Genomics of alternative sulfur utilization in ascomycetous yeasts

Tomas Linder

Department of Microbiology, Swedish University of Agricultural Sciences, Box 7050, SE-750 07, Uppsala, Sweden

Thirteen ascomycetous yeast strains with sequenced genomes were assayed for their ability to grow on chemically defined medium with 16 different sulfur compounds as the only significant source of sulfur. These compounds included sulfoxides, sulfones, sulfonates, sulfamates and sulfate esters. Broad utilization of alternative sulfur sources was observed in Komagatella pastoris (syn. Pichia pastoris), Lodderomyces elongisporus, Millerozyma farinosa (syn. Pichia sorbitophila), Pachysolen tannophilus, Scheffersomyces stipitis (syn. Pichia stipitis), Spathaspora passalidarum, Yamadazyma tenuis (syn. Candida tenuis) and Yarrowia lipolytica. Klyuyveromyces lactis, Saccharomyces cerevisiae and Zygosaccharomyces rouxii were mainly able to utilize sulfonates and sulfate esters, while Lachancea thermotolerans and Schizosaccharomyces pombe were limited to aromatic sulfate esters. Genome analysis identified several candidate genes with bacterial homologues that had been previously shown to be involved in the utilization of alternative sulfur sources. Analysis of candidate gene promoter sequences revealed a significant overrepresentation of DNA motifs that have been shown to regulate sulfur metabolism in Sac. cerevisiae.

INTRODUCTION

Sulfur is essential for life. Within the living cell, sulfur is predominantly found within the amino acids methionine and cysteine, the cysteine-derived redox regulator glutathione as well as the methyl donor S-adenosyl methionine (AdoMet). Sulfur is also an essential component of a number of cofactors including biotin, coenzyme A, lipoic acid and thiamine. Our current knowledge regarding how fungi assimilate sulfur from the environment is mainly limited to inorganic sulfur and amino acids. Yet the predominant form of sulfur in aerobic soils, a prime habitat for many fungi, is non-amino acid organosulfur compounds such as sulfate esters and sulfonates (Autry & Fitzgerald, 1990). Previous studies have shown that bacteria are able to utilize a wide variety of alternative sulfur sources such as sulfate esters, sulfamates, sulfonates, sulfones, sulfoxides and thioethers (reviewed by Kertesz, 2000). When starved of favoured sulfur sources such as sulfate or sulfur-containing amino acids, bacteria induce a set of enzymes and uptake systems for alternative sulfur compounds. Fungi are well known for their metabolic versatility and one would expect that they too would have the capacity to assimilate a wide range of sulfur sources. However, until now, sulfur utilization has not been investigated in a systematic fashion in fungi. The two main questions that remain to be answered are what range of sulfur compounds can be utilized by fungi and what genes are involved in this process?

The common yeast model system Saccharomyces cerevisiae has been shown to assimilate some alternative sources of sulfur including cysteate, isethionate, nitrobenzenesulfate and a number of aliphatic sulfate esters (Autry & Fitzgerald, 1990; Hogan et al., 1999; Uria-Nickelsen et al., 1993). A strain of the basidiomycetous yeast Cryptococcus humicolus isolated from wastewater sludge has been shown to metabolize DMSO in manner reminiscent of the oxidative bacterial pathway (Murakami-Nitta et al., 2003). Cryptococcus humicolus has also been shown to assimilate other organosulfur compounds, including methanesulfonate, dimethylsulfide (DMS) and diethylsulfide. Sac. cerevisiae does not appear to assimilate DMSO but rather reduces it into the more volatile DMS (Hansen, 1999). The ascomycetous yeast Candida digboiensis has been shown to desulfurise the heterocyclic organosulfur compound dibenzothiophene (DBT), although it not known whether this yeast can utilize DBT as its sole source of sulfur (Sood & Lal, 2009). Strains of the basidiomycetous yeast Rhodospirillum toruloides can utilize DBT as the sole sulfur source by directly adhering to the surface of DBT crystals in solution (Baldi et al., 2003). A number of studies have demonstrated the ability of lignin-degrading fungi to desulphurise DBT and various other heterocyclic organosulfur compounds such as diphenylsulfide.
and thiophene (Faison et al., 1991; Ichinose et al., 2002; Van Hamme et al., 2003). In some cases it has been shown that these fungi are able to use these compounds as their sole source of sulfur (Ichinose et al., 2002).

Very few genes involved in alternative sulfur source utilization in fungi have been described to date. Arylsulfatases in filamentous fungi were first described nearly a century ago (Neuberg & Kurono, 1923), and were subsequently shown to be involved in sulfur assimilation (Harada & Spencer, 1962). Only two alternative sulfur utilization genes have been described in yeast. Both of these genes are found in Saccharomyces cerevisiae, the sulfonate dioxygenase JLP1 (Hogan et al., 1999) and the alkylsulfatase BDS1 (Hall et al., 2005). Laccases and cytochrome P450 enzymes have been implicated in the desulfurization of heterocyclic organosulfur compounds (Ichinose et al., 2002; Schlenk et al., 1994; Sood & Lal, 2009), but no direct biochemical or genetic evidence has identified specific genes. Little is known about the detailed regulatory mechanisms of these genes. Generally utilization of alternative sulfur compounds appears to be repressed by the presence of sulfur-containing amino acids or sulfate (Harada & Spencer, 1962; Uria-Nickelsen et al., 1993) and induced under sulfur-limiting conditions (Boer et al., 2003; Hébert et al., 2011; Lee et al., 2010).

This study took a systematic approach to address both the substrate diversity of sulfur compound utilization among ascomycetous yeasts and the identification of potential candidate genes involved in this process. Thirteen ascomycetous yeasts with sequenced genomes were assayed for their ability to grow on chemically defined media with 16 different compounds as their main source of sulfur. Growth profiles could then be correlated with gene content in order to identify probable candidate genes for alternative sulfur source utilization.

### METHODS

**Yeast strains.** The strains Kluyveromyces lactis CBS 2359, Konagataella pastoris CBS 704 (syn. Pichia pastoris), Lachancea thermotolerans CBS 6340, Lodderomyces elongisporus CBS 2605, Millerzyma farinosa CBS 7064 (syn. Pichia sorbitophila), Pachysolen tannophilus CBS 4044, Saccharomyces cerevisiae S288c, Scheffersomyces stipitis CBS 6054 (syn. Pichia stipitis), Spasothpora passalidarum CBS 10155, Yamaadzyma tenuis CBS 615 (syn. Candida tenuis), Yarrowia lipolytica CBS 7504 and Zygosaccharomyces rouxii CBS 732 were purchased from Centraalbureau voor Schimmelcultures (Utrecht, the Netherlands). Schizosaccharomyces pombe 972h was a generous gift from Dr Pernilla Björling (Uppsala University, Sweden). Strains were maintained on rich media agar composed of 3 g yeast extract 1⁻¹, 3 g malt extract 1⁻¹, 5 g peptone 1⁻¹, 10 g glucose 1⁻¹ and 20 g agar 1⁻¹.

**Sulfur utilization growth assays.** All sulfur-containing chemicals were purchased from Sigma Aldrich. A sulfur-limited medium (SLD) with only trace amounts of sulfate was used for assay growth on individual sulfur-containing compounds (residual sulfate concentration was estimated to be less than 6 μM). SLD medium consisted of 1.2 g yeast nitrogen base 1⁻¹ without amino acids, ammonium sulfate or magnesium (Formedium), 4 g ammonium chloride 1⁻¹, 0.84 g magnesium chloride hexahydrate 1⁻¹ and 20 g glucose 1⁻¹. Prior to the sulfur utilization assay, individual yeast strains were pre-cultured in 3 ml minimal glucose medium (MMID) consisting of 6.7 g Difco yeast nitrogen base 1⁻¹ without amino acids (Becton, Dickinson and Company) and 20 g glucose 1⁻¹. Pre-cultures were washed twice in sterile deionized water before being resuspended in 2.97 ml SLD to a final OD₆₀₀ of 0.005 in a 50 ml tube. Individual sulfur-containing compounds were added as 30 μl of 10 mM stock solution, making a final concentration of 0.1 mM. A non-supplemented sample with 30 μl deionized water was used as a control. Chloramphenicol (15 μg ml⁻¹) was included to prevent bacterial contamination. Samples were incubated for 6 days at 30 °C in a rotary shaker set to 200 r.p.m. before OD₆₀₀ measurement. To rule out suspected cases of contamination by other yeasts, total culture DNA was isolated and the internal transcribed spacer (ITS) of the rDNA gene cassette was amplified by PCR using primers 5'-GAG CTG CAT TCC CAA ACA AC-3' and 3'-CAG ACT TGG TCA TTT AGA GG-3', analysed by restriction enzyme cleavage and compared with the predicted restriction pattern based on the genomic sequence of the yeast species in question. OD₆₀₀ measurements were carried out with a 1 cm pathlength using an Ultrospec 1100 pro spectrophotometer (GE Healthcare). Cultures were typically diluted 20- to 50-fold in deionized water during measurements depending on cell density.

**Sequence alignment and phylogenetic analysis.** All BLASTP searches applied an expected value cut-off of 10⁻⁷ with the low-complexity region filter enabled. BLASTP searches against the Ko. pastoris CBS 704 and Pa. tannophilus CBS 4044 genomes were carried out at the Pichia pastoris genome browser (http://www.pichiagenome.org/) and the Joint Genome Institute (JGI) Pachysolen tannophilus project homepage (http://genome.jgi-psf.org/Pacta1_2/Pacta1_2.home.html), respectively. Protein sequences were aligned in Multiple Alignment based on Fast Fourier Transform (MAFFT) (Katoh et al., 2005; http://mafft.cbrc.jp/alignment/server/index.html) and subsequently formatted using BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). Selection of sequence positions suitable for phylogenetic analysis was carried out in G Blocks (Castresana, 2000; http://molevol.cmima.csic.es/tools/gblocks_server.html). The resulting amino acid positions were then analysed in MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003). The data were fitted to a model that assumed a proportion of invariant sites and gamma-distributed substitution rates (I + Γ) combined with a mixed amino acid substitution model consisting of 10 individual models with equal prior probability. Analyses were run until the average standard deviation of split frequencies fell below 0.01. The consensus trees were visualized in FigTree v. 1.0 (http://tree.bio.ed.ac.uk/software/figtree/). Branches with posterior probabilities less than P=0.50 were collapsed.

### RESULTS

**Ascomycetous yeasts can utilize a wide spectrum of alternative sulfur sources.** Sulfur assimilation in yeasts has received much less attention than assimilation of either carbon or nitrogen and has not
been addressed in a systematic fashion. This study aimed to take advantage of the recent wealth of available yeast genomic sequence information to explore alternative sulfur source utilization and attempt to identify its genetic components. Thirteen yeast species with sequenced genomes were chosen for this study, 12 of which span a wide taxonomic range of the subphylum Saccharomycotina, sometimes also referred to as hemiascomycetes (Kurtzman, 2011). The 13th yeast species, the fission yeast \textit{Schi. pombe}, belongs to the basal subphylum Taphrinomycotina of the ascomycetes and was included for its evolutionary distance as well as its common use as a model system.

The 12 hemiascomycetous yeasts were divided into three groups based on their taxonomy. The first group was the family \textit{Saccharomycetaceae}, which consisted of the common baker’s yeast \textit{Sacc. cerevisiae} and the three related species \textit{Kl. lactis}, \textit{La. thermotolerans} and \textit{Z. rouxii}. The six yeasts \textit{Lo. elongisporus}, \textit{Mi. farinosa}, \textit{Pa. tannophilus}, \textit{Sche. stipitis}, \textit{Spat. passalidarum} and \textit{Yam. tenuis} all use an alternative nuclear genetic code, where the codon CUG/CTG is translated as serine rather than leucine (Ohama \textit{et al.}, 1993), and were grouped together in this study as the ‘CTG clade’. The two remaining hemiascomycetous yeasts, \textit{Ko. pastoris} and \textit{Yar. lipolytica}, belong to separate divergent lineages with few described species, but for convenience they were grouped together under the Saccharomycetales of uncertain placement (\textit{incertae sedis}) in this study.

A total of 16 sulfur-containing compounds were selected for this study (Fig. 1). An effort was made to include different classes of sulfur compounds in terms of both the sulfur bond to be broken (S–C, S–N or S–O) and the structure of the carbon side-chain (long/short aliphatic or aromatic). Fairly low concentrations (0.1 mM) of the sulfur compounds were used in growth assays to avoid problems with toxicity and solubility. Although regular yeast nitrogen base has a significantly higher sulfate concentration (approximately 43 mM), 0.1 mM sulfate was found to be sufficient for robust growth in all 13 yeasts. One difficulty with assaying yeast sulfur utilization is the ability of most yeasts to grow for a number of generations with little or no sulfur supplementation. Therefore, simple spot assays on solid agar medium do not always give clear-cut results (Hall \textit{et al.}, 1993).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{sulfur_compounds.png}
\caption{Sulfur compounds tested for utilization by yeast in this study.}
\end{figure}
et al., 2005). Growth assays in liquid medium allowed for more exact and reproducible measurements, as differences in optical density between a particular sample and a non-supplemented control could be subjected to direct statistical testing. Optical density was measured 6 days after inoculation and each assay was carried out at least three times.

All strains assayed in this study displayed a minor degree of growth in the non-supplemented control samples, which contained only trace amounts of sulfate (6 μM or less). Similar results have been reported in completely sulfur-free medium and are likely due to the utilization of internal sulfur stores in the form of glutathione and AdoMet as well as a transition to a low-sulfur amino acid proteome, so-called sulfur sparing (Fauchon et al., 2002). Further addition of either a carbon source (glucose) or a nitrogen source (ammonium chloride) did not affect growth of the sulfur non-supplemented controls. The Saccharomycetaceae yeasts grew predominantly on aliphatic sulfonates and sulfate esters (Fig. 2). Z. rouxii was the only member of the Saccharomycetaceae that displayed (barely) significant growth on DMSO ($P = 0.048$) and marginal growth on sulfones that did not exceed the $P = 0.05$ significance threshold. The CTG clade yeasts as well as Ko. pastoris and Yar. lipolytica all displayed robust growth on the majority of sulfur compounds, while Sch. pombe only showed marginal growth on one alternative sulfur compound, potassium 4-nitrobenzenesulfate. Growth assays were generally very reproducible, even though experimental replicates were carried out several days apart and in some cases several weeks apart. One notable exception was the growth of Kl. lactis on isethionate. This particular assay was repeated six times, and in roughly half of the cases growth was barely distinguishable from that of the non-supplemented control, while the other half would display growth slightly weaker or similar to that on sulfate. Contamination by another yeast was ruled out by restriction analysis of the ITS region as well as by microscopy. One possible explanation for this inconsistency is that Kl. lactis has the metabolic capacity in place to desulfurize isethionate, but the regulatory mechanism is not fully tuned but primed to activate the necessary gene(s). Thus during the course of a growth assay there is an underlying probability that some Kl. lactis cells will activate the required genes for isethionate catabolism.

Generally all yeasts displayed normal morphology when growing on alternative sulfur compounds with the exception of Sche. stipitis cultured on sulfolane. Sche. stipitis grew well on sulfolane but displayed a severe cell aggregation phenotype that could not be dispersed by the addition of 50 mM EDTA. Ko. pastoris and Yar. lipolytica were the only two yeasts able to utilize dibutylsulfone in this study, while Ko. pastoris, Pa. tannophilus and Yam. tenuis were the only species that showed significant growth on sulfamate. Of the yeasts tested in this study, no significant growth was detectable after 6 days with either sodium benzenesulfonate or methyl phenylsulfoxide as the principal sulfur source. However, there still remained the possibility of slower adaptive responses to alternative sulfur sources that would be observable later than 6 days after inoculation. A smaller set of assays were conducted in which growth on 0.1 mM dibutylsulfone, benzenesulfonate or methyl phenylsulfoxide was monitored in all 13 yeasts for 16 days with OD600 readings every 24 h (see Fig. S1 available with the online version of this paper). Non-supplemented SLD media as well as sulfate-supplemented (0.1 mM) media were included for comparison. After 16 days no significant growth was detectable in any of the 13 yeasts with either benzenesulfonate or methyl phenylsulfoxide as the principal sulfur source as compared with the non-supplemented control, while only Ko. pastoris and Yar. lipolytica displayed significant growth on dibutylsulfone.

Even though low concentrations of sulfur compounds were used, there still remained a possibility that some of these compounds were inhibitory to growth even at lower levels. To avoid misinterpreting these results as a failure to utilize a particular compound, two of the compounds, dibutylsulfone and methyl phenylsulfoxide, were subjected to further analysis. A subset of eight yeasts (Ko. pastoris, Lo. elongisporus, Mi. farinosa, Pa. tannophilus, Sac. cerevisiae, Sche. stipitis, Spat. passalidarum and Yar. lipolytica) were grown in mixtures of 0.1 mM of a utilisable sulfur source (either sulfate or isethionate) together with 0.1 mM of either dibutylsulfone or methyl phenylsulfoxide. Some instances of statistically significant inhibitory effects ($P < 0.05$) were observed by the addition of either dibutylsulfone or methyl phenylsulfoxide (Fig. S2). However, no instances of severe growth inhibition were observed, and so it was concluded that the lack of significant growth was due to the failure to assimilate the two sulfur sources rather than toxicity.

Bacterial genes involved in the assimilation of alternative sulfur sources are conserved in yeast

The BDS1 and JLP1 genes in Sac. cerevisiae are the only genes in yeast that have been implicated in the utilization of alternative sulfur sources (Hall et al., 2005; Hogan et al., 1999). Bacterial assimilation of alternative sulfur compounds has been studied in much greater detail and has led to the elucidation of assimilatory pathways and identification of individual enzymes (Fig. 3a, b). Organosulfur compounds containing S–C bonds proceed through a step-wise oxygen-dependent pathway whereby sulfoxides are first oxidized into sulfones and then sulfonates through the eventual cleavage of the first of the two S–C bonds. Flavin mononucleotide (FMNH2)-dependent monooxygenases (Pfam accession no. PF00296) have been shown to catalyse the oxidation of sulfones into sulfonates and may act on sulfoxides as well. The remaining S–C bond within the sulfonate group is subsequently cleaved, which releases sulfite and the remaining side-chain as an aldehyde (Fig. 3a). This desulphonation reaction can be carried out by a number of different enzymes including FMNH2-dependent monooxygenases as well as 2-oxoglutarate-dependent dioxygenases (PF02668) and Rieske iron–sulfur-domain proteins.
monooxygenases (PF00355/PF00848) (Fig. S3a, c, d). Sulfite is then reduced into sulfide before incorporation into amino acids. Sulfate esters can be hydrolysed by either arylsulfatases (PF00884) or metallo-lactamase (MBL)-like sulfatases (PF00753) to release sulfate and an alcohol (Figs 3b and S3e). Some 2-oxoglutarate-dependent dioxygenases can also catalyse this reaction an oxygen-dependent manner to release sulfate and an aldehyde (Fig. S3b). All five protein families had credible homologues in the genomes of the yeasts assayed in this study (Table 1).

Fig. 2. Growth of yeasts on selected sulfur sources. Bars represent the mean OD_{600} value after 6 days incubation in 3 ml chemically defined medium containing 0.1 mM of the indicated sulfur source. Growth assays were carried out in triplicate; error bars, SD. Significant differences between a particular sulfur source compared with the non-supplemented control were evaluated using Student's t test (*P<0.05; **P<0.01).
2-Oxoglutarate-dependent dioxygenases desulfurize both sulfate esters and sulfonates (Fig. S3a, b) in a reaction that, aside from 2-oxoglutarate, also requires oxygen and Fe(II). The two best-characterized bacterial members of this family are the *Pseudomonas putida* AtsK alkylsulfatase, which is specific for aliphatic sulfate esters, and the *Escherichia coli* TauD taurine dioxygenase (Kahnert & Kertesz, 2000), which acts mainly on taurine and to a lesser extent some longer-chain alkyl sulfonates (Eichhorn et al., 1999; van der Ploeg et al., 1996). The *Sacc. cerevisiae* JLP1 gene belongs to this protein family and is involved in the desulfonation of aliphatic sulfonates such as isethionate and MOPS (Hogan et al., 1999). Putative 2-oxoglutarate-dependent dioxygenases were present in all the yeasts in this study with the exception of *La. thermotolerans*, and were often present in several copies (Table 1, Fig. S4). Phylogenetic analysis of 2-oxoglutarate-dependent dioxygenases revealed a fairly well-supported clade specific to the four sequences in the *Saccharomycetaceae*, while the remaining sequences appeared to have undergone several duplications, forming several smaller clades (Fig. 4a).

The FMNH$_2$-dependent monooxygenases include the *E. coli* SsuD alkanesulfonate monooxygenase (Eichhorn et al., 1999), the *Ps. putida* dimethylsulfone monooxygenase SfnG (Endoh et al., 2005) and methanesulfonate monooxygenase SsuD (Endoh et al., 2003), the *Pseudomonas aeruginosa* methanesulfonate monooxygenase MsuD (Kertesz et al., 1999) and the *Rhodococcus erythropolis* DszA DBT monoxygenase (Ohshiro et al., 1999). Putative FMNH$_2$-dependent monooxygenases were found in the genomes of all yeasts in this study apart from *Sacc. cerevisiae* and *Schi.*
pombe (Table 1, Fig. S5). Phylogenetic analysis of FMNH$_2$-dependent monooxygenases in the remaining yeasts showed separation into three distinct clades (Fig. 4b), one for *Saccharomyces cerevisiae*, one for the CTG-clade yeasts and *Ko. pastoris*, and one clade specific for *Yar. lipolytica*.

Bacterial FMNH$_2$-dependent monooxygenases are often physically associated with NAD(P)H-dependent FMN reductases (PF03358), which supply the monooxygenase with reduced FMN (Fig. S3c) and tend to be co-expressed within the same operon. (Eichhorn *et al.*, 1999; Endoh *et al.*, 2003; Kertesz *et al.*, 1999). FMN reductases were present in one or more copies in all the genomes in this study (Table 1, Fig. S6). FMN reductases also participate in other cellular processes, although there is little functional data from yeast.

Rieske iron–sulfur-domain monooxygenases are used by the methylotrophic bacteria *Marinosulfonomonas methylo tropha* (Baxter *et al.*, 2002) and *Methylosulfonomonas methylovora* (de Marco *et al.*, 1999) for assimilation of methanesulfonate. The catalytic subunit MsmA consists of an N-terminal [2Fe–2S]-type centre (PF00355) and a C-terminal aromatic ring hydroxylase domain (PF00848). Homologues of MsmA were found in yeast species of the CTG clade with the exception of *Spat. passalidarum* (Fig. S7). Three additional subunits, MsMB, MsMC and MsMD, are found in the bacterial enzyme, but these lack credible homologues in fungi. As bacterial MsMA is also homologous to plant choline monooxygenase (Rathinasabapathi *et al.*, 1997), this may be the function of the yeast homologues rather than sulfur assimilation.

The arylsulfatase family was originally described in fungi (Neuberg & Kurono, 1923) and despite their name have a wide substrate spectrum. The best-characterized members among bacteria include the *Ps. aeruginosa* AtsA arylsulfatase (Beil *et al.*, 1995) and the *Sinorhizobium meliloti* BetC choline sulfatase (Osterás *et al.*, 1998). Putative arylsulfatases in yeast tended to be single-copy genes, with the exception of *Kl. lactis* and *Mi. farinosa* (Table 1). In addition, one of the *Kl. lactis* and two of *Mi. farinosa* sequences formed a distinct group, as evident from the sequence alignment (Fig. S8).

Like the arylsulfatases, MBL-like sulfatases cleave the sulfate ester bond through hydrolysis, producing sulfate and the corresponding alcohol (Fig. S3e). A small number of MBL-like sulfatases have been biochemically characterized and include the alkylsulfatases SdsA from *Pseudomonas* sp. ATCC 19151 (Davison *et al.*, 1992) and SdsA1 from *Ps. aeruginosa* PAO1 (Hagelueken *et al.*, 2006). A small number of yeasts within the *Saccharomyces cerevisiae* contain MBL-like sulfatases (Table 1), which were acquired from bacteria through a horizontal gene transfer event (Hall *et al.*, 2005). These include a single gene in *La. thermotolerans* and the BDS1 gene in *Sac. cerevisiae* (Fig. S9).

Little is known about microbial catabolism of sulfamate. A sulfamate hydrolase activity has been described in a

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**Table 1. Conservation of candidate gene families in yeast genomes**

<table>
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<tr>
<th>Species name and strain</th>
<th>2-Oxoglutarate-dependent dioxygenases (PF02668)</th>
<th>FMNH$_2$-dependent monooxygenases (PF00296)</th>
<th>NAD(P)H-dependent FMN reductase (PF03358)</th>
<th>Rieske iron–sulfur-domain monooxygenases (PF00355/PF00848)</th>
<th>Arylsulfatases (PF00884)</th>
<th>MBL-like sulfatases (PF00753)</th>
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Fig. 4. Phylogenetic analysis of (a) 2-oxoglutarate-dependent dioxygenases and (b) FMNH2-dependent monooxygenases. Nodes are shaded according to posterior probability.
Mycobacterium soil isolate (Fulton & Cooper, 2005), but the corresponding gene or genes were never cloned. S–N bonds in heparan sulfate can be cleaved by the NSulf heparan N-sulfatase from Pedobacter heparinus (Myette et al., 2009) and by human sulfamidase (Scott et al., 1995), both of which are members of the arylsulfatase family (Fig. S8). However, both Ko. pastoris and Pa. tannophilus, which are able to utilize sulfamate, also lack putative arylsulfatasas (Table 1). Therefore a candidate enzyme or enzymes responsible for sulfamate utilization in these two yeasts remain to be identified.

Putative sulfur-regulatory motifs are overrepresented in the promoter sequences of candidate genes for alternative sulfur source utilization

We currently have a good understanding of the regulatory circuits in Sacc. cerevisiae that govern assimilation of inorganic sulfur as well as sulfur-containing amino acids. The MET4 gene encodes the central transcriptional activator of sulfur metabolism, although the Met4 protein cannot bind DNA and instead relies on three DNA-binding factors for promoter recruitment, Cbf1, Met31 and Met32 (reviewed by Thomas & Surdin-Kerjan, 1997). These three DNA-binding factors in turn lack the ability to activate transcription on their own and serve as adapters for Met4 recruitment. Cbf1 is a basic helix–loop–helix factor that binds the consensus sequence CACGTGAC as a homodimer, while the two closely related zinc finger proteins Met31 and Met32 bind the consensus sequence AAACGTGGCC. Promoters of sulfur metabolism genes in Sacc. cerevisiae tend to be enriched in these two motifs (Boer et al., 2003; Lee et al., 2010). Cbf1 has been shown to act more as a general activator rather than sulfur metabolism-specific activator in Sacc. cerevisiae, and also binds within the centromere (Thomas & Surdin-Kerjan, 1997), which is not the case in the CTG clade yeast Candida albicans (Lavoie et al., 2010). Conversely, Cbf1 co-regulates the expression of ribosomal protein genes in Candida albicans but not in Sacc. cerevisiae (Lavoie et al., 2010). The Met31 and Met32 transcription factors are thought to be more specific for the regulation of sulfur metabolism genes in Sacc. cerevisiae, but have also been shown to regulate other pathways that are dependent on an adequate supply of sulfur-containing metabolites. These genes are involved in such processes as metal homeostasis, Fe–S cluster biogenesis and polynime synthesis (Petti et al., 2012).

Assimilation of alternative sulfur compounds in Sacc. cerevisiae is repressed when preferred sulfur sources such as methionine and sulfate are present (Uria-Nickelsen et al., 1993). However, when preferred sources of sulfur become limiting, Sacc. cerevisiae cells induce genes involved in the uptake and metabolism of alternative sulfur sources, which include the 2-oxoglutarate-dependent dioxygenase JLP1 and the MBL-like sulfatase BDS1 (Boer et al., 2003). It therefore seemed reasonable that Cbf1- and Met31/32-binding sites in promoter regions of candidate genes in other yeasts would provide further support for their role in sulfur source utilization.

It is not known to what degree the Met4/Cbf1/Met31/Met32 regulatory system is conserved in other yeasts. Sequence similarity searches identified credible homologues (E-value <10^-6) of all four Sacc. cerevisiae proteins in the other 12 yeasts included in this study (data not shown). The next step was to determine the degree of conservation of the corresponding DNA motifs of Cbf1 and Met31/32 by promoter analysis of conserved sulfur metabolism genes. The promoter analysis was restricted to nine of the 13 yeasts whose intergenic regions were accessible through the Entrez Gene database: Kl. lactis, Ko. pastoris (strain GS115), La. thermotolerans, Lo. elongisporus, Sacc. cerevisiae, Sch. stipitis, Sch. pombe, Yar. lipolytica and Z. rouxii. A set of six genes were chosen that were conserved across the nine yeasts and whose Sacc. cerevisiae homologues had already been shown to contain both Cbf1 and Met31/32 motifs (Lee et al., 2010). The genes selected were ATP sulfurylase (encoded by the MET3 gene in Sacc. cerevisiae), l-homoserine-O-acetyltransferase (MET2), cobalamin-independent methionine synthase (MET6), one of the two isozymes of AdoMet synthetase in Sacc. cerevisiae (SAM2), cystathionine γ-lyase (CYS3) and O-acetyl homoserine/O-acetyl serine thiolase (MET17). These six genes are all single-copy genes in the eight species, including AdoMet synthetase (Table S1). Separate analyses of the six promoter regions from each yeast species in MEME showed a barely significant (E=0.01–10) but detectable overrepresentation of both motifs in all species except Schi. pombe (Fig. S10), indicating that both motifs are conserved in subphylum Saccharomycotina but not in subphylum Taphrinomycotina. When all sequences except those of Schi. pombe were pooled into a single input set, both motifs showed a highly significant overrepresentation (Fig. 5a). It was therefore concluded that both Cbf1- and Met31/32- like motifs are conserved within the Saccharomycotina.

The next question was whether these motifs were significantly overrepresented in the promoter sequences of the different families of candidate genes. At this stage the promoter analysis was extended to include nine further yeasts with intergenic sequences accessible through the Entrez Gene database: Ashbya gossypii, Candida albicans, Candida dubliniensis, Candida tropicalis, Clavispora lusitaniae, Debaryomyces Hansenii, Eremothecium cymbalariae, Meyeromyza guillermondii and Torulaspora delbrueckii. Promoter sequences were analysed by protein family, which included 55 2-oxoglutarate-dependent dioxygenases, 27 FMNH2-dependent monoxygenases, seven Rieske iron–sulfur-domain monoxygenases and 14 arylsulfatasas.

The analysis showed that Met31/32-like motifs (but not Cbf1) were significantly overrepresented in the promoter sequences of the 2-oxoglutarate-dependent dioxygenases, the FMNH2-dependent monoxygenases and the arylsulfatasas (Fig. 5b). 2-Oxoglutarate-dependent dioxygenases
Neither the Met31/32 motif nor the Chf1 motif was found to be overrepresented in the Rieske iron–sulfur-domain monooxygenases, which suggests that these genes probably have functions unrelated to sulfur assimilation and may be involved in choline metabolism, like their plant homologues (Rathinasabapathi et al., 1997).

**DISCUSSION**

Several decades of research on the assimilation of sulfate and sulfur-containing amino acids in yeast have given us a solid understanding of the biochemistry and regulation of this process. The assimilation of alternative sulfur sources has received much less attention and overall only a handful of genes have been described in fungi, which in turn have not been characterized in any great depth. The same is not true for bacteria, where the biochemistry and genetics of alternative sulfur utilization have been relatively well characterized. This study set out to fill this gap in our knowledge by focusing on the ascomycetous yeasts, a group of fungi that not only includes two of the best-understood eukaryotic model systems, *Sacc. cerevisiae* and *Schi. pombe*, but also contains a large proportion of species with fully sequenced genomes. The first question to be addressed was what range of sulfur compounds could be utilized by ascomycetous yeasts, something that had never been investigated in a systematic fashion. This study demonstrated that ascomycetous yeasts display notable versatility in sulfur source utilization (Fig. 2), although this was not the case for either of the model organisms, *Sacc. cerevisiae* and *Schi. pombe*. This may, in part, explain why this process has not received more attention.

The established body of work in alternative sulfur utilization in bacteria made it possible to identify several classes of candidate genes based on their homology to bacterial genes (Table 1, Figs S4–S9). Generally, the diversity of genes seemed to correlate very well with the versatility of sulfur source utilization. However, in some cases gene content was less predictive of the ability to utilize various sulfur sources. For example, the *La. thermotolerans* genome contains three candidate genes, one FMNH2-dependent monooxygenase, one arylsulfatase and one MBL-type sulfatase. Yet the only alternative sulfur source on which *La. thermotolerans* cells showed significant growth was 4-nitrobenzenesulfate. At the other extreme, *Ko. pastoris* was able to utilize a wide variety of sulfur compounds, despite the fact that the *Ko. pastoris* CBS 704 genome contains only two 2-oxoglutarate-dependent dioxygenases and a single FMNH2-dependent monooxygenase (Table 1). This result could be explained either by a particularly broad substrate range of the three *Ko. pastoris* genes or by the more likely possibility of as-yet-uncharacterized genes being involved in this process. Other possible candidate genes include cytochrome P450 oxidases, which have been implicated in the desulfurization of heterocyclic organosulfur compounds (Sood & Lal, 2009; Van Hamme et al., 2003). However, although CTG clade yeasts have several putative cytochrome P450 oxidases similar to Yar.
lipolytica n-alkane monooxygenases, the Ko. pastoris genome contains only three P450-family genes, which are close homologues of the Sac. cerevisiae ergosterol biosynthetic genes ERG3 and ERG11, and are thus likely to have the same function.

Generally, members of the Saccharomycetaceae grew poorly on alternative sulfur sources (Fig. 2), which in part could be explained by the lower diversity of candidate genes for desulfurization (Table 1). Two other factors that are likely to influence the ability to utilize alternative sulfur sources are dedicated transporters for efficient import of the sulfur compounds into the cell and efficient regulation of the genes that encode both transporters and the desulfurizing enzymes. Specialized transporters have been described in bacteria (Endoh et al., 2003; van der Ploeg et al., 1996), but remain to be identified in fungi. Analysis of hemiascomycete promoter sequences demonstrated the broad conservation of the Cbf1 and Met31/32 promoter elements previously identified in Sac. cerevisiae sulfur metabolism (Figs 5a and S10). Although both elements are conserved within the subphylum Saccharomycotina, only the Met31/32 motif appears to be associated with the utilization of alternative sulfur sources (Figs 5b and S1–S13). How the frequency and arrangement of this motif will affect the activation of the genes encoding putative desulfurizing enzymes and how that in turn will affect the overall ability of a particular yeast to utilize alternative sulfur sources remain to be established. It has been suggested that other, as-yet-unidentified, promoter elements may also play a role in the regulation of these genes (Lee et al., 2010).

As sulfonates, sulfur esters and other non-amino acid organosulfur compounds are the predominant forms of sulfur in aerobic soils (Autry & Fitzgerald, 1990), one would expect that the ability to assimilate these compounds would be vital to soil-living bacteria and fungi. In an experiment involving the soil-living bacterium Ps. putida, it was shown that a mutant without the ability to utilize these compounds did in fact display lower survival in soil (Mirleau et al., 2005). Another aspect of sulfur distribution in soil is the interaction between plants and micro-organisms in the rhizosphere. Plants are unable to assimilate alternative sulfur compounds and appear to rely on micro-organisms in soil to convert these compounds into inorganic sulfur that the plants can then assimilate (Kertesz & Mirleau, 2004). Despite the importance of mycorrhizal fungi for plant growth, very little is known about the extent to which these fungi make alternative forms of sulfur available to the host plant. Sulfur deficiency in crops is a growing problem in agriculture, and it is estimated that as much as 345 Tg of sulfur was used for the manufacture of fertilizer in 2008 alone (Till, 2010). A better understanding of how soil fungi assimilate alternative sources of sulfur and make it available to plants may lead to novel and more efficient ways of managing crop sulfur supply.

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