks containing 100 ml of minimal medium (MM) supplemented with 2 mM sulfate (Paszewski and Grabski, 1973) and according to the auxotrophic requirements of the strain. The mycelia were grown up to 1 g of wet weight, harvested by filtration, washed with distilled water, dried on filter paper, weighed, frozen in liquid nitrogen and kept at –70°C. If exogenous stress factors were tested the flasks were supplemented with sodium sulfide, hydrogen peroxide or sodium chloride up to the final concentration 5 mM, 10 mM or 1 M, respectively, and mycelia were kept at 30°C for 10 min with shaking before harvesting.

Resistance of the mutants to sulfide, methylglyoxal, cadmium and menadione were carried out on solid MM medium with stress agents added in different concentrations, at 37°C for 2 days.

### 2.2 Microarray analysis

Mycelia were powdered in liquid nitrogen and total RNA was isolated using TRI Reagent (Molecular Research Center) according to the manufacturer's protocol (Chomczynski, 1993) and subsequently precipitated with lithium chloride added to a final concentration of 3.42 M. Samples of total RNA (50µg) were subjected to the first strand cDNA synthesis in reaction using RevertAid<sup>TM</sup> H Minus M-MuLV Reverse Transcriptase (Fermentas) according to the manufacturer's protocol. The nascent cDNA was simultaneously

labeled using fluorescent dyes DY-547-aadUTP or DY-647-aadUTP (Dyomics, Germany). Labeled probes were hybridized to *A. nidulans* 70-mer oligonucleotide microarrays (FGSC\_A4, version 2) obtained from the Pathogen Functional Genomics Resource Center (PFGRC; J. Craig Venter Institute). They carried 11,481 probes spotted in duplicate, representing verified genes, contigs and ESTs. A total of 10580 genes (99 % of the *A. nidulans* genome ORFs) were represented. Following hybridization the microarrays were scanned with an Axon GenePix 4000B microarray scanner and analyzed with GenePix Pro 6.1. Raw data were normalized and analyzed further with Acuity 4.0 software. Additional manipulations were done with Microsoft Excel. Each comparison of mutant versus wild-type transcriptome was done in three biological replicates and each replicate had two technical replicates with dye swap.

A gene was considered to be differentially expressed between a mutant strain and the wild type if its transcript level differed between the two strains at least twofold ( $|\log_2\text{Ratio}| > 1$ ) and the probability of such a difference by chance was less than 0.1 ( $p < 0.1$ ). The resulting lists of differentially expressed genes were subjected to further bioinformatics analysis. Spot information was obtained from the PFGRC annotation file and updated to version 4 with information found in the Supplementary Download from the *Aspergillus* Comparative Sequencing Project, Broad Institute of Harvard and MIT (<http://www.broadinstitute.org/>). Additional information was found in the GPL5138 annotation file (Breakspear and Momany, 2007). Oligonucleotides unassigned to known genes were used as a query in a BLAST (Altschul et al., 1990) version 2.2.17 search using NOBLAST 2.1 extensions (Lagnel et al., 2009) against the *A. nidulans* FGSC A4 genome version s06-m01-r07 downloaded from the *Aspergillus* Genome Database (<http://www.aspgd.org/>). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE54130 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE54130>).

### 2.3 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 1) were designed by 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- $\mu$ l reaction mixture containing 5  $\mu$ l of LightCycler® 480 SYBR Green I Master mix (Roche

Laboratories), two primers (3 pmol of each) and an equivalent amount of diluted template cDNA. cDNA was synthesized from 5 µg of total RNA treated with DNaseI (Roche) using RevertAid<sup>TM</sup> H Minus M-MuLV Reverse Transcriptase kit (Fermentas) according to supplier's protocol.

The actin gene 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 used to calculate relative expression with respect to that of actin.

#### 2.4 Determination of sugar and polyol contents

Extracts from conidia for determination of sugar and polyol contents were prepared according to Hallsworth and Magan (1997) with modifications. *A. nidulans* strains were cultivated on solid minimal medium for 3 days at 30°C, and then conidia were washed off with deionized water, collected by centrifugation and weighed. Next, the conidia were resuspended in deionized water and opened with two freeze-thaw cycles using liquid nitrogen followed by grinding the suspension in a mortar with liquid nitrogen. The homogenate was boiled for 6 minutes and spun down to remove debris. Mycelia were powdered in liquid nitrogen and homogenized in deionized water, then boiled for 6 minutes and centrifuged. Extracts were deproteinized by overnight precipitation at -20°C after addition of three volumes of 96% ethanol. The precipitate was removed by centrifugation and the supernatant was dried under vacuum and dissolved in deionized water.

Sugars and sugar alcohols were determined in the supernatants diluted 100x by high performance anion-exchange chromatography on a Dionex ICS-3000 Ion Chromatography system with a Carbo Pac PA10 analytical column. Neutral compounds were eluted with 18 mM NaOH at 0.25 ml/min (Zdebska and Koscielak, 1999).

#### 2.5 Bioinformatics analysis

Sequence databases were searched using BLAST programs (Altschul et al., 1990) running on the *Aspergillus* Genome Database server. Information about categories of the Functional Catalogue (Ruepp et al., 2004) assigned to all *A. nidulans* protein coding genes was exported from the p3\_p40559 file of the PEDANT database (<http://pedant.gsf.de/>; Walter et al., 2009). Standard spreadsheet operations were applied to identify functional categories assigned to the differentially expressed genes. Venn diagrams were generated with the BioInfoRx software ([http://apps.bioinformx.com/bxaf6/tools/app\\_overlap.php](http://apps.bioinformx.com/bxaf6/tools/app_overlap.php))

### 3. Results and Discussion

#### 3.1 Categories of Functional Catalogue

Microarray analysis showed that the levels of 882 transcripts were more than twofold elevated and of 339 more than twofold decreased in at least one of the *cysB*, *sconB* and *sconC* mutants relative to the wild-type strain. The sets of genes with transcript levels altered in individual mutants overlap: 125 transcripts are up-regulated and 18 transcripts down-regulated in all three of them (Fig. 2). This indicates that the three regulatory mutations disturbing sulfur metabolism, in addition to large groups of genes unique to each of the regulators, also affect expression of a common subset of genes. All significantly up- and down-regulated genes ( $|\log_2\text{Ratio}| > 1$ ) are listed in the Supplementary Table 2. Many of them have no assigned function.

To look for possible physiological relevance of the observed transcriptome changes we searched the Functional Catalogue categories (Ruepp et al., 2004) assigned to all the genes with expression changed in the mutants. For further analysis we selected categories containing at least three similarly regulated genes in at least one mutant and also at least three-fold enriched among the differentially regulated genes compared to the whole genome. Eight of the selected functional categories enriched in up-regulated genes (Fig. 3) belong to the main category Metabolism (01), four to Cell rescue, Defence and Virulence (32), and three to Energy (02). This distribution indicates that disturbances in sulfur metabolism influence diverse cellular functions.

#### 3.2 Metabolism of sulfur

Consistently with our previous results (Paszewski et al., 1994; 2000), sulfur metabolism-related genes were found among those showing elevated expression in the mutants studied. They have been assigned to three functional categories: biosynthesis of homocysteine (01.01.06.05.01.01), sulfur metabolism (01.02.03) and sulfate assimilation (01.02.03.01), and collectively are ten times enriched among the up-regulated genes (Fig. 3, Supplementary Table 3). They include those coding for homocysteine synthase (*cysD*), and sulfite reductase (AN1752, AN7600) and adenosine 3-phosphate 5-phosphosulfate (PAPS) reductase (*sA*) involved in sulfate assimilation (Paszewski et al., 1994). The sulfur metabolism FunCat contains also a choline sulfatase gene (AN5449) and three genes coding for arylsulfatases (AN11149, AN6847 and AN8341). The sulfur metabolism-linked categories are enriched with up-regulated genes in the *cysB* and *sconB* mutants but not in *sconC* (see Supplementary Table 3). These results are coherent with our earlier results (Natorff et al.,

1993) showing that enzymes of the alternative pathway of cysteine synthesis are only slightly elevated in the *sconC* mutant.

### 3.3 Dysregulation of sulfate assimilation pathway

The derepression of the sulfate assimilation pathway in the mutants leads to dissipation of ATP and NADPH and overproduction of sulfide (Fig. 1A). The latter, being reactive and toxic (Reiffenstein et al., 1992), could be a primary signal evoking multiple stress reactions. This assumption is supported by the fact that a gene coding for an enzyme involved in sulfide detoxification (sulfide:quinone reductase, AN1825) is the most highly up-regulated one in all three mutants (Supplementary Table 2). Sulfide:quinone reductases are found in many organisms including cyanobacteria, fungi and vertebrates (Griesbeck et al., 2000). The *S. pombe hmt2* gene encoding this enzyme is necessary for cadmium tolerance (Vande Weghe and Ow, 2001).

The AN1825 gene is adjacent in a divergent orientation in the *Aspergillus* genome to another strongly up-regulated gene (AN1826) and both apparently form a small regulon. The latter gene encodes a putative metallo- $\beta$ -lactamase, which is similar to glyoxalases that take part in methylglyoxal detoxification. The AN1825 and AN1826 genes are at least sixteen-fold induced in all three mutants, as determined by microarray analysis. However, validation of this result by qPCR showed over two thousand-fold up-regulation of these genes in the mutants (Table 1). The very high induction of the AN1825 gene indicates that its regulation must be very sensitive to the intracellular sulfide concentration and therefore could serve as a marker of the sulfate assimilation pathway dysregulation.

Sulfide acting as a common stress factor could explain why the sets of genes induced overlap substantially or at least belong to the same functional categories. The mechanism of sulfide toxicity could depend on its binding to heme aa3 (Nicholls, 1975), which is a cofactor of cytochrome c oxidase. Thus, if sulfide does indeed accumulate in the mutants, it could block the respiratory chain. Such a possibility is supported by the finding that some genes induced by anaerobic stress, e.g., *alcC* (Kelly et al., 1990), are up-regulated in the mutants.

### 3.4 Genes involved in signal transduction

Notably, the two-component signal transduction system FunCat (30.05.01.10) is the most enriched one (eight to fifteen times) among the up-regulated genes (Fig. 3). Fifteen genes encoding receptor histidine kinases are present in the *A. nidulans* genome (Kanamaru, 2011) and four of them (*phkB*, *hk-8-1*, *hk-8-2* and *hk-8-3*) are highly induced in all three

mutants, and three others (*tcsB*, *hk2*, and *hk-8-5*) in the *sconC* mutant only. Because the gene coding for the single histidine-containing phosphotransfer protein YpdA is also up-regulated (Supplementary Table 2), this indicates that the whole two-component signal transduction pathway is stimulated in the mutants. This observation is reinforced by the fact that other stress related FunCats are also enriched among the up-regulated genes. These functional categories often comprise overlapping sets of genes, like the osmosensing and response FunCat (34.11.03.13) which shares nine genes (*gfdB*, *hogA*, *ypdA*, *hk-8-1*, *hk-8-2*, *hk-8-3*, *hk-8-5*, AN1274 and AN1767) with osmotic and salt stress response (32.01.03).

Particularly interesting is the *hogA* transcript, which is significantly elevated in all three mutants. The HogA (high osmolarity glycerol) protein is a mitogen-activated protein kinase (MAPK), a core protein of a regulatory kinase cascade called the HOG pathway. The HogA kinase can phosphorylate transcription factors in the response to osmotic stress and also to heat, cold, hypoxia, arsenite, low pH, and other stresses (Saito and Posas, 2012 and references therein). Interestingly, several genes encoding transcription factors were also up-regulated. Notably, the *atfA* gene, required for resistance of *Aspergillus* conidia to heat and hydrogen peroxide treatment (Hagiwara et al., 2008), was up-regulated in the three mutants. The AtfA and HogA proteins physically interact in response to stress and during conidiophore development (Lara-Rojas et al., 2011). Other genes coding for transcription factors also exhibit elevated transcript levels: *hapB* involved in regulation of carbohydrate, nitrogen and secondary metabolism (Steidl et al., 1999) is up-regulated in the *cysB* and *sconB* mutants, *rosA* engaged in sexual development (Vienken et al., 2005) is elevated in the *cysB* mutant, and the *flbB* gene, which takes part in conidiophore development (Etxebeste et al., 2008) is induced in the *sconC* strain.

### 3.5 Genes involved in environmental stress response

Numerous FunCats significantly enriched among the up-regulated genes in the three mutants are related to stress responses including osmotic and salt stress response, osmosensing and response, heat shock response, temperature perception and response, chemical agent resistance, oxygen and radical detoxification (Fig. 3, Supplementary Table 3). The FunCat oxygen and radical detoxification (32.07.07) contains the thioredoxin (*trxA*) and peroxiredoxin (AN10223) genes up-regulated in the *cysB* mutant but not in the *scon* mutants. These two proteins decompose peroxides and are important for maintaining redox potential (Kalinina et al., 2008) so the elevated transcription of the corresponding genes could be caused by the low glutathione level in the *cysB* mutant leading to oxidative stress.

A striking finding is an elevated level of transcripts encoding small heat shock proteins which belong to the FunCat heat shock response (32.01.05). The *A. nidulans* genome contains five genes encoding such proteins and two of them (*hsp20* and AN7892) exhibit elevated transcript levels in all three mutants while the remaining three (*hsp30*, AN3555 and AN5781) are up-regulated in the *sconB* mutant only. Their orthologs in *S. cerevisiae* (*HSP26* and *HSP42*) are induced by various stress agents including heat, salt, hydrogen peroxide or carbon source starvation (Amoros and Estruch, 2001). Transcription of the *A. nidulans awh11* gene coding for a small membrane-associated heat shock protein is also up-regulated in the mutants along with the *hsp70* and *hsp104* genes up-regulated in *cysB* and *sconB*.

Three genes connected with membrane metabolism up-regulated in the mutants belong to the FunCat temperature perception and response (34.11.09): *pilA* encoding a primary component of eisosomes, a gene coding for an ortholog of the *S. cerevisiae* ER membrane protein Wsc4p (AN4674), and AN10452. The latter codes for alkaline dihydroceramidase which preferentially hydrolyzes dihydroceramide to a free fatty acid and dihydrosphingosine. Remarkably, the level of dihydrosphingosine increases tenfold in heat stressed yeast cells, which suggests importance of dihydroceramidase in heat stress response (Jenkins et al., 1997).

Chemical agent resistance (32.05.01.03) is another stress-related FunCat enriched two- to five-fold among the up-regulated genes (Supplementary Table 3). This category contains drug:H<sup>+</sup> antiporters (AN0601 and AN9370) and ATP-binding cassette (ABC) transporters (*atrA* and AN8892) that may remove toxins and xenobiotics from cells. Genes encoding two arrestin domain proteins (*artB* and *apyA*) also exhibit elevated transcript levels in the mutants. Their orthologs in *S. cerevisiae* (Aly1p and Rod1p, respectively) have been proposed to regulate the endocytosis of plasma membrane proteins by recruiting the ubiquitin ligase Rsp5p to its targets (Lin et al., 2008).

The main category 20 Cellular Transport includes two categories enriched among the genes up-regulated in the mutants: amine/polyamine transport (20.01.11) and proton driven antiporter (20.03.02.03.01). Both of them include six cell-membrane polyamine transporters, two of which are orthologs of the *S. cerevisiae TPO3* gene (AN5540 and AN8122) and four of *TPO1* (AN1243, AN4119, AN5329 and AN7295) (Uemura et al., 2005). Polyamines confer resistance to cold, drought and salt stress in plants (Alcazar et al., 2010). Thus, it seems likely that the increased expression of polyamine transporters in the mutants could be part of a stress response.

### 3.6 Exogenous stress agents

Since our data show up-regulation of numerous stress response genes it was of interest whether similar responses can be evoked in the wild type strain by exogenous agents. Therefore regulation of selected transcripts by sulfide, hydrogen peroxide or salt was tested (Table 2). Both sulfide:quinone reductase (AN1825) and metallo- $\beta$ -lactamase (AN1826) are significantly up-regulated by sulfide and hydrogen peroxide. Transcripts of the *hogA* gene and three genes for histidine kinases (*hk-8-1*, *hk-8-2* and *hk-8-3*) are also elevated under conditions tested. Sodium chloride treatment increases levels of these transcripts though not as strongly as redox stress agents. These results support assumption that accumulation of sulfide could evoke a redox stress in the studied mutants.

The high expression of stress resistance genes suggests that the sulfur metabolism mutants could be more resistant to external stress agents than the wild type strain. Indeed, we found that the *sconB* and *sconC* mutants are more resistant to sulfide, cadmium and methylglyoxal than is the wild type strain; in contrast, the *cysB* mutant is more sensitive (Fig. 4). The high resistance of the *scon* mutants to methylglyoxal could result from a high level of glutathione, which reacts non-enzymatically with methylglyoxal and the adduct formed is subsequently metabolized by glyoxalases I and II (Rabbani and Thornalley, 2010). Glutathione is also a substrate for biosynthesis of phytochelatins providing cadmium resistance (Mendoza-Cozatl et al., 2005). Since the *cysB* mutant, being deficient in glutathione, is highly sensitive not only to cadmium and methylglyoxal but also to oxidative stress agents such as menadione (Fig. 4) it appears that the resistance of the tested strains to the stress agents mentioned above correlates with their glutathione content but not with the activity of sulfide:quinone reductase (AN1825).

Our microarray results suggest that overproduction of sulfide leads to redox stress in the mutants. This stress might be either oxidative or reductive. The latter stress induced with 2mM dithiotreitol causes a twofold increase of the TRX2 gene transcript coding for thioredoxin in yeast (Trotter and Grant, 2002). It is worth noting that the *A. nidulans* ortholog of this gene (*trxA*) is also up-regulated in the *cysB* mutant but down-regulated in *sconC*.

Oxidative stress may be induced by a variety of factors, e.g., menadione or diamide. Interestingly, the pyruvate decarboxylase gene (*pdca*), significantly up-regulated in all three mutants tested in this study, was down-regulated by menadione stress at both the transcriptional and protein abundance levels (ANID\_04888.1 in Pusztahelyi et al., 2011). The *gsdA* and *idpA* genes involved in NADP<sup>+</sup> metabolism (see section 3.7 below) were induced by menadione (Pusztahelyi et al., 2011) but are not significantly affected by the sulfur

metabolism mutations. These results suggest that the cellular NADPH stores are less affected by the mutations studied than by the menadione treatment. Thus, the nature of the stress observed in the sulfur metabolism mutants seems to differ from that induced by menadione.

Farnesol is another stress agent and induces many genes that are also up-regulated in the mutants studied here, e.g., a gene coding for a small heat shock protein (AN7892), putative apoptosis-inducing factor (AIF)-like mitochondrial oxidoreductase (*aifA*), FAD-dependent glycerol 3-phosphate dehydrogenase (AN1396) and alternative NADH dehydrogenase (*ndeA*) (Savoldi et al., 2008).

### 3.7 Carbohydrate and energy metabolism genes

Numerous FunCats enriched among the up-regulated genes in the mutants studied belong to the main categories Metabolism (01) and Energy (02) which are functionally linked because production and storage of energy is connected with carbohydrate metabolism. The FunCats sugar, glucoside, polyol and carboxylate catabolism (01.05.02.07) and sugar, glucoside, polyol and carboxylate anabolism (01.05.02.04) include the highest number of up-regulated genes (36 and 15, respectively, compared with 233 and 93 overall assigned to these categories).

Expression of genes encoding subunits of trehalose synthase/phosphatase complex, AN10533 (large subunit), AN8639 (synthase subunit) and the *tpsA* gene encoding  $\alpha,\alpha$ -trehalose-phosphate synthase, are elevated in the mutants. Trehalose is involved in *A. nidulans* resistance to a variety of stresses, including heat and oxidative stress. It is also required for the survival of conidia during prolonged storage (Fillinger et al., 2001). Interestingly, transcript of the *treA* gene encoding acid trehalase is also up-regulated in the *sconC* mutant. This enzyme is localized to the conidial cell wall and required for utilization of extracellular trehalose during germination (d'Enfert and Fontaine, 1997). Thus, genes encoding enzymes involved in both synthesis and degradation of trehalose are up-regulated in the *sconC* strain. Simultaneous induction of genes involved in synthesis (*TPS1*, *TPS2* and *TSL1*) and in degradation (*NTH1* and *ATH1*) of trehalose occurs in response to multiple stresses in *S. cerevisiae* (Gasch, 2002).

Transcripts of two genes encoding NADP<sup>+</sup>-specific glycerol dehydrogenases are up-regulated in the *sconC* mutant (AN10499 and AN1274). In this strain transcript for the NAD<sup>+</sup> dependent glycerol 3-phosphate dehydrogenase (*gfdB*) is also elevated while the transcript of the FAD-dependent mitochondrial isozyme (AN1396) is up-regulated in the *cysB* mutant.

Up-regulation of the *gpdC* gene encoding the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase in the mutants suggests an accelerated rate of glycolysis. On the other hand, the *acuG* gene coding for fructose-1,6-bisphosphatase, a key regulatory enzyme in the gluconeogenesis pathway, also exhibits an elevated transcript level. The simultaneous activation of these genes may appear contradictory but it is a characteristic feature of environmental stress response that both catabolic and anabolic pathways are activated concomitantly at the transcriptional level because their activities are finally controlled at the posttranslational levels (Gasch, 2002). In this context one should mention two interesting genes *pfkZ* and AN1761, both significantly up-regulated in all three mutants. AN1761 codes for an ubiquitin-conjugating enzyme and its *S. cerevisiae* homolog Ubc8p negatively regulates fructose-1,6-bisphosphatase (Schule et al., 2000). The *pfkZ* gene codes for 6-phosphofructo-2-kinase, the enzyme responsible for the synthesis of fructose-2,6-bisphosphate, an allosteric regulator controlling glycolysis and gluconeogenesis (Okar et al., 2001). Thus, genes coding for proteins controlling the activity of key enzymes of glycolysis and gluconeogenesis are also up-regulated.

Elevated levels of transcripts coding for  $\alpha$ -amylase (*amyD*) and 1,4- $\alpha$ -glucan branching enzyme (AN2314) could indicate a fast metabolism of glycogen in the mutants resulting from a high requirement for energy. This notion is supported by elevated levels of transcripts for cellulose-degrading enzymes (e.g.,  $\beta$ -1,4-endoglucanase AN2388 and  $\beta$ -glucosidases *bglJ* and AN10124). In addition, elevated levels of transcripts for enzymes degrading mannans, pectins and glucuronides ( $\beta$ -1,4-endomannanase AN3358, arabinan endo-1,5- $\alpha$ -L-arabinosidase *abnD* and  $\beta$ -glucuronidase AN3200) also suggest that mutant mycelia are prepared for utilization of alternative sources of energy.

The induction of genes involved in energy metabolism could result from an elevated requirement for NADPH or ATP consumed by the up-regulated sulfate assimilation pathway (Fig. 1A). It is worth noting that two genes coding for NADP<sup>+</sup>-specific glycerol dehydrogenases (AN1274 and AN10499) are up-regulated in the *sconC* mutant. This could indicate increased synthesis of glycerol or elevated NADP<sup>+</sup> metabolism. The main sources of cellular NADPH are the pentose phosphate pathway and NADP<sup>+</sup>-linked isocitrate dehydrogenase (van Dijken and Scheffers, 1986). However, neither the *gsdA* transcript encoding the key enzyme of the pentose phosphate pathway (glucose-6-phosphate dehydrogenase) nor the *idpA* transcript encoding NADP<sup>+</sup>-linked isocitrate dehydrogenase (Szewczyk et al., 2001) is elevated in the mutants. This indicates that genes responsible for

production of NADPH are not activated in the mutants and suggests that the oxidation of NADPH by the sulfate assimilation pathway is not a primary stress agent but stress responses are more likely evoked by overproduction of sulfide as was put forward in section 3.3.

### 3.8 Sugar and polyol levels in conidia

The marked changes in the expression of numerous genes connected with sugar metabolism prompted us to check the content of sugars and sugar alcohols in the mycelia and conidia of the mutants, especially considering the role of sugars and sugar alcohols in environmental stress response. Trehalose is a major protectant of yeast cells against heat-induced damage (Attfield, 1987) and mannitol is required for stress tolerance in *Aspergillus niger* conidiospores (Ruijter et al., 2003). Levels of these compounds were not significantly changed in the mutant mycelia (results not shown) but they did differ in the mutant conidia from the ones in the wild type strain. The levels of mannitol and trehalose, the most abundant soluble compounds, were slightly elevated in the *cysB* conidia and strongly decreased in the *scon* conidia (Fig. 5). Statistically significant ( $P < 0.05$ ) decrease of mannitol concentration in conidia of the *scon* strains could be a result of stress responses exploiting cellular stores of mannitol as an energy reserve. This polyol is metabolized by NADP-dependent mannitol dehydrogenase (AN7590), which is up-regulated in the three mutants. The levels of glycerol in mutant conidia do not differ significantly from the wild type strain levels. However, erythritol is lowered while arabitol accumulates in the *sconC* mutant conidia (Fig. 5). The latter change seems relevant because arabitol, in addition to glycerol, has been shown to accumulate in response to osmotic stress in *A. nidulans* (Beever and Laracy, 1986).

### 3.9 Alcohol fermentation

The FunCat alcohol fermentation (02.16.01) is another functional category related to sugar and energy metabolism enriched among the up-regulated genes in the mutants. This FunCat includes several alcohol dehydrogenase genes induced in the mutants (AN2470, AN3030, AN3873, AN5355, AN8628 and *alcC*). The latter gene is expressed in response to anaerobic stress and required for anaerobic survival (Kelly et al., 1990). Its ortholog in *S. cerevisiae* codes for mitochondrial alcohol dehydrogenase isozyme III involved in ethanol production and shuttling of the mitochondrial NADH to the cytosol under anaerobic conditions (Bakker et al., 2000). The elevated levels of the *alcC* transcript suggest that ethanol production could be enhanced in the mutants. This FunCat includes also the *pdcA* gene encoding pyruvate decarboxylase, which is induced by anaerobic growth conditions

(Lockington et al., 1997). Since both the *alcC* and *pdca* genes are induced by anaerobic conditions it suggests that such conditions may exist in the mutant mycelia.

Another gene up-regulated in all three mutants is *ndeA*. Its ortholog in *S. cerevisiae* (*NDE2*) codes for the alternative NADH dehydrogenase which enables mitochondrial oxidation of cytosolic NADH (Rigoulet et al., 2004). There are two main pathways for cytosolic NADH oxidation, the alternative NADH dehydrogenase mentioned above, and the glycerol-3-phosphate dehydrogenase shuttle (Rigoulet et al., 2004). Some components of this shuttle are also up-regulated in the mutants: AN1396 in *cysB* and *gfdB* in *sconC*. Thus, the both main pathways for mitochondrial oxidation of cytosolic NADH are induced, which suggests that NADH metabolism is accelerated in the mutants. These two routes transfer electrons to ubiquinone, and their derepression suggests that complex I of the respiratory chain cannot cope with NADH oxidation and these two ancillary pathways are switched on to help regenerate  $\text{NAD}^+$ . Another rationale for derepression of alternative NADH dehydrogenase gene is bypassing respiratory chain complex I which is implicated in generation of reactive oxygen species (Joseph-Horne et al., 2001). This could be particularly important under conditions of redox stress.

### 3.10 The Krebs cycle bypasses

The FunCat C-2 compound and organic acid metabolism (01.05.06.07) includes five genes up-regulated in the mutants (Supplementary Table 3). One of them is the isocitrate lyase gene (*acuD*) catalyzing the first reaction of the glyoxylate bypass and competing with isocitrate dehydrogenase for their common substrate, isocitrate (El-Mansi et al., 2006). Unlike the Krebs cycle enzyme, isocitrate lyase does not require oxidized  $\text{NAD}^+$ , which may be important under hypoxic conditions (see section 3.9 above). In this context, it seems interesting that glyoxylate pathway is induced in rice by hypoxia (Lu et al., 2005). Concomitant up-regulation of the malate synthase gene (*acuE*) indicates activation of the glyoxylate bypass and suggests an elevated requirement for Krebs cycle intermediates, which are used by various biosynthetic pathways. One of them could be the biosynthesis of homocysteine, the carbon backbone of which originates from oxaloacetate.

Another category enriched among the up-regulated genes is degradation of glutamate (01.01.03.02.02). It includes six up-regulated genes, among them those encoding glutaminase (AN4901), glutamate dehydrogenase (AN7451), two genes coding for glutamate decarboxylase (AN5447 and AN7278) and that for succinic semialdehyde dehydrogenase (AN3829). The latter enzyme can use both  $\text{NAD}^+$  and  $\text{NADP}^+$  as cofactors (Coleman et al.,

2001) and can thus contribute to NADPH production. Glutamate decarboxylase is also linked with maintaining redox potential because its overexpression confers resistance to oxidative stress (Coleman et al., 2001). Both enzymes belong to the metabolic pathway called the GABA shunt because it bypasses two steps of the Krebs cycle. The GABA shunt carries a substantial portion of carbon flux under normal conditions and is additionally induced under stress (Fait et al., 2008).

The elevated expression of genes encoding enzymes of the glyoxylate bypass and the GABA shunt suggests major changes in the central metabolism. It is possible that these pathways are activated due to a higher requirement for metabolites like GABA and oxaloacetate. The other possibility is that the Krebs cycle does not function effectively, which could happen due to the respiratory chain blockage by accumulated sulfide. Another possibility could be up-regulation of the sulfate assimilation pathway enhancing requirement for ATP and NADPH. Activation of the two auxiliary pathways parallel with the Krebs cycle and production of additional intermediate metabolites by the glyoxylate bypass could speed up the central metabolism of the cell.

### *3.11 Numerous down-regulated genes encode membrane proteins*

All the functional categories enriched among the genes down-regulated in the *cysB*, *sconB* and *sconC* mutants are shown in Fig. 6 (details in Supplementary Table 4). Many of them belong to the main categories Metabolism (01) and Cellular Transport (20). However, the most enriched is the subcategory vacuolar protein degradation (14.13.04.02) of the main category Protein Fate (14). Four genes involved in vacuolar protein degradation, encoding carboxypeptidases (AN1426 and AN7231), aminopeptidase Y (AN8445) and dipeptidyl aminopeptidase (AN6438), are down-regulated, especially in the *sconC* mutant. Though such an effect of sulfur metabolism dysregulation seems surprising it is worth mentioning that vacuolar protein-sorting defects have been observed in mutants sensitive to diamide (Thorpe et al., 2004). Because diamide depletes the cellular pool of glutathione (Kosower and Kosower, 1995), the results of Thorpe et al. (2004) and ours could both indicate some interaction between glutathione metabolism and vacuolar function.

The most numerous sets of down-regulated genes are those assigned to the categories C-compound and carbohydrate transport (20.01.03) and virulence, disease factors (32.05.05). Among them are seven genes (AN0178, AN2044, AN2249, AN3395, AN5312, AN7270 and AN7774) down-regulated in the *cysB* and *sconC* mutants. These genes are similar to the *Magnaporthe grisea Pth11* gene which codes for a protein important for differentiation of

appressoria in response to inductive substrate cues (DeZwaan et al., 1999). Pth11 belongs to a superfamily of G-protein-coupled receptors (Oldham and Hamm, 2008). The Pth11 protein family is widely represented in filamentous fungi like *M. grisea* and *N. crassa* but is absent in the yeast *S. cerevisiae* and *S. pombe* (Kulkarni et al., 2005). The lowered expression of genes encoding Pth11 family proteins, which are localized to the plasma membrane, endocytosed and targeted to the vacuole (DeZwaan et al., 1999), suggests a possible link with the decreased expression of the proteinases involved in vacuolar protein degradation discussed above.

Besides the *Pth11* family genes, the FunCat virulence, disease factors contains also the *easB* gene encoding putative polyketide synthase required for the biosynthesis of emericellamide. Seven of the down-regulated genes from the C-compound and carbohydrate transport category code for sugar and glycerol permeases (AN4148, AN6669, AN6804, AN8400, AN8972 and AN9289).

Many other genes encoding cell membrane proteins are also down-regulated in the mutants, including three riboflavin transporters (*dbaD*, AN0528 and AN8366). These orthologs of the *S. cerevisiae* *MCH5* gene exhibit significantly lowered levels of transcripts in the *sconC* mutant. One finds here also three genes (AN2043, AN3304 and AN3345) encoding putative  $\gamma$ -aminobutyric (GABA) transporters (as annotated by EnsemblFungi; <http://fungi.ensembl.org/index.html>). GABA can be produced from glutamate or putrescine and GABA synthesis is stress-induced, similarly to polyamines (Shelp et al., 2012). Therefore down-regulation of the putative GABA symporters with simultaneous up-regulation of polyamine antiporters (section 3.5 above) appears to be an adaptation to stress in the sulfur metabolism mutants.

Among the down-regulated genes are also those encoding diverse membrane proteins assigned to various categories (Supplementary Table 4). An interesting example are nitrate and nitrite transporters (*nrtB* and *nitA*, respectively), assigned to FunCat anion transport (20.01.01.07), and down-regulated in the *sconB* mutant. The FunCat transport ATPases (20.03.22) includes down-regulated genes coding for various transport proteins, of which three are involved in steroid transport (*atrA*, AN7877 and AN10949). The FunCat photoperception and response (34.11.01) contains genes encoding two putative retinol dehydrogenases (*pkfC* and AN6931) down-regulated in the *sconC* mutant, and a short-chain dehydrogenase (AN10815) down-regulated in the *cysB* and *sconB* mutants, which is similar to the *N. crassa* light-regulated Bli-4 protein (Bruchez et al., 1996). The FunCat phospholipid metabolism (01.06.02.01) groups genes encoding membrane located proteins involved in lipid

metabolism. Four genes from this group, encoding 1-acyl dihydroxyacetone phosphate reductase (AN10399 and AN3276) and two phospholipases (AN5402 and AN7792), are down-regulated in the *cysB* and *sconC* mutants.

It is worth noting that the genes described in this section are regulated oppositely to arrestins. While several genes coding for arrestin-domain proteins are up-regulated (section 3.5) many genes encoding membrane proteins, which are putative targets of arrestins, are down-regulated.

### 3.12 Secondary metabolism is down-regulated

Numerous genes related to secondary metabolism are down-regulated in the studied mutants. The most interesting of them are from the FunCat metabolism of peptide antibiotics (01.20.37.03) and include three genes *acvA*, *ipnA* and *aatA*, members of the penicillin biosynthesis cluster (Martin, 1992). These three genes and AN8433 coding for a non-ribosomal peptide synthetase exhibit strongly decreased expression in the *sconC* mutant and slightly lowered one in the *cysB* and *sconB* mutants.

The FunCat biosynthesis of phenylalanine (01.01.09.04.01) includes two genes (*CYP573A3* and *CYP631B1*), encoding cytochrome P450-family proteins (Kelly et al., 2009), down-regulated in the *sconC* mutant. The *CYP631B1* gene has recently been shown to take part in aspernidine A biosynthesis and it has been named *pkfB* (Yaegashi et al., 2013).

Finally, two genes (AN2574 and AN5550) from the FunCat metabolism of alkaloids (01.20.17.09) are down-regulated in the *sconC* mutant. These two genes encode 6-hydroxy-D-nicotine oxidase, which is involved in nicotine degradation by *Arthrobacter nicotinovorans* (Brandsch, 2006) and has also been found among proteins secreted by *Penicillium chrysogenum* (Jami et al., 2010).

### 3.13 Other down-regulated genes

Two groups of down-regulated genes encode proteins that are neither membrane-located nor connected with secondary metabolism. The FunCat aminosaccharide catabolism (01.05.09.07) includes three genes (AN5076, AN6470 and AN8969) coding for lysozyme (muramidase) involved in bacterial cell wall degradation, which are most strongly down-regulated in the *sconC* mutant.

Three genes assigned to the FunCat alcohol fermentation (02.16.01) are down-regulated in the *sconB* mutant (*alcA*, *aldA* and AN3573). These genes are different from the up-regulated ones related to alcohol fermentation (section 3.9). Four genes encoding short

chain dehydrogenases/reductases (AN1677, AN3312, AN4691 and AN5547) and one encoding hexokinase 2 (*hxcC*), all down-regulated in the *sconB* and *sconC* mutants, belong to the FunCat sugar, glucoside, polyol and carboxylate anabolism (01.05.02.04).

### 3.14 Differences between mutants

The mutants investigated here have different defects in sulfur metabolism but they exhibit altered expression of overlapping groups of genes. This similarity is even more apparent after the FunCat analysis because the same functional categories are enriched among the up- or down-regulated genes in the three mutants. Nevertheless, many genes show altered levels of transcripts in individual mutants only. Thus, *sconC* exhibits decreased expression of 160 genes specifically in this mutant while 80 genes are also down-regulated in the *cysB* or *sconB* mutant (Fig. 2). Another apparent difference concerns the functional category temperature perception and response (34.11.09), which contains several genes connected with membrane metabolism and up-regulated by stresses. Four genes from this category are up-regulated in the *sconC* mutant while only one is significantly up-regulated in *cysB* and none in *sconB*. Some other functional categories contain more genes up-regulated in the *sconC* mutant than in *cysB* or *sconB*, e.g., sugar, glucoside, polyol and carboxylate metabolism, metabolism of energy reserves, and two-component signal transduction system (Supplementary Table 3). Thus, it appears that stress responses are induced in the *sconC* mutant more widely than in the other two mutants tested. This could result from *sconC* coding for a constant component of the SCF ubiquitin ligases so its mutation is likely to affect a wider set of genes than the mutation in the *sconB* gene coding for a protein conferring the specificity of the SCF<sup>SconB</sup> complex to the MetR protein.

Some of the down-regulated genes are also specifically affected by particular mutations. While *sconB* affects a group of genes assigned to the FunCat alcohol fermentation (02.16.01), the *sconC* mutation uniquely down-regulates several groups of genes involved in secondary metabolism (section 3.12 above).

Additionally, screening of the microarray data for genes with transcript levels altered in opposite directions revealed a considerable number of genes oppositely regulated in the *sconC* mutant than in the other two: 23 genes up-regulated in the *cysB* strain but down-regulated in *sconC*, eleven genes elevated in the *sconB* mutant but down-regulated in *sconC*, and eight genes up-regulated in the *sconC* strain but down-regulated in *cysB* (Supplementary Table 5). These differences support the assumption that *sconC* encoding a constant component of the SCF ligase may have other target(s) beyond the MetR protein.

#### 4. Conclusions

This study has shown that dysregulation of sulfur metabolism leads to activation of general stress responses resulting in elevated transcription of genes involved in reaction to heat shock, osmotic and salt stress, chemical agents, oxygen and radical-induced stresses. Several functional categories involved in such responses are highly enriched among the genes up-regulated in the mutants. Since all the mutants were cultivated in a constant environment, the source of primary stress must have been endogenous and – obviously – due to the mutations studied. In spite of having different defects in sulfur metabolism, they share important features – all show derepression of the sulfate assimilation pathway leading to dissipation of ATP and NADPH and overproduction of sulfide (Fig. 1A). These could be the primary signals evoking stress responses and could explain why the sets of induced genes overlap substantially in all three strains or at least belong to the same functional categories. Notably, these categories comprise genes coding for proteins central in the cell metabolism, for instance histidine kinases of the two-component signal transduction system and a large group of enzymes of carbohydrate and energy metabolism. The very high induction of the gene coding for sulfide:quinone reductase involved in sulfide detoxification indicates that its regulation must be very sensitive to intracellular sulfide concentration and may serve as a marker of sulfate assimilation dysregulation.

Among the down-regulated genes the largest groups encode diverse membrane proteins including peptidases, permeases and various transporters as well as proteins involved in lipid metabolism (described in section 3.11). Interestingly, several genes involved in secondary metabolism, including synthases of peptide antibiotics, are also down-regulated.

Apart from genes showing similar expression changes in all three mutants, many others show differently altered transcripts levels. In this respect the *sconC* strain is strikingly different from the other two. The stress response in *sconC* involves more genes and is often more pronounced. This is not surprising because the *sconC* gene codes for a constant component of the SCF ubiquitin ligases so its mutation could affect a wider set of genes than mutation in *sconB*. The *cysB* mutation causes cysteine shortage which renders the SCF<sup>SconB</sup> complex inactive, therefore its effects on gene expression resemble that caused by *sconB*. Another source of the differences between the transcriptional patterns of the three mutants could lie in the markedly different levels of cellular thiols, particularly glutathione and cysteine.

In short, our results show that mutations dysregulating sulfur metabolism evoke a broad stress response resulting in considerable remodeling of cell metabolism.

### **Acknowledgements**

This work was supported by the State Committee for Scientific Research, grant number 2762/B/P01/2009/37 to A. P. The microarray chips were obtained through NIAID's Pathogen Functional Genomics Resource Center. We thank Michał Koper for helpful hints regarding quantitative PCR.

## Figure Legends

### Fig. 1

Sulfur metabolism (A) and its regulation (B) in *Aspergillus nidulans*. Genes and encoded enzymes of sulfur amino acids metabolism: *cysB* - cysteine synthase, *cysD* - homocysteine synthase, *mecA* - cystathionine  $\beta$ -synthase, *mecB* - cystathionine  $\gamma$ -lyase, *metB* - cystathionine  $\gamma$ -synthase, *metG* - cystathionine  $\beta$ -lyase, and sulfate assimilation pathway: *sA* - PAPS reductase, *sB* - sulfate permease, *sC* - ATP sulfurylase, *sD* - APS kinase, *sF* -  $\alpha$  subunit of sulfite reductase and AN7600 –  $\beta$  subunit of sulfite reductase. Metabolites: APS - adenosine-5'-phosphosulfate, PAPS – 3'-phosphoadenosine-5'-phosphosulfate, PAP- 3'-phosphoadenosine-5'-phosphate, Ado-Hcy - S-adenosylhomocysteine, Ado-Met - S-adenosylmethionine.

### Fig. 2

Numbers of genes up- or down-regulated in *Aspergillus nidulans* *cysB*, *sconB* and *sconC* mutants. Total number of genes differentially expressed in a given mutant is shown in parentheses.

### Fig. 3

Functional categories overrepresented among genes up-regulated in the *cysB*, *sconB* and *sconC* mutants are shown as horizontal bars. Average enrichment ratios calculated for the mutants indicated on the left are shown below the bars. Only categories including at least three up-regulated genes and enriched at least threefold were selected (details in Supplementary Table 3).

### Fig. 4

Resistance of sulfur metabolism mutants to stress agents - sulfide, methylglyoxal, cadmium and menadione.

### Fig. 5

Content of sugar alcohols and trehalose in conidia of the *cysB*, *sconB* and *sconC* mutants compared to the wild type strain. The results are average from three (wild type and *cysB*) or four (*scon* mutants) independent experiments. Sugar and polyol contents significantly different ( $P < 0.05$ ) from wild type values are marked with an asterisk (★).

## Fig. 6

Functional categories overrepresented among genes down-regulated in the *cysB*, *sconB* and *sconC* mutants are shown as horizontal bars. Average enrichment ratios calculated for the mutants indicated on the left are shown below the bars. Only categories including at least two down-regulated genes and enriched at least twofold were selected (details in Supplementary Table 4).

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Table 1. Validation of transcript levels of selected highly regulated genes using qPCR.  $\gamma$ -actin gene (AN6542) was used as a reference.

ORF Number	Gene	fold change relative to WT					
		<i>cysB102</i>		<i>sconB2</i>		<i>sconC3</i>	
		qPCR	$\mu$ Array	qPCR	$\mu$ Array	qPCR	$\mu$ Array
AN1825 <sup>a</sup>		278	26	1862	60	844	39
AN1826 <sup>b</sup>		304	16	2866	24	1664	45
AN1017	<i>hogA</i>	3.2	2.5	9.4	6.1	13.7	6.1
AN2581	<i>hk-8-1</i>	8.8	6.5	18.1	8.6	41.3	9.2
AN4113	<i>hk-8-2</i>	3.4	6.1	8.5	2	23.5	12.1
AN6820	<i>hk-8-3</i>	5.5	4.9	14.3	3.3	24.4	9.2

<sup>a</sup> -AN1825 gene codes for sulfide:quinine reductase

<sup>b</sup> -AN1826 gene codes for metallo- $\beta$ -lactamase

Table 2. Regulation of stress response genes by exogenous stress factors in *Aspergillus nidulans* wild type strain. The fold change is normalized to the  $\gamma$ -actin gene transcript (AN6542).

ORF	Gene	Sulfide	Hydrogen peroxide	Sodium chloride
AN1825 <sup>a</sup>		16.4	13.7	3.1
AN1826 <sup>b</sup>		18.8	8.9	2.5
AN1017	<i>hogA</i>	2.6	1.9	1.9
AN2581	<i>hk-8-1</i>	1.3	3.4	1.6
AN4113	<i>hk-8-2</i>	0.9	3.9	1.4
AN6820	<i>hk-8-3</i>	1.9	3.9	2.4

<sup>a</sup>, <sup>b</sup> – see footnotes in Table 1

Figure 1

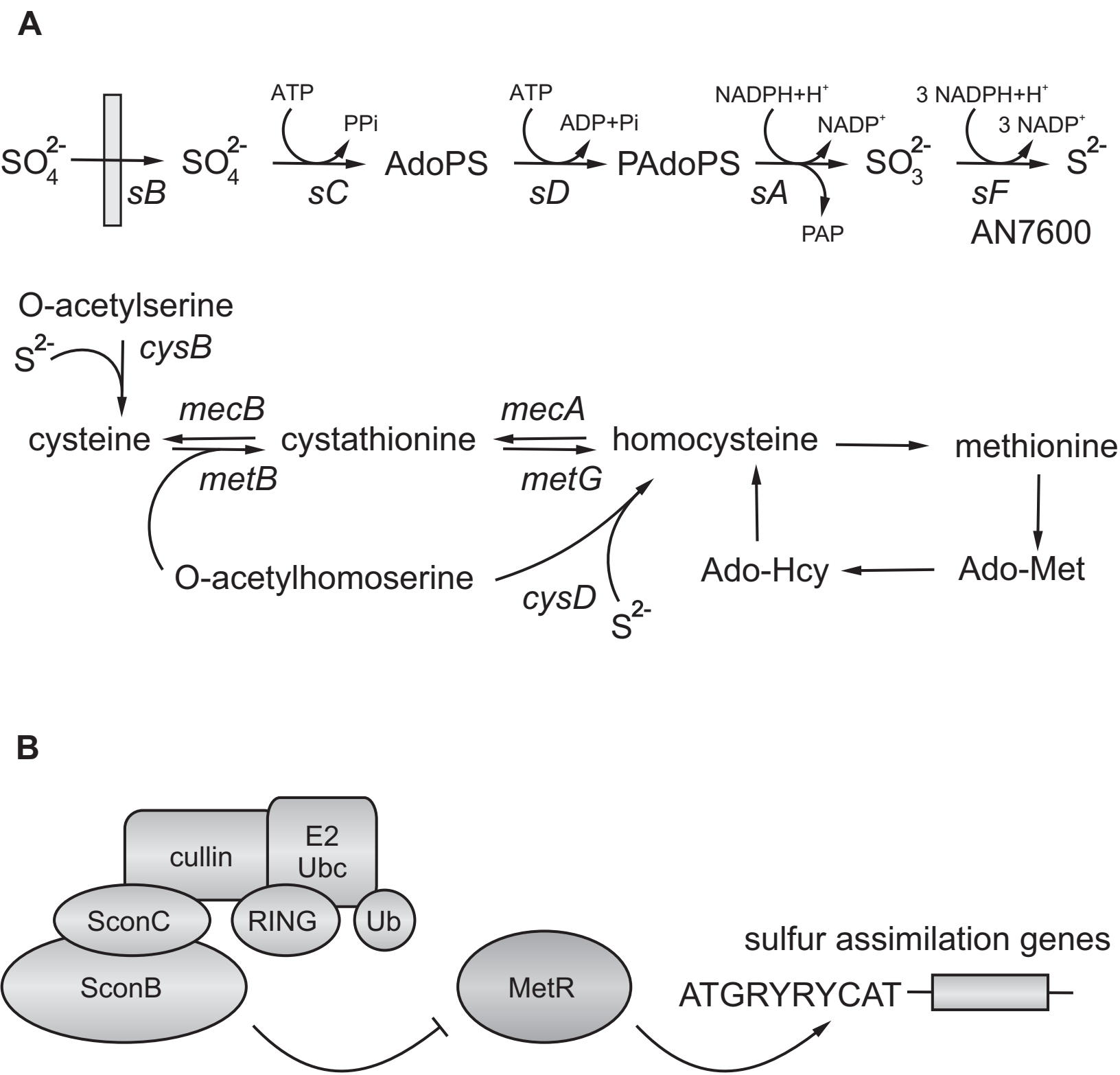
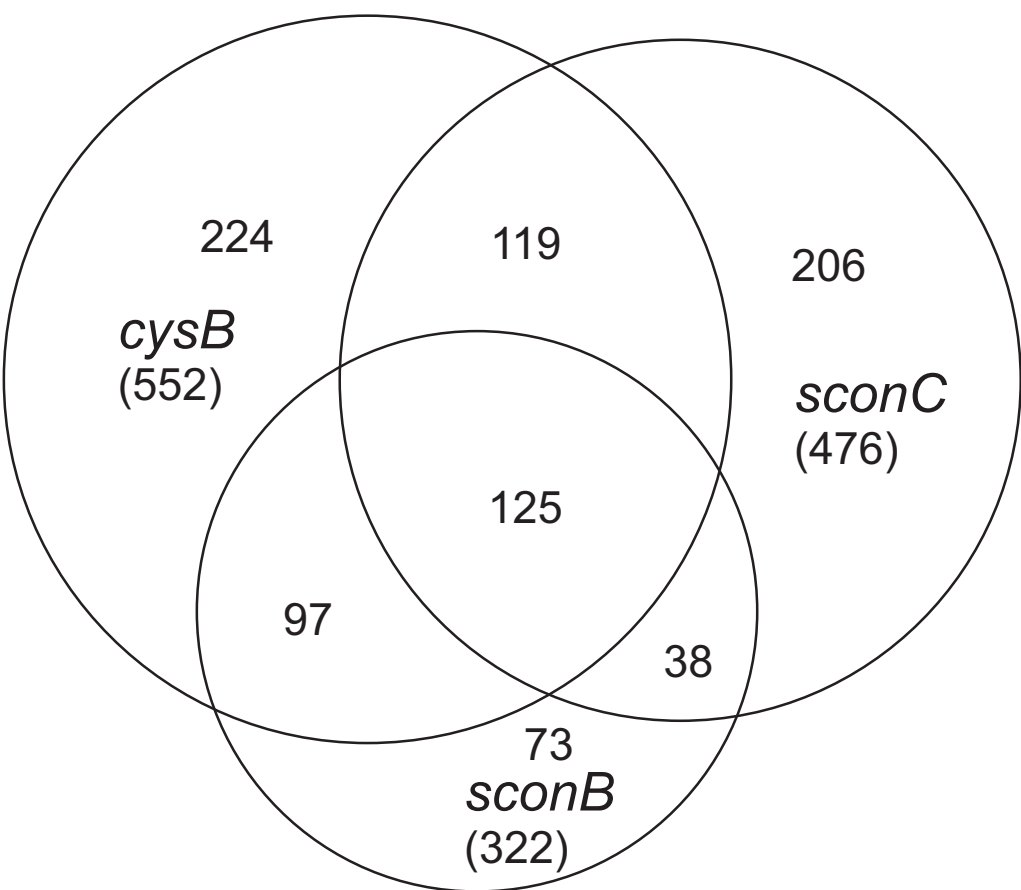


Figure 2

## up-regulated genes



## down-regulated genes

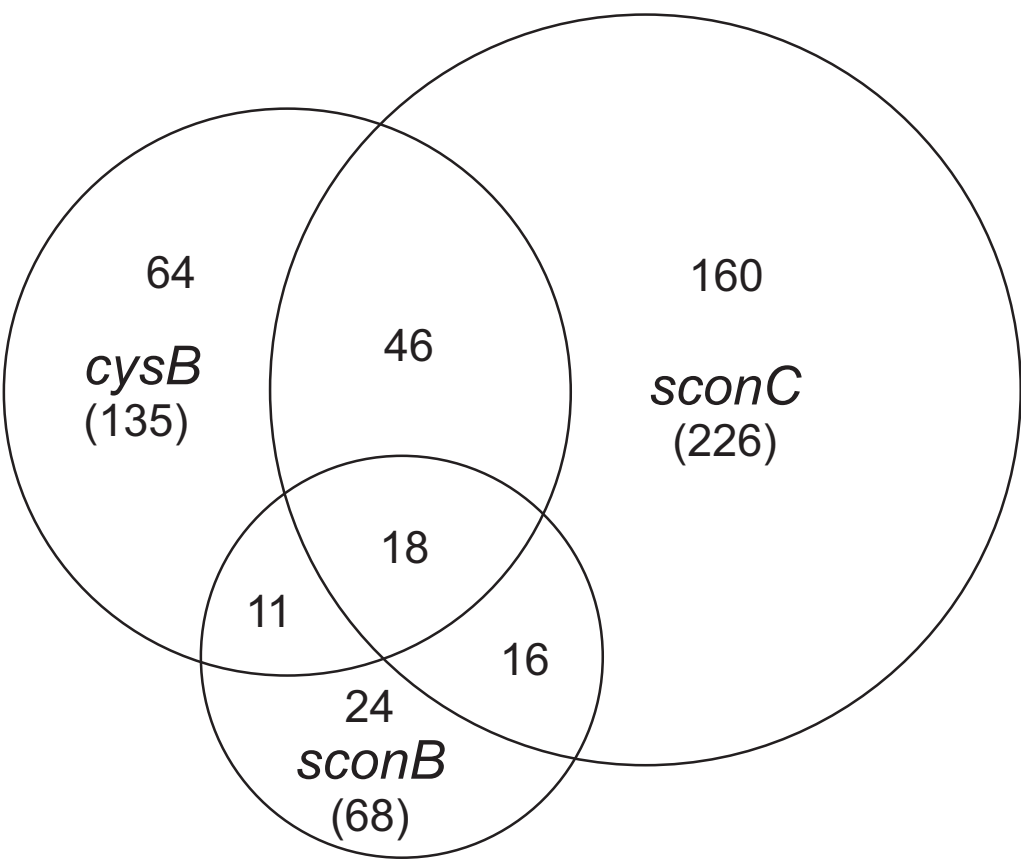


Figure 3

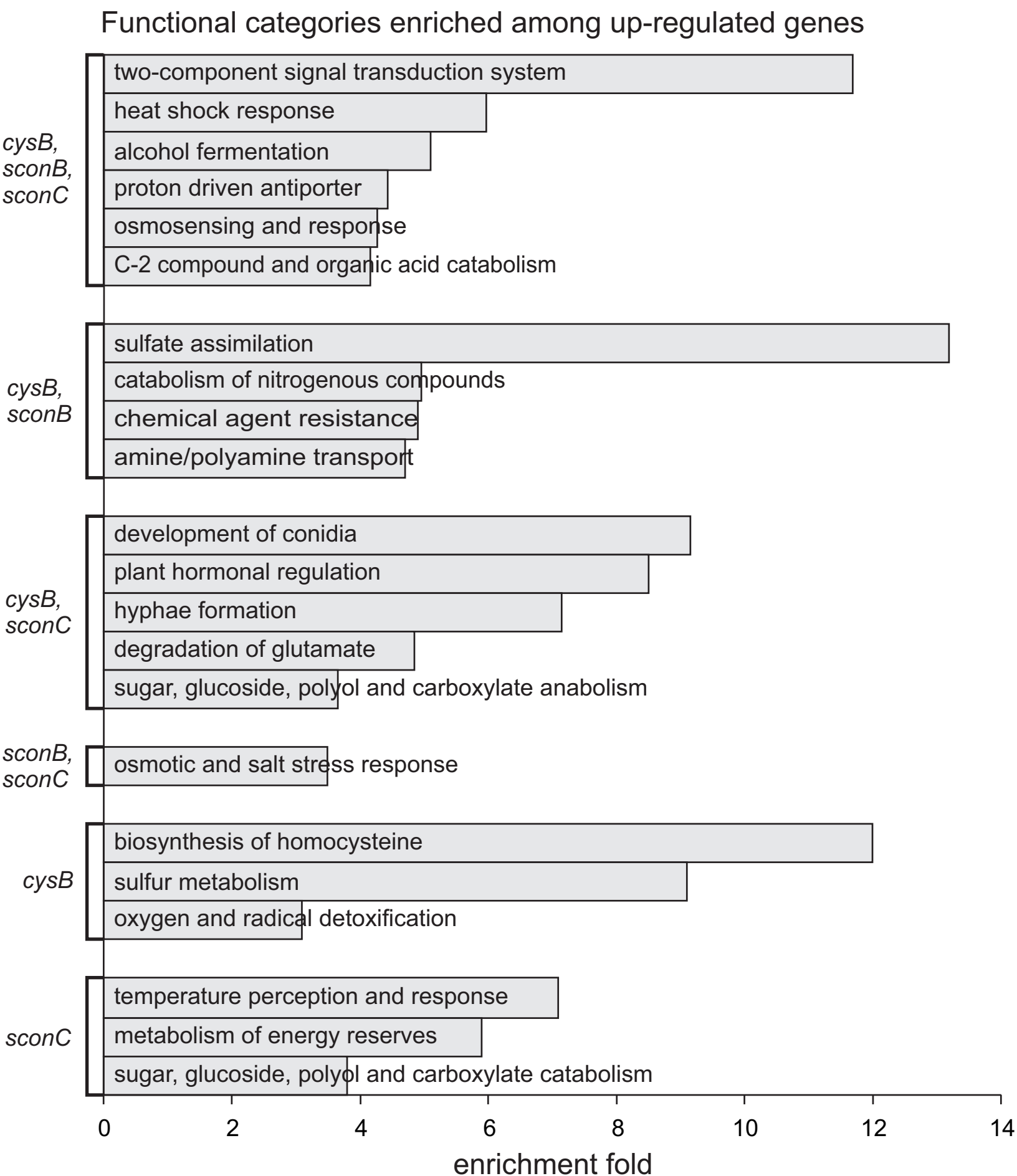


Figure 4

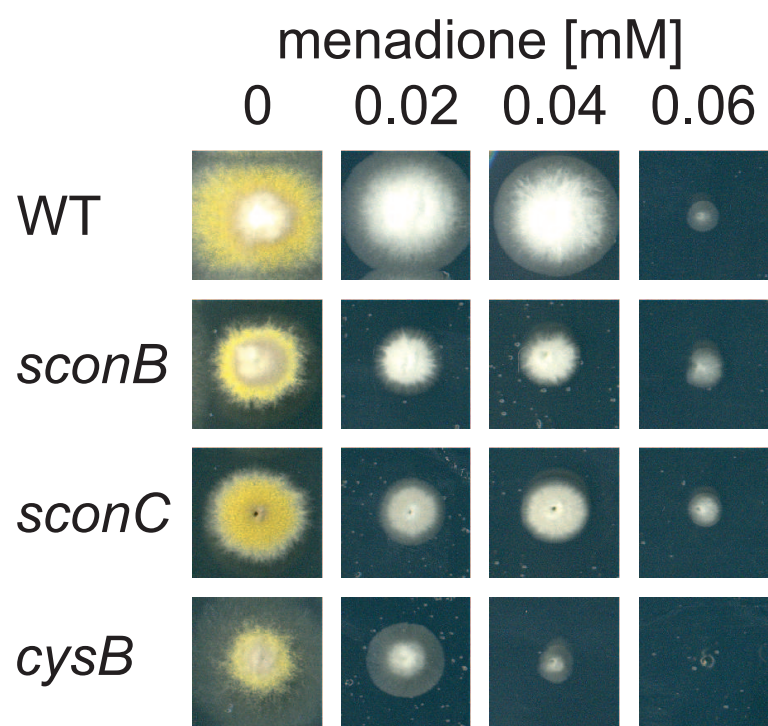
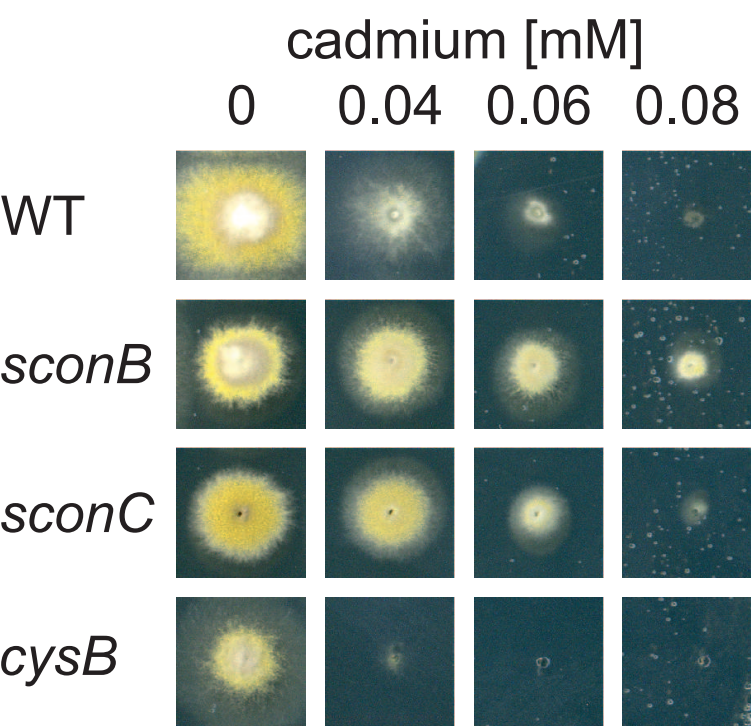
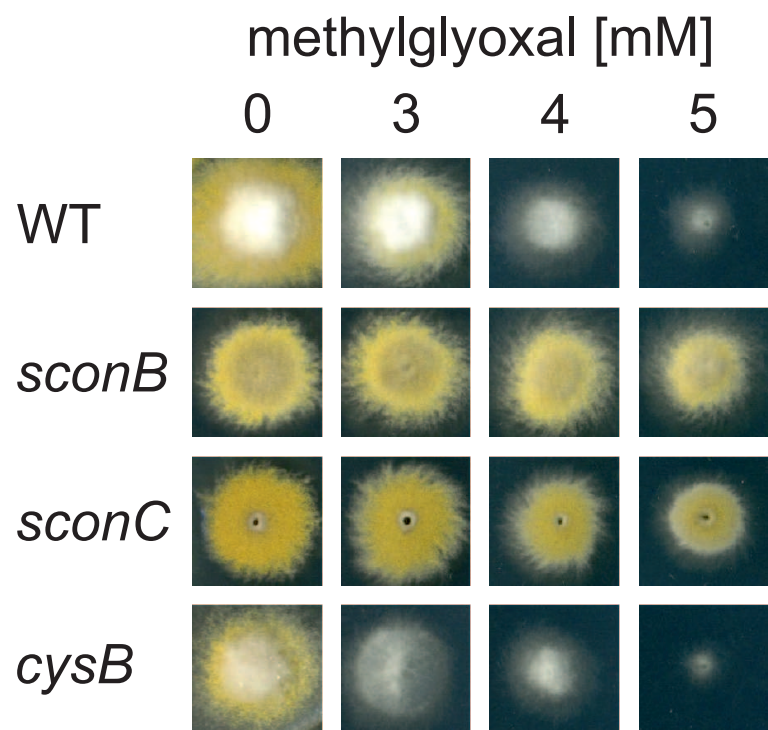
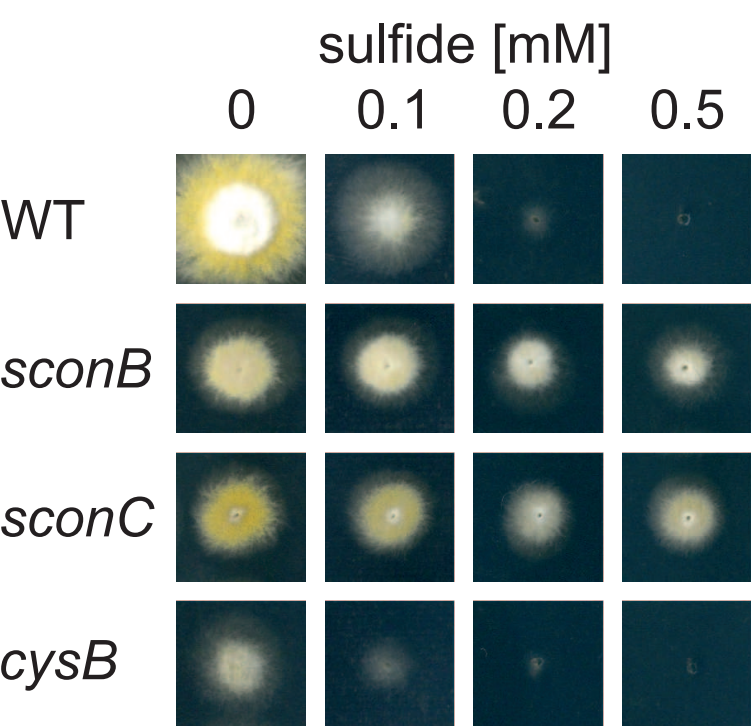


Figure 5

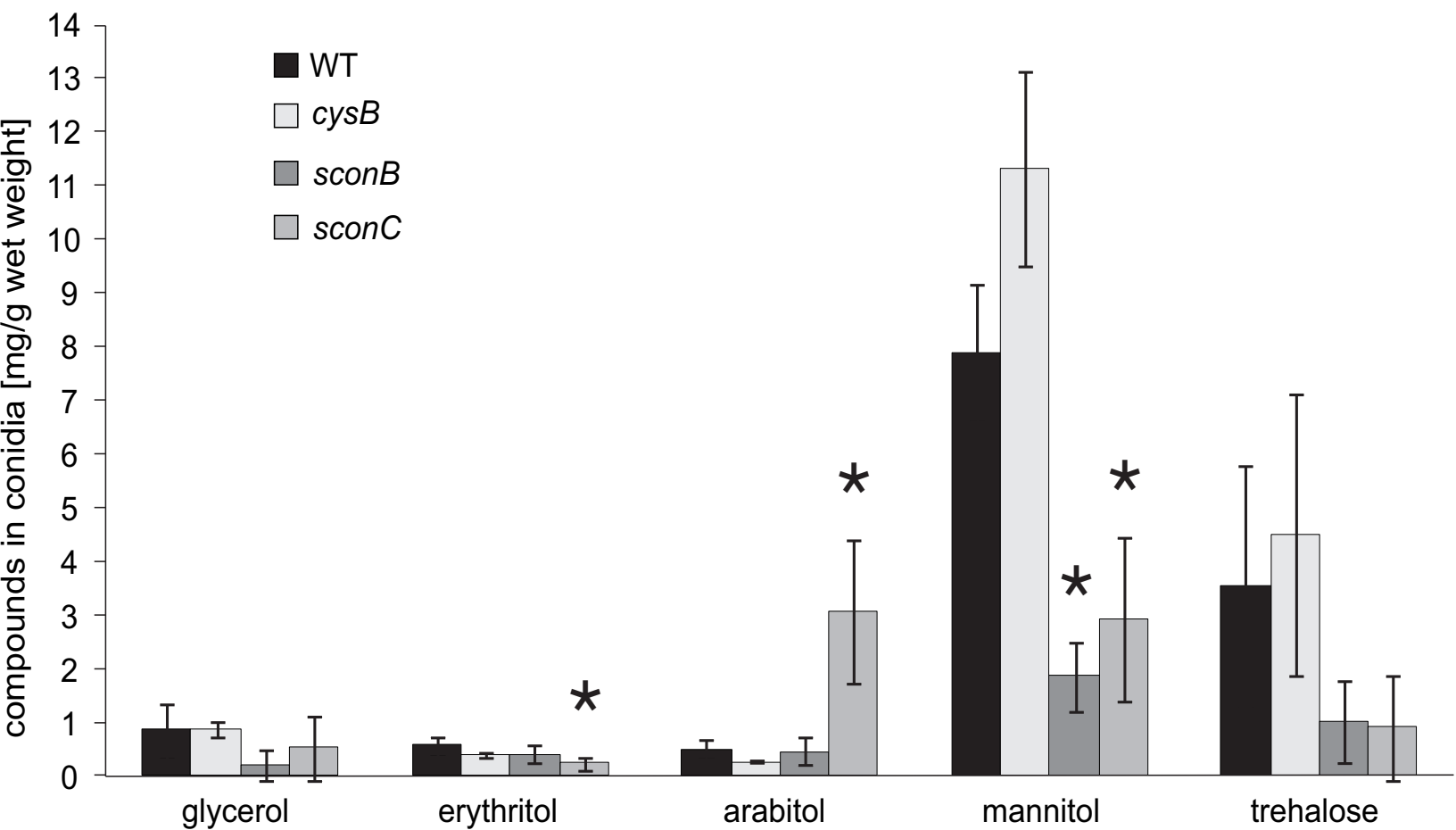


Figure 6

