Transcriptional regulation of methionine synthase by homocysteine and choline in *Aspergillus nidulans*

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Roles played by homocysteine and choline in the regulation of MS (methionine synthase) have been examined in fungi. The *Aspergillus nidulans metH* gene encoding MS was cloned and characterized. Its transcription was not regulated by methionine, but was enhanced by homocysteine and repressed by choline and betaine. MS activity levels were regulated in a similar way. The repression by betaine was due to its metabolic conversion to choline, which was found to be very efficient in *A. nidulans*. Betaine and choline supplementation stimulated growth of leaky *metH* mutants apparently by decreasing the demand for methyl groups and thus saving methionine and *S*-adenosylmethionine. We have also found that homocysteine stimulates transcription of MS-encoding genes in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*.

Key words: betaine, choline, homocysteine, methionine synthase gene, regulation.

INTRODUCTION

MS (methionine synthase) catalyses the formation of methionine by the transfer of a methyl group from methyltetrahydrofolate to homocysteine. The homocysteine methylation reaction is the point of convergence of two metabolic domains, i.e. sulphur metabolism and one-carbon unit metabolism. This reaction is the last step in methionine biosynthesis and, indirectly, also serves, among others, to generate the methyl group of SAM (*S*-adenosylmethionine), a substrate required for more than a hundred biological methylation reactions.

Two types of this enzyme are found: cobalamin-dependent MS (EC 2.1.1.13) and cobalamin-independent MS (EC 2.1.1.14), which share no similarity and have different evolutionary origins [1]. The former type is found in bacteria, protozoa and metazoa. Bacteria and protozoa may also possess cobalamin-independent MS, which is the only enzyme found in fungi and plants.

Cobalamin-independent MS uses mainly a triglutamate derivative of methyltetrahydrofolate as a methyl group donor. The GenBank® database contains more than 40 sequence codes for these enzymes. However, the regulation of their synthesis has been studied in relatively few organisms.

The *Escherichia coli* MS encoded by the *metE* gene is regulated by the MetR transcription factor, which is activated by homocysteine or homocysteine thiolactone [2,3]. It is repressed by the *metJ* gene product with SAM acting as a co-repressor [4]. In plants, the MS level varies in different organs and changes at day/night periods [5,6]. An increase in the MS transcript, but not protein level, was observed in *Catharanthus roseus* and *Solanum tuberosum* under stress induced by sucrose [6,7]. Interestingly, in *Chlamydomonas reinhardtii*, cobalamin-independent MS was induced severalfold during adhesion-induced activation of gametes, which involves a cAMP-dependent cascade of signal transduction [8]. This may result from an increased demand for SAM (and also for methionine) for carboxy methylation of signalling molecules and for the synthesis of polyamines involved in chromatin restructuring.

Among fungi, methionine synthesis has been most extensively studied in *Saccharomyces cerevisiae*. The MS-encoding gene *MET6* [9] is repressed in the presence of methionine in the growth medium, but apparently by a different mechanism when compared with that regulating other *MET* genes [10,11]. Moreover, the enzyme level is significantly decreased after osmotic [12] and oxidative [13] shocks. In contrast with *S. cerevisiae*, MS is up-regulated under heat conditions and during the yeast-to-hyphal transition in the human pathogen *Candida albicans* [14]. Burton and Metzenberg [15] observed slightly increased levels of MS in *Neurospora crassa* mutants defective in SAM or folylpolyglutamate synthesis. The enzyme level was decreased when growth medium was supplemented with choline.

In *Aspergillus nidulans*, despite extensive studies of sulphur amino acid metabolism, no investigation of an MS gene and regulation of its expression have been performed so far. In this fungus, biosynthesis and metabolism of sulphur amino acids comprises a rich combination of metabolic options (see Figure 1, reviewed in [16]). Several sulphur metabolic genes, particularly those involved in sulphate assimilation, are regulated by a cascade of negatively acting *scon* (sulphur controller) genes [17] and the positively acting *metR* gene, encoding a sulphur-specific transcription factor [18] forming the cysteine-mediated SMR (sulphur metabolite repression) system. The *scon* mutations render sulphur metabolism insensitive to SMR, whereas *metR* mutants are methionine-requiring auxotrophs.

Two mutants, *metH2* and *metH10* (formerly *metD10*), growing on methionine but not on homocysteine, apparently blocked in the homocysteine methylation step, have been known for many years [19]. The mutants contain twice as much cystathionine as the wild-type strain and accumulate some homolanthionine, a higher homologue of cystathionine [20], which results from an increased homocysteine pool. Genetic tests pointed to a very close

Abbreviations used: BHMT, betaine:homocysteine methyltransferase; MM, minimal medium; MS, methionine synthase; RT, reverse transcriptase; SAM, *S*-adenosylmethionine; SMR, sulphur metabolite repression.

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The nucleotide sequence data reported will appear in the GenBank® Nucleotide Sequence Database under the accession number AF275676.

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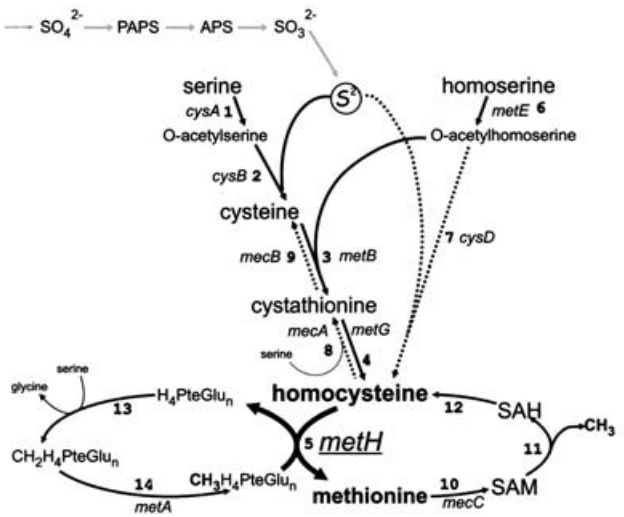

Figure 1 An outline of methionine synthesis and methyl group metabolism in *A. nidulans*

1, Serine acetyltransferase; 2, cysteine synthase; 3, cystathionine γ-synthase; 4, cystathionine β-lyase; 5, MS; 6, homoserine acetyltransferase; 7, homocysteine synthase; 8, cystathionine β-synthase; 9, cystathionine γ-lyase; 10, SAM synthetase; 11, various methyltransferases; 12, *S*-adenosylhomocysteine hydrolase; 13, serine hydroxylmethyltransferase and 14, methyltetrahydrofolate reductase. ·····, Alternative pathway of sulphur-containing amino acids synthesis, which is repressed under normal conditions and depressed in *scon* strains and *cysA* and *cysB* mutants [16]. Symbols of the genes encoding particular enzymes are indicated. $H_4PteGlu_n$, tetrahydrofolate; SAH, *S*-adenosylhomocysteine.

linkage of these mutations [21], but *metH10* responded not only to methionine but also to betaine. Therefore it was not clear whether these mutations affect the same or adjacent loci.

In the present study, we report cloning and characterization of the *metH* gene, which was shown to code for a cobalamin-independent MS. The gene is mutated in both *metH2* and *metH10* mutants. Its transcription was found to be strongly enhanced by homocysteine and some increase in the transcript level was also observed on SAM shortage. Betaine and choline had a repressing effect, whereas methionine had none. Evidence was obtained indicating reduction of betaine to choline *in vivo*, a process that, for a long time, was thought not to take place in nature, but recently its occurrence has gained some experimental support [22]. Since choline synthesis consumes most of the methyl groups derived from methionine, MS may be considered as the last enzyme in the methionine and first in the choline biosynthetic pathways. Therefore the effect of choline on *metH* expression appears to be a typical negative feedback regulation.

EXPERIMENTAL

Organisms, media and growth conditions

The following strains of *A. nidulans* from our collection which carry standard markers [23] were used: *pyroA4*,*yA2*, used as a reference wild-type strain; *metH2*,*pyroA4*,*yA2*; *metH10*,*pyroA4*, *yA2*; *metH2*,*argB2*,*nicA2*,*biA1*,*yA2*; *mecC13*,*anA1*,*biA1*; *mecA1*, *pyroA4*,*yA2*; *metG55*,*pyroA4*,*yA2*; *adF9*,*choA1*,*pabaA2*,*yA2*; *pyrG89*,*pabaA1*; *sconB2*,*pyroA4*,*yA2* [17]; *metR1*,*pyroA4yA2* [18]. The conditional *metH* null mutant *ΔmetH:pyr4AlcA-metH*, *pyrG89*,*pabaA1* was constructed as described by May [24]. The *Bam*HI–*Bam*HI fragment from a cDNA clone of the *metH* gene containing 184 nt upstream from ATG codon was introduced into the pAL4 *A. nidulans* expression vector containing the *AlcA* ethanol inducible promoter [25] and the *pyr4* marker which complements *A. nidulans pyrG89* mutation. The resulting pAH41 plasmid was transformed into *pyrG89*, *pabaA1* and integrated into the *metH* locus giving a conditional, ethanol-dependent *metH*$^-$ mutant.

A. nidulans strains were grown on MM (minimal medium) or complete medium as described by Cove [26] for 16–18 h at 37 °C with shaking. Solid media contained 1.5 % (w/v) agar. Supplements added to the media were betaine, choline, DL-homocysteine and L-methionine. They were purchased from Sigma.

S. cerevisiae strains, BY4742 (*MATα his3-Δ1 leu2-Δ0 lys2-Δ0 ura3-Δ0*), BY4742*Δmet6* (*MATα his3-Δ1 leu2-Δ0 lys2-Δ0 ura3-Δ0 met6-Δ::KAN*) and BY4742*Δmet15* (*MATα his3-Δ1 leu2-Δ0 lys2-Δ0 ura3-Δ0 met15-Δ::KAN*) were purchased from EUROSCARF. The wild-type strain Σ1278b was from the Laboratory of Microbiology (Free University of Brussels, Brussels, Belgium). The *Schizosaccharomyces pombe* strain *h+ ade6* was from the Institute of Microbiology (University of Wroclaw, Wroclaw, Poland). Yeast cells were grown on YPD medium [2 % (w/v) glucose/2 % (w/v) peptone/1 % (w/v) yeast extract] or W0 medium (1 % carbon source/0.57 % yeast nitrogen base without amino acids) with 20 μg/ml of the supplements required for growth, at 30 °C.

The *E. coli* strains used were DH5α F′ for plasmid amplification and XL1 Blue (Stratagene) as a host strain for the *A. nidulans* λZAP cDNA library.

Transformation of *A. nidulans*

Transformation of *A. nidulans* was performed as described by Sienko et al. [27].

Cloning and sequencing of *metH* gene

Since the *metH* gene was localized on chromosome III [28], the *metH2*,*argB2*,*nicA2*,*biA1*,*yA2* strain was co-transformed with DNA isolated from a fraction of the *A. nidulans* cosmid chromosome-specific library [29] covering this chromosome (divided into subpools) and the Arp1 helper plasmid, which increases transformation efficiency as much as 200-fold. The subpool that gave met$^+$ transformants was further subdivided and one cosmid (W25A01) restoring methionine prototrophy was found. It was digested with several restriction endonucleases and used for subsequent transformation. The cosmid digested with *Xba*I gave the highest number of transformants. Individual DNA fragments were isolated from agarose gel and used for transformation. Finally, a 5.1 kb *Xba*I–*Xba*I-transforming fragment was cloned into the pUC19 vector giving pLX2. This fragment was sequenced on both strands using several subclones and standard primers for pUC19 and pBluscriptIIKS + vectors.

cDNA isolation and splicing analysis

A cDNA copy of the *metH* gene was isolated from the *A. nidulans* λZap cDNA library (constructed by R. Aramayo, Fungal Genetics Stock Center, Kansas City, KS, U.S.A.) using UNI-ZAP^{TM}XR Cloning kit (Stratagene). Plaque hybridization with DIG-labelled probe was performed using Dig High Prime DNA-labelling kit (Roche, Rotkreuz, Switzerland) according to the manufacturer's instructions. A PCR fragment, obtained with SM1UP 5′-TTCTGTGCTTGCATTGTTTA-3′ and SM1LO 5′-ACGGGGCGGGTGACGATAC-3′ primers, was used as a probe. DNA inserts in pBluescriptSK were excised from the λZapII

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vector with a helper phage, according to the method prescribed by Stratagene.

mRNA analysis

The transcription start point was determined using 5′-full RACE (rapid amplification of cDNA ends) Core Set (Takara, Osaka, Japan) following the manufacturer's method. The SM3LO primer was phosphorylated at the 5′-end with T4 kinase (Gibco BRL, Paisley, Renfrewshire, Scotland, U.K.) and it was used to amplify the 5′-terminal fragment of *metH* mRNA. Amplification of the PCR product containing the 5′-end of the SM3LO 5′-GTAACCGGTAAGACGCTCAC-3′ primer linked to the first nucleotide of the mRNA was achieved using the SM3UP 5′-AGCTCTCTTCTCCACGTTCC-3′ and SM6LO 5′-TTCTCGTAGGCAGGCTTGAA-3′ primers. The resulting PCR fragments were used as a template for second amplification with the SM4UP 5′-AGCTCGGCCTTGACGTTCTG-3′ and SM7LO 5′-TTCTTGAGGTCACGGAGCTT-3′ primers. The final PCR fragment was sequenced on both strands.

The *metH* mRNA sequence in the intron–exon region was determined by sequencing the RT (reverse transcriptase)–PCR product obtained using total RNA, primers SM1UP and SM1LO and SuperScript™ One-Step RT–PCR System (Gibco BRL).

Isolation of RNA

Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH, U.S.A.) according to the manufacturer's instructions. For isolation of RNA from *A. nidulans*, mycelia were grown for 16 h at 37 °C in 100 ml of liquid MM with supplements. Mycelia (200 mg) were immediately frozen in liquid nitrogen and ground in a mortar with powdered glass and 1.5 ml of TRIzol®. *S. cerevisiae* and *Sch. pombe* were grown in 100 ml of W0 medium until the cultures reached A_{600} 0.8. After centrifugation and washing with water, 2 ml of TRIzol® was added to the pellet and the cells were disrupted by vigorous vortex-mixing with glass beads. The RNA samples for 5′-transcript termini mapping and RT–PCR were dissolved in RNase-free water and treated with RNase-free DNaseI (Roche).

Northern blotting

Northern-blot analysis was performed as described in [30] with some modifications. The sample containing 20 μg of total RNA was glyoxylated [in 24 ml of buffer containing 15 mM sodium phosphate buffer (NaPi), pH 6.5/0.025 % Bromophenol Blue/5 % (v/v) glycerol/0.05 % ethidium bromide/50 % (v/v) DMSO/7 % (w/v) glyoxal] at 55 °C for 1 h followed by fractionation in 1 % agarose in 15 mM NaPi (pH 6.5). Ethidium bromide-stained 28 S rRNA was used to standardize the amounts of RNA loaded on to the gel. It was then capillary-transferred overnight to a nylon membrane (Hybond N^+; Amersham Biosciences) in 25 mM NaPi (pH 6.5). The filters were prehybridized in 250 mM NaPi (pH 7.5) with 7 % (w/v) SDS and 2 % blocking reagent (Roche) for 3 h at 68 °C. DNA probes were α-^{32}P-labelled by random priming with the HexaLabel Plus DNA labelling kit (MBI Fermentas, Munich, Germany) and purified on SigmaSpin Post Reaction Purification Columns (Sigma). Hybridization was performed in the same buffer similar to prehybridization at 68 °C, overnight. Filters were washed with 20 mM Na_2HPO_4, 2 mM EDTA and 1 % SDS for 10 min, five times at 65 °C. Standard autoradiography was performed with a PhosphorImager system (Molecular Dynamics). The intensity of gene expression during induction or repression was estimated by quantification of the transcript level using the Image Quant system (Molecular Dynamics). In every case, at least three independent expositions and two independent experiments were performed.

For determining MS transcript levels, PCR fragments were synthesized with the following primers: for *A. nidulans*, SM3UP (see above) and SM4LO 5′-ACTTGCTGTTCTCGATGGAC-3′; for *S. cerevisiae*, SCUP1 5′-CCAAGCCGCCATTAAGAAGG-3′ and SCLO1 5′-CTTAACGGCAGCATCGTGGA-3′; and for *Sch. pombe*, SPUP1 5′-CCATCACCAATGTTGAGTCC-3′ and SPLO1 5′-GCAAGAGGTATAGCGGCTAA-3′.

Extract preparation

A. nidulans strains were cultured in 100 ml of MM with supplementation as indicated for 16–18 h at 37 °C. Mycelia (500 mg) were then homogenized in a chilled mortar with powdered glass and 1–2 ml of 10 mM potassium phosphate buffer (KPi; pH 7.2). The extracts were centrifuged at 13000 ***g*** and supernatants were used for the enzyme assay. Protein concentration in the extracts was measured with Bio-Rad protein assay and adjusted to 2 mg/ml with the buffer.

MS assay

MS was assayed as described by Drummond et al. [31]. The reaction was linear with respect to time and concentration of protein, until the concentration of tetrahydrofolate reached 9.1 μM (A_{350} 0.350). At this concentration tetrahydrofolate probably inhibits the enzyme activity. There were no differences in specific activities between crude extracts and extracts purified by filtration through Sephadex G-25.

Betaine:homocysteine methyltransferase (BHMT) assay

BHMT assay was performed as described by Skiba et al. [32]. In the reaction, 100 μg of total protein was used.

Isolation of lipids and TLC

A. nidulans strains were grown for 18 h in 50 ml of liquid MM containing 100 μM radiolabelled [glycine-1-^{14}C]-betaine (50000 c.p.m./μmol; Moravek Biochemicals, Brea, CA, U.S.A.) or [methyl-^{14}C]betaine (Biotrend Chemikalien, Cologne, Germany). Mycelia were ground in a mortar with powdered glass and 5 ml of the mixture chloroform/methanol/water (25:12.5:10, by vol.). Cell debris was removed by centrifugation; 1.25 ml of chloroform and 1.25 ml of 0.9 % NaCl were added to the supernatant and the resulting phases were separated. The organic phase was dried. The residue containing lipids was dissolved in 500 μl of methanol. Lipid solution (100 μl) was applied on a Silica gel 60 F_{254} plate (Merck) and subjected to two-dimensional chromatography. The first development was in methanol/chloroform/ammonium hydrate (14:6:1, by vol.) and the second development was in chloroform/methanol/acetone/acetic acid/water (14:6:6:3:1, by vol.). Lipid spots were visualized using iodine vapour and identified by co-chromatography with standards. The radioactive metabolites were visualized by autoradiography.

RESULTS

Cloning and analysis of *metH* gene

The 5.1 kb *Xba*I–*Xba*I fragment of the W25A01 cosmid, which restored the met^+ phenotype in the *metH2* mutant was cloned

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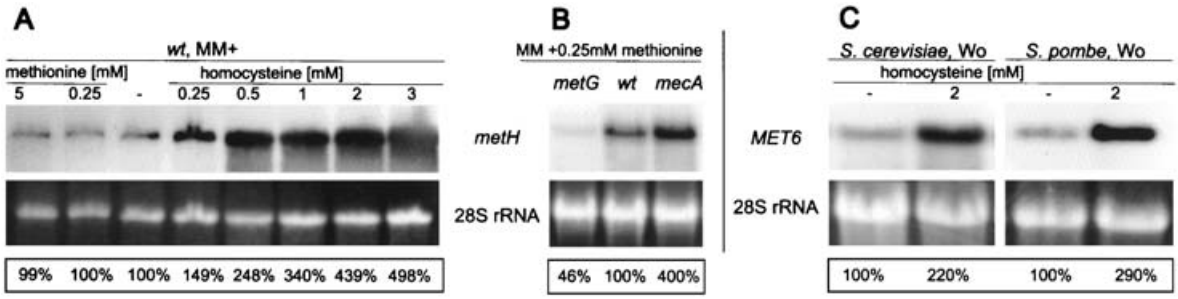

Figure 2 Homocysteine-induced *metH* transcription

Northern-blot analysis. (**A**) Wild-type strain was grown on MM with different concentrations of methionine or homocysteine. (**B**) *metH* transcript level in *metG55* and *mecA1* mutants bearing mutations in cystathionine β-lyase and cystathionine β-synthase genes respectively (see Figure 1), growing on MM with 0.25 mM methionine. The amounts of *metH* transcript level relative to its content in the wild-type strain grown on MM with 0.25 mM methionine are shown at the bottom (numbers are means from at least two independent experiments). (**C**) Induction of the MS-encoding gene transcription by homocysteine in yeast strains. The amounts of induced transcript levels relative to its content under a non-inducing condition are shown at the bottom. Percentage increases in transcript level have been corrected for variations in the levels of 28 S RNA.

into the pUC19 vector giving the pLX2 plasmid. The sequence of 4323 bp from this fragment was submitted to the GenBank® under the accession no. AF275676. It contains an open reading frame encoding a protein of 774 amino acids showing 61 % identity with *S. cerevisiae* cobalamin-independent MS and 47 % with both *A. thaliana* and *E. coli* enzymes. As revealed by comparison with the cDNA copy, the open reading frame is interrupted by one 65 bp intron, beginning at 16th nucleotide downstream from the putative ATG initiation codon. The fragment also comprises 1123 nt upstream from the coding sequence and a 720 nt downstream from the stop codon.

This gene was also sequenced in the *metH2* and *metH10* strains, and was found to be mutated in both of them: *metH2* has a tyrosine to cysteine transition causing a Y635 → H substitution, whereas *metH10* has a mutation located at the 5′-splice site of the intron; the wild-type sequence C/GTATGC is changed in the third position to C/GTTTGC which may impair the splicing process. These findings confirmed that the cloned MS gene was *metH* and not a suppressor of the *metH2* mutation.

Since both the *metH2* and *metH10* strains are leaky and show an appreciable MS activity (see below) we attempted to determine whether *metH* is an essential gene. The gene was disrupted and the resulting strain was a tight auxotroph. It was viable on methionine showing that *metH* is not an essential gene.

The *S. cerevisiae Δmet6* mutant, lacking MS, grew on galactose-supplemented medium when transformed with a plasmid carrying *metH* cDNA under the yeast *GAL1* promoter form pYES2 vector (Stratagene). This indicated that the *A. nidulans* enzyme is functional in *S. cerevisiae*.

Homocysteine increases MS mRNA levels

The nearest transcription start point of the *metH* gene is positioned at −174 nt from the predicted ATG-initiation codon as determined by 5′-RACE analysis (see the Experimental section). The regulation of gene transcription under various growth conditions was studied by Northern-blot analysis. It was found that the transcript level was affected neither by concentration of sulphate (0.1–2.0 mM) and methionine (0.25–5.0 mM) nor by the regulatory mutations *sconB2* and *metR1* (results not shown).

On the other hand, the transcription was strongly increased in cells grown in the presence of homocysteine (Figure 2A), even at a concentration as low as 0.25 mM. At least 400 % increase in the transcript level was repeatedly observed in experiments with 3 mM homocysteine. The inducing effect of homocysteine was

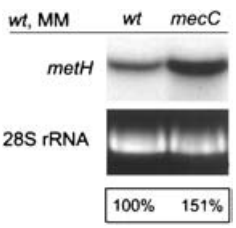

Figure 3 Increase in MS transcript level owing to *S*-adenosylmethionine shortage in *mecC13* strain

Northern-blot analysis of strains grown on MM. The amounts of *metH* transcript level relative to its content in the wild-type strain grown on MM are shown at the bottom (means from three experiments). Percentage increases in transcript levels have been corrected for variations in the levels of 28 S RNA.

confirmed in the mutants impaired in sulphur metabolism (Figure 2B). As compared with the wild-type, a lower level (by at least 50 % of the transcript level) was observed in the *metG55* strain, lacking cystathionine β-lyase and accumulating cystathionine, whereas it was higher by approx. 4-fold in the *mecA1* strain impaired in cystathionine β-synthase, which should be expected to have a higher level of homocysteine.

It is of interest to determine whether the transcriptional regulation of MS-encoding genes by homocysteine is a more general phenomenon, at least in fungi. As shown in Figure 2(C), homocysteine in the growth medium stimulated transcription of the MS genes both in *S. cerevisiae* and *Sch. pombe*.

A slight increase (from 140 to 160 %) in MS transcript level was observed in the *mecC13* mutant partially blocked in SAM synthase (Figure 3) leading to reduction of cellular SAM to 30 % of the wild-type [33]. This suggested that a shortage of SAM stimulates *metH* expression.

A very low transcript level was found in *metH10* (Figure 4). RT–PCR fragments obtained with RNA isolated from the mutant had a sequence identical with those obtained for RNA isolated from the wild-type. This showed that the intron was spliced correctly but with a low efficiency. As a consequence, *metH10* makes a normal enzyme but in a low amount which is responsible for the met$^-$ phenotype. Unspliced or wrongly spliced mRNA was probably degraded. Its level in the mutant, even in the presence of homocysteine in the growth medium never exceeded 60 % of the control. In the *metH2* mutant grown on a medium containing

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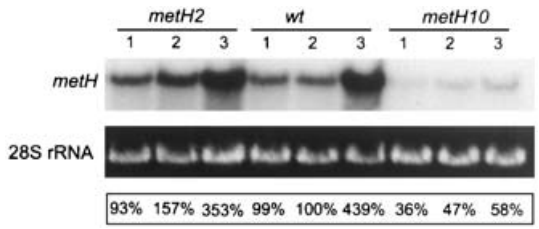

Figure 4 Expression of *metH* gene in *metH* mutants

Mycelia were grown on MM with: 1, 5 mM methionine; 2, 0.25 mM methionine and 3, 0.25 mM methionine and 2 mM homocysteine. The amounts of *metH* transcript level relative to its content in the wild-type strain grown on MM with 0.25 mM methionine are shown at the bottom (means from two experiments). Percentage increases in transcript levels have been corrected for variations in the levels of 28 S RNA.

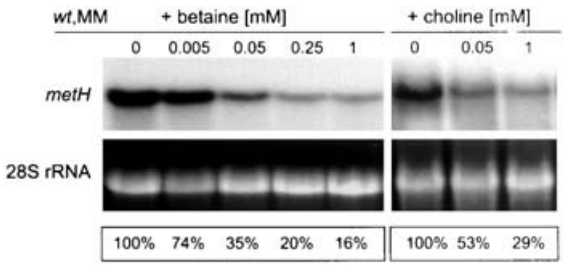

Figure 6 Repression of *metH* gene transcription by betaine as well as choline

Northern-blot analysis of *metH* transcription in the wild-type grown on MM supplemented with different concentrations of betaine or choline. The amounts of *metH* transcript level relative to its content in the wild-type strain grown on MM with 0.25 mM methionine are shown at the bottom. Percentage increases in transcript levels have been corrected for variations in the levels of 28 S RNA.

low methionine concentration, the transcript level was 1.5-fold higher than in the wild-type, probably due to the accumulation of homocysteine. This was not observed at high concentrations of methionine since sulphate assimilation and homocysteine synthesis are repressed in this condition.

Homocysteine stimulates MS expression at the protein activity level

MS activity was assayed in the wild-type strain and several mutants grown under various conditions of sulphur supplementation. The results shown in Figure 5 indicate that, in contrast with its effect on transcription, methionine has some suppressing effect on the enzyme level whereas homocysteine enhances it more than 3-fold. A similar effect of homocysteine was observed in *metH2*, which showed approx. 50 % of the wild-type activity (surprisingly high, by taking into account the slight leakiness of the mutant). There were also no significant differences between the K_m values for MS in these strains: 60.4 ± 6.5 and $87.7 \pm 10.3\ \mu$M for $CH_3H_4PteGlu_3$ and 6.2 ± 0.9 and $6.7 \pm 1.6\ \mu$M for homocysteine for the wild-type and *metH2* strains respectively.

Regarding other mutant strains affected by sulphur metabolism, an increased enzyme activity was found in *mecA1*, which corresponded to the increased transcript level observed in this strain. However, the *metG55* strain showed MS activity similar to that of the wild-type despite a lower transcript level. In contrast, the high transcript level in the *mecC13* mutant did not coincide with an increase in enzyme activity.

Regarding regulatory mutants, a higher MS activity than in the wild-type was observed in the *sconB2* strain although the transcript level was not affected, but the *metR1* mutation had no effect on either the transcript or enzyme levels.

No MS activity was found in the strain containing a disrupted *metH* gene. This indicated that the enzyme activity observed in the *metH* mutants could not be attributed to an isoenzyme encoded by another MS gene.

Betaine negatively regulates the expression of MS on mRNA and protein levels

As mentioned earlier, betaine stimulates growth of *metH10*. This raised the question: what is the mechanism by which this compound stimulates growth of this strain? It does so in spite of the fact that it strongly represses *metH* transcription (Figure 6) and enzyme activity (Figure 5). Betaine (1 mM) always led to > 80 % repression of *metH* transcript level. Betaine is known

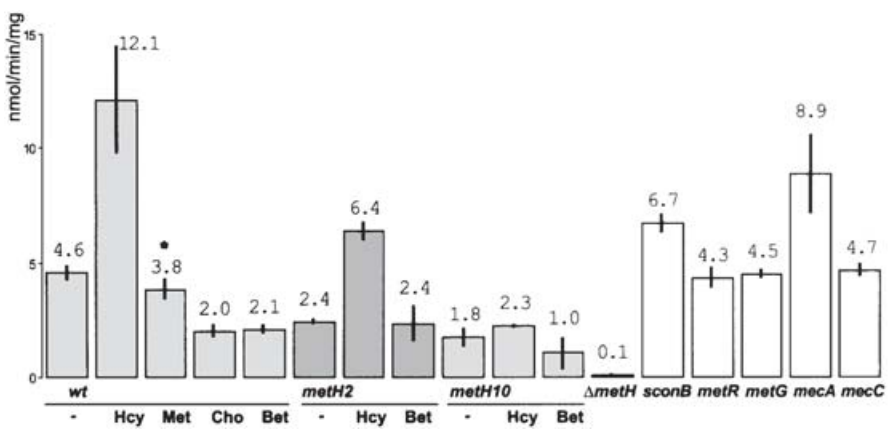

Figure 5 MS-specific activity in the wild-type and mutant strains

Mycelia grown on MM with 0.25 mM methionine and the following supplements: Hcy, 3 mM homocysteine; Met, 5 mM methionine; Cho, 3 mM choline; Bet, 3 mM betaine. The numbers given above the columns represent mean activity (nmol of product · min^{-1} · mg of total $protein^{-1}$ in the cell extract) from at least three independent experiments. S.D. are shown as bars. The repression by methionine was statistically significant ($^*P < 0.05$).

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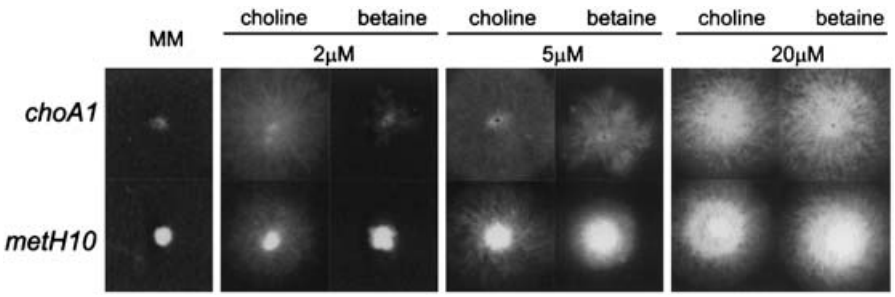

Figure 7 Growth of *metH10* and *choA1* mutants on MM supplemented with different concentrations of betaine and choline

Strains were grown for 48 h at 37 °C on MM supplemented with the indicated concentrations of betaine and choline.

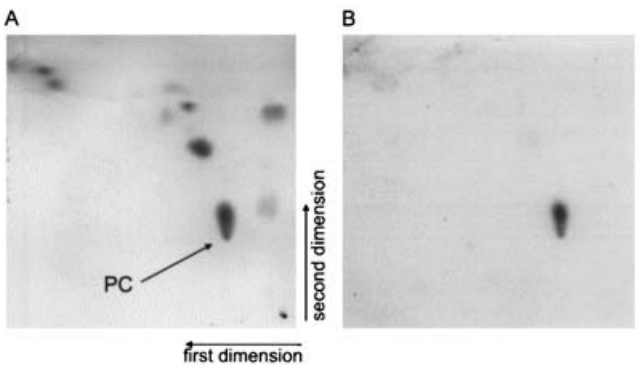

Figure 8 Choline formed from betaine is incorporated into phosphatidylcholine (PC) in *A. nidulans*

(**A**) Total lipid extract, isolated from wild-type mycelium grown in the presence of [$^{14}CH_3$]betaine, was fractionated by TLC, visualized by iodine and identified by co-chromatography with standards. (**B**) Autoradiography of (**A**).

to be a donor of a methyl group for methylation of homocysteine in a reaction catalysed by BHMT. Such an enzyme is known to be expressed in mammalian liver and kidney cortex [34] and BHMT activity has also been reported in halophylic bacteria [35,36]. We looked for this activity in *A. nidulans* mycelial extracts but the results were negative (a previous report of BHMT activity in *A. nidulans* [42] was incorrect).

Experiments using choline-supplemented media and choline-requiring mutant *choA1* (Figure 7) have shed light on the role of betaine. First, choline could substitute for betaine in stimulating the growth of *metH10*. This was not surprising since choline is known to be a precursor of betaine. An unexpected finding, however, was the growth of the *choA1* strain on betaine-supplemented medium. This suggested that betaine can be converted into choline.

To verify this possibility, the *metH10* strain was grown in the presence of [$^{14}CH_3$]betaine. The lipids were then extracted from the mycelium and separated by TLC. The lipid fraction contained approx. 40% of the total incorporated radioactivity and practically all of that was found in one radioactive spot corresponding to phosphatidylcholine (Figure 8). Identical results were obtained with betaine labelled in the glycine backbone (results not shown), indicating that betaine does not serve directly or indirectly as a methyl donor for ethanolamine methylation.

Therefore it appears that the observed effects of betaine on growth and transcription are, in fact, brought about by choline made from it or its derivative. As shown in Figures 5 and 6, supplementing the growth medium with choline leads to a decrease in both the transcript and enzyme levels.

DISCUSSION

The present study describes a novel type of regulation of the MS in *A. nidulans*: the enzyme synthesis is positively regulated by homocysteine and negatively regulated by choline, both at the transcriptional level. Both mRNA and active enzyme levels were increased by supplementation with homocysteine. Supplementing with choline resulted in repression of *metH* transcription and lower levels of MS enzyme. In contrast with MS genes from other organisms, the gene was not regulated by methionine. Also no regulation by the cysteine-mediated SMR system was found.

The regulation of MS gene expression by homocysteine, already observed in *S. typhimurium* and *E. coli* [3,4], may be also common in fungi, since we have observed it not only in *A. nidulans* but also in two yeast species *S. cerevisiae* and *Sch. pombe*. It appears that this regulation of *metH* transcription is subtle, as demonstrated by the different transcript levels in mutant strains depending on whether they accumulate the amino acid or experience a shortage of it. This precise regulation may also result from a need to remove homocysteine, which is toxic at higher concentrations.

Homocysteine is known to be toxic to *A. nidulans* [37] and *S. cerevisiae* [3,38] mutants impaired in cystathionine β-synthase and therefore unable to metabolize it to cysteine (Figure 1). This toxicity was attributed to the conversion of homocysteine to homocysteine thiolactone, which unproductively consumes considerable amounts of ATP [38]. This conversion is performed by methionyl-tRNA synthase, which misactivates homocysteine and then edits it by cyclization of homocysteine adenylate [38]. In addition, homocysteine thiolactone, being a chemically reactive compound, forms adducts with the ε-amino group of protein lysine residues, increasing the detrimental effect of homocysteine on cell growth [39].

It is possible that, for the same reasons, the addition of homocysteine is toxic for the *metH2* and *metH10* strains. Neither metabolize homocysteine effectively owing to decreased levels of MS activity and therefore contain relatively high homocysteine and low methionine levels. Lower activities of the enzyme in the mutants are caused by different mechanisms. The *metH10* strain produces low levels of wild-type enzyme, even after homocysteine supplementation, owing to a reduced amount of MS transcript. The *metH2* strain makes a normal or even increased amount of the transcript, so it is expected to produce a normal amount

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of less-active enzyme. The MS activity in the mutant grown in the presence of homocysteine is 2-fold lower when compared with that in the wild-type strain grown under the same conditions.

SAM starvation led to an increase in *metH* transcription, but no induction was observed in the enzyme level in contrast with the observation made by Burton and Metzenberg [15] in *N. crassa*. In accordance with the results obtained with *N. crassa*, repression of the enzyme activity was brought about by choline. Betaine exerted the same effect and both compounds repressed the *metH* gene transcription.

Experiments with *A. nidulans* strains grown in the presence of radiolabelled betaine showed that a large proportion of the radioactivity incorporated into the mycelium was found in phosphatidylcholine. Identical results were obtained with ^{14}C-betaine labelled on the methyl group and on the glycine backbone. The straightforward interpretation of these results is that betaine is reduced to choline. Conversion of betaine to choline had been commonly believed to be an irreversible process, but recent observations in *Penicillium fellutanum* made by Park et al. [22] strongly suggested such a possibility. Intermediates in this pathway remain to be determined. Park et al. [22] suggested that the carboxy group of betaine may be phosphorylated and then reduced to betaine aldehyde, as it is for the L-threonine biosynthetic pathway, where aspartate is phosphorylated to aspartyl-β-phosphate and subsequently reduced to aspartyl-β-semialdehyde. Another possibility is that some multi-specific dehydrogenase may perform the reduction of betaine to betaine aldehyde since such a reduction has been demonstrated *in vitro* by glycolaldehyde dehydrogenase (EC 1.2.1.21) in *E. coli* [40].

The homocysteine-dependent induction and choline (betaine)-dependent repression of MS gene transcription represents a novel type of regulation in the sulphur metabolic network in *A. nidulans* besides the SMR and methionine-mediated systems. It remains to be determined if such a complex regulation of MS genes also takes place in other organisms. The repression by choline is not surprising since MS can be regarded not only as the last enzyme in methionine synthesis but also as the first in choline synthesis. The latter process consumes a major fraction of the methyl groups of methionine. It was found that, in plants, 46% of MS activity contributes to the synthesis of phosphatidylcholine [41]. Thus, when choline is supplied, the demand for methyl groups, and therefore for methionine, is reduced. In such a situation, choline exerts feedback inhibition on *metH* transcription. This explanation is supported by the fact that only leaky *metH* mutants, including partial revertants of the *metH2* strain (results not shown), responded to betaine and choline. These compounds are ineffective when the synthesis of methionine falls below the threshold needed for protein synthesis, since they cannot replace the amino acid in this role.

It is worth noting that there is no strict correlation between transcript level and the enzyme activity. The presence of methionine in the growth medium, which does not repress the transcript level, decreases the enzyme-specific activity by approx. 20%. Moreover, the 5-fold induction of transcription by homocysteine leads only to a 3-fold induction of activity. Similarly, more than 3-fold repression of transcript level by betaine causes only a 2.5-fold decrease in the enzyme level. A mutant with impaired homocysteine synthesis (*metG55*), in which *metH* transcript level is low, shows a wild-type level of MS, whereas the increased homocysteine level in the *mecA1* strain, causing transcription induction, also increases enzyme activity. An increased activity of the enzyme was also observed in the *sconB* mutant despite the fact that the transcript level did not differ from that in the wild-type. On the other hand, the increased transcript level in the *mecC13* mutant, caused not by homocysteine but by SAM deficit, did not lead to increased MS-specific activity. These results strongly suggest that the synthesis of MS in *A. nidulans* is also regulated post-transcriptionally.

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