**CMO1** encodes a putative choline monooxygenase and is required for the utilization of choline as the sole nitrogen source in the yeast *Scheffersomyces stipitis* (syn. *Pichia stipitis*)

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Sixteen yeasts with sequenced genomes belonging to the ascomycete subphyla Saccharomycotina and Taphrinomycotina were assayed for their ability to utilize a variety of primary, secondary, tertiary and quartenary aliphatic amines as nitrogen sources. The results support a previously proposed pathway of quaternary amine catabolism whereby glycine betaine is first converted into choline, which is then cleaved to release trimethylamine, followed by stepwise demethylation of trimethylamine to release free ammonia. There were only a few instances of utilization of *N*-methylated glycine species (sarcosine and *N*,*N*-dimethylglycine), which suggests that this pathway is not intact in any of the species tested. The ability to utilize choline as a sole nitrogen source correlated strongly with the presence of a putative Rieske non-haem iron protein homologous to bacterial ring-hydroxylating oxygenases and plant choline monooxygenases. Deletion of the gene encoding the Rieske non-haem iron protein in the yeast *Scheffersomyces stipitis* abolished its ability to utilize choline as the sole nitrogen source, but did not affect its ability to use methyamine, dimethylamine, ethylamine, diethylamine, ethanolamine or glycine as nitrogen sources. The gene was named *CMO1* for putative choline monooxygenase 1. A bioinformatic survey of eukaryotic genomes showed that *CMO1* homologues are found throughout the eukaryotic domain.

**INTRODUCTION**

The vast majority of studies on the underlying biochemistry and genetics of yeast nitrogen assimilation has been confined to the common baker’s yeast *Saccharomyces cerevisiae*. Consequently, the catabolic pathways of nitrogen sources not assimilated by *Sacch. cerevisiae* are still poorly understood (Large, 1986). This is exemplified by aliphatic amines (or alkylamines), which can be used as nitrogen sources by most yeasts within the subphylum Saccharomycotina but not by *Sacch. cerevisiae* (van der Walt, 1962; Brady, 1965; LaRue & Spencer, 1968; van Dijken & Bos, 1981; Green *et al.*, 1982). Aliphatic amines are ubiquitous in nature and play a wide variety of functions in living cells. Ethanolamine and choline are both important components of phospholipids. Zwitterionic alkylamines, such as trimethylamine N-oxide, glycine betaine, choline o-phosphate and choline o-sulfate, often act as compatible solutes to protect cells against environmental stress.

In yeast cells, primary (mono-alkylated) amines are metabolized by peroxisomal copper-containing amine oxidases (EC 1.4.3.6) to release ammonia, hydrogen peroxide and the corresponding alkylaldehyde:

\[
\text{RCH}_2\text{NH}_2 + O_2 + H_2O \rightarrow \text{NH}_3 + \text{RCHO} + H_2O_2
\]

Amine oxidases are so far the only enzymes in the alkylamine assimilation pathway for which the corresponding genes have been identified. Although there are currently over 60 sequenced yeast genomes, the current understanding on how yeasts catabolize multi-alkylated amines remains fragmentary. The catabolism of secondary (di-alkylated) and tertiary (tri-alkylated) amines involves two distinct cytochrome P450-like monooxygenase activities (Green & Large, 1983, 1984; Fattakhova *et al.*, 1991):

\[
(RCH_2)_2\text{N} + \text{NAD(P)}H + H^+ + O_2 \rightarrow (RCH_2)_2\text{NH} + \text{NAD(P)}^+ + \text{RCHO} + H_2O
\]
(RCH₂)₄ NH + NAD(P)H + H⁺ + O₂ →
RCH₂NH₂ + NAD(P)⁺ + RCHO + H₂O

Choline, which is a quaternary (tetra-alkylated) amine, is thought to be assimilated through four sequential de-alkylation reactions to release free ammonia (Zwart et al., 1983). Candida tropicalis grown on choline as the sole nitrogen source was shown to convert choline into trimethylamine and ethylene glycol (Mori et al., 1988), which suggests that the first de-alkylation step removes the ethanol group. Trimethylamine can then rapidly be demethylated via dimethylamine and methylamine to free ammonia (Zwart et al., 1983).

This study set out to revisit the utilization of aliphatic amines as nitrogen sources by ascomycetous yeasts in the post-genomic era. Fourteen budding yeasts (subphylum Saccharomycotina) and two fission yeasts (subphylum Taphrinomycotina), all with sequenced genomes, were assayed for the ability to use twelve aliphatic amines as nitrogen sources. The strategy was to identify likely candidate genes for the utilization of multi-alkylated amines by correlating amine-utilization profiles with gene content.

METHODS

Yeast strains. The strains Cyberlindnera jadinii CBS 5609 (syn. Candida utilis), Kluyveromyces lactis CBS 2359, Komagataella pastoris CBS 704 (syn. Pichia pastoris), Lipomyces starkeyi CBS 1807, Lodderomyces elongisporus CBS 2605, Ogaetaea arabinofermentans CBS 8468 (syn. Candida arabinofermentans), Pachysolen tannophilus CBS 4044, Sacch. cerevisiae S288C, S. bayanus D4 (syn. S. paradoxus), Scheffersomyces stipitis CBS 6054 (syn. Pichia stipitis), Spalthaspora passalidarum CBS 10155, Tortispora caseinolytica CBS 7881 (syn. Candida caseinolytica), Yarrowia lipolytica 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 Bjerling (Uppsala University, Sweden).

Nitrogen-utilization assays. Hydrochloride salts of methylvamine, dimethylamine, trimethylamine, ethylvamine, diethylamine, triethylamine, glycine, sarcosine, N,N-dimethylglycine, glycine betaine and choline were purchased from Sigma Aldrich. A nitrogen-limited glucose medium (NLD), which contains only trace amounts (approx. 16 µM) of nitrogen in the form of vitamins, was used for assaying growth on individual amines. NLD medium is composed of 1.7 g Difco yeast nitrogen base without amino acids or ammonium sulfate 1 l⁻¹ (Becton Dickinson), 5.37 g sodium sulfate 1 l⁻¹ and 20 g glucose 1 l⁻¹. Prior to the nitrogen-utilization assay, individual yeast strains were pre-cultured in 3 ml minimal glucose medium (MMD) consisting of 6.7 g glucose medium (NLD), which contains only trace amounts (approx. 0.05 µM) of nitrogen in the form of vitamins, was used for assaying growth on individual amines. NLD medium is composed of 1.7 g Difco yeast nitrogen base without amino acids or ammonium sulfate 1 l⁻¹ (Becton Dickinson), 5.37 g sodium sulfate 1 l⁻¹ and 20 g glucose 1 l⁻¹. Prior to the nitrogen-utilization assay, individual yeast strains were pre-cultured in 3 ml minimal glucose medium (MMD) consisting of 6.7 g Difco yeast nitrogen base without amino acids or ammonium sulfate 1 l⁻¹ (Becton Dickinson), 5.37 g sodium sulfate 1 l⁻¹ and 20 g glucose 1 l⁻¹. Prior to the nitrogen-utilization assay, individual yeast strains were pre-cultured in 3 ml minimal glucose medium (MMD) consisting of 6.7 g Difco yeast nitrogen base without amino acids or ammonium sulfate 1 l⁻¹ (Becton Dickinson), 5.37 g sodium sulfate 1 l⁻¹ and 20 g glucose 1 l⁻¹. Pre-cultures were washed twice in sterile deionized water before being resuspended in 2.97 ml NLD to a final OD₆₀₀ of 0.005 in a 50 ml tube. Individual nitrogen sources were added as 30 µl of a 1 M stock solution, making a final concentration of 10 mM. A non-supplemented sample with 30 µl deionized water was used as a control. Chloramphenicol (final concentration 15 mg l⁻¹) was included to prevent bacterial contamination. Samples were incubated at 30 °C in a rotary shaker set to 200 r.p.m. with OD₆₀₀ measurements after 6, 12 and 18 days. OD₆₀₀ measurements were carried out with a 1 cm path length using an Ultrospec 1100 pro spectrophotometer (GE Healthcare).

Preparation of transformation constructs. The PCR primers used in this study are listed in Table 1. The wild-type Schef. stipitis HIS3 gene was amplified from Schef. stipitis CBS 6054 genomic DNA with primers ShS3 fwd and ShS3 rev. Prior to transformation the Schef. stipitis HIS3 PCR product was purified into sterile water using a QIAquick PCR purification kit (Qiagen). An integration vector for transformation of a Schef. stipitis HIS3 gene was created by first amplifying the full-length HIS3 gene from Debaryomyces hansenii CBS 767 genomic DNA with primers DhHIS3 fwd and DhHIS3 rev, and then inserting the BamHI/PstI-cut DkHIS3 PCR product into BamHI/PstI-cut pUC19 to produce the plasmid pUC19-DkHIS3. The genomic regions flanking the Schef. stipitis CMO1 gene were then amplified and inserted into the pUC19-DkHIS3 vector to enable targeted gene replacement. The Schef. cmo1 upstream region was amplified from Schef. stipitis CBS 6054 genomic DNA with primers ShCMO1 5' rev and ShCMO1 3' rev, and then inserted into

<table>
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<th>Table 1. Primers used in this study</th>
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<tr>
<td><strong>Primer name</strong></td>
</tr>
<tr>
<td>ShS3 fwd</td>
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<tr>
<td>ShS3 rev</td>
</tr>
<tr>
<td>DhHIS3 fwd</td>
</tr>
<tr>
<td>DhHIS3 rev</td>
</tr>
<tr>
<td>ShCMO1 5' fwd</td>
</tr>
<tr>
<td>ShCMO1 5' rev</td>
</tr>
<tr>
<td>ShCMO1 3' fwd</td>
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<tr>
<td>ShCMO1 3' rev</td>
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<tr>
<td>ShCMO1 control fwd</td>
</tr>
<tr>
<td>ShCMO1 control rev</td>
</tr>
<tr>
<td>pUC control fwd</td>
</tr>
<tr>
<td>YtCMO1 656 5' fwd</td>
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<tr>
<td>YtCMO1 1007 5' fwd</td>
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<tr>
<td>YtCMO1 311 3' rev</td>
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<tr>
<td>YtCMO1_498 3' rev</td>
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BamHI/Smal-cut pUC19-DhHIS3 to produce the intermediate plasmid pUC19-DhHIS3-CMO1 5′. The SsCMO1 downstream region was amplified from Schef. stipitis CBS 6054 genomic DNA with primers SsCMO1 3′ fwd and SsCMO1 3′ rev, digested with Smal and Swal, and inserted into Smal/Swal-cut pUC19-DhHIS3-CMO1 5′ to produce the plasmid pUC19-DhHIS3-Δcmo1 (Fig. 1a). In order to substitute the endogenous Schef. stipitis CMO1 gene with the homologous gene in Yam. tenuis, the Yam. tenuis CMO1 gene was amplified using two primer sets producing a shorter (2332 bp) and longer (2870 bp) form. The short form consisted of the YtCMO1 coding sequence flanked by 656 bp upstream and 311 bp downstream (GenBank accession no. NW_006281246, residues 2117786–2120117) and was amplified using primers YtCMO1_656 5′ fwd and YtCMO1_311 3′ rev. The long form consisted of the YtCMO1 coding sequence flanked by 1007 bp upstream and 498 bp downstream (GenBank accession no. NW_006281246, residues 2117435–2120304) and was amplified using primers YtCMO1_1007 5′ fwd and YtCMO1_498 3′ rev. The amplification products of both the short and long forms of YtCMO1 (from now on referred to as YtCMO1656/311 and YtCMO11007/498, respectively) were digested with PstI and inserted into PstI-cut pUC19-DhHIS3-Δcmo1 to produce the plasmids pUC19-DhHIS3-Δcmo1-YtCMO1656/311 and pUC19-DhHIS3-Δcmo1-YtCMO11007/498, respectively. Restriction digest analysis was carried out to select plasmid clones where both variants of the YtCMO1 gene had been inserted in the same orientation as the upstream DhHIS3 gene. Prior to transformation, the pUC19-DhHIS3-Δcmo1, pUC19-DhHIS3-Δcmo1-YtCMO1656/311 and pUC19-DhHIS3-Δcmo1-YtCMO11007/498 plasmids were digested with Swal (Fig. 1b) and purified into sterile water using a QIAquick PCR purification kit (Qiagen).

Schef. stipitis transformation. The genetically modified yeast strains used in this study are listed in Table 2. The transformation methodology used in this study is based on previously described protocols (Dohmen et al., 1991; Maassen et al., 2008). The Schef.

![Diagram](http://mic.sgmjournals.org)
results & discussion

Although the assimilation of aliphatic amines by yeasts has been known for about decades (van der Walt, 1962; Brady, 1965; LaRue & Spencer, 1968; van Dijken & Bos, 1981), very little is known about the actual genes involved. Thirty years ago three possible pathways were proposed for the catabolism of choline in yeasts (Zwart et al., 1983), which also include several potential entry points for the assimilation of other aliphatic amines (Fig. 2). The first option involves the initial conversion of choline into glycine betaine via a betaine aldehyde intermediate (Fig. 2, reactions A3 and A4). Glycine betaine is then demethylated in three enzymic steps (Fig. 2, reactions B1–B3) to produce glycine, which is then subsequently deaminated to release ammonia (reaction B4). This pathway is known to exist in animal cells (Frisell & MacKenzie, 1962) and many bacteria (Wargo et al., 2008; Wargo, 2013). A glycine betaine biosynthetic pathway was recently described in Aspergillus fumigatus (Lambou et al., 2013), which suggests that in filamentous ascomycete fungi (subphylum Pezizomycotina), choline is also assimilated through a glycine betaine intermediate followed by stepwise demethylation along pathway B. In A. fumigatus the flavin-dependent choline oxidase ChoA (GenPept accession no. XP_747216, EC 1.1.3.17), catalyses the oxidation of choline to glycine betaine with betaine aldehyde as an intermediate (Lambou et al., 2013). However, the A. fumigatus choline oxidase is only moderately conserved in the budding yeasts and the closest homologues to the A. fumigatus ChoA protein were a number of already characterized alcohol oxidases (Szamecz et al., 2005) and formate oxidases (Maeda et al., 2008). Biochemical studies on extracts of amine-grown yeasts (Zwart et al., 1983; Mori et al., 1988) have suggested that utilization of choline instead proceeds through an initial cleavage of choline to release trimethylamine and ethylene glycol (Fig. 2, reaction D1) followed by demethylation of trimethylamine to produce dimethylamine (reaction D2), methylamine (reaction D3) and then finally release free ammonia (reaction D4). A third pathway was also proposed (Zwart et al., 1983) involving stepwise demethylation of choline directly to ethanolamine (reactions C1–C3), which can then be deaminated to release ammonia (reaction C4). Although this pathway has yet to be demonstrated in yeast, current data does not exclude its existence (Zwart et al., 1983; Mori et al., 1988). A proposed pathway for catabolism of mono-, di- and tri-ethylated amines has been included for completeness (Fig. 2, reactions E1–E3).

Table 2. Genetically modified yeast strains used in this study

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<tr>
<th>Strain no.</th>
<th>Relevant genotype</th>
<th>Source</th>
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<tr>
<td>SF1</td>
<td>his3-1 trp5-10 Ayku80::ScTRP5</td>
<td>U. Klinner; Maassen et al. (2008)</td>
</tr>
<tr>
<td>TLS0001</td>
<td>HIS3 trp5-10 Ayku80::ScTRP5</td>
<td>This study</td>
</tr>
<tr>
<td>TLS0002</td>
<td>his3-1 trp5-10 Ayku80::ScTRP5 Δcmo1::pUC19-DhHIS3</td>
<td>This study</td>
</tr>
<tr>
<td>TLS0003</td>
<td>his3-1 trp5-10 Ayku80::ScTRP5 Δcmo1::pUC19-DhHIS3-YCMO1&lt;sub&gt;1007/498&lt;/sub&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>TLS0004</td>
<td>his3-1 trp5-10 Ayku80::ScTRP5 Δcmo1::pUC19-DhHIS3-YCMO1&lt;sub&gt;1536/311&lt;/sub&gt;</td>
<td>This study</td>
</tr>
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</table>

stipitis SF1 strain, which lacks a functional non-homologous end-joining DNA repair pathway (Maassen et al., 2008), was kindly provided by Ulrich Klinner (Aachen University, Germany). Briefly, a SF1 pre-culture was diluted in 100 ml fresh YMPD (3 g yeast extract l<sup>-1</sup>, 3 g malt extract l<sup>-1</sup>, 5 g peptone l<sup>-1</sup>, 10 g glucose l<sup>-1</sup>, 15 mg chloramphenicol l<sup>-1</sup>) to a final OD<sub>600</sub> of 0.1 and incubated at 30 °C with shaking at 200 r.p.m. until the OD<sub>600</sub> reached 0.5–0.6. Cells were collected by centrifugation (1730 g, 3 min) and washed once in 10 ml transformation buffer 1 [10 mM Bicine-NaOH (pH 8.35), 1.5 M KCl, 30 mM CaCl<sub>2</sub>, 3 % ethylene glycol]. The washed cells were centrifuged once more and resuspended in 600 μl transformation buffer 1. DMSO (27.5 μl) was added to the cell suspension, which was then split into five 105.5 μl aliquots. An aliquot (10 μl) of the wild-type Sch. stipitis HIS3 gene PCR product (to make strain TLS0001), Swal-cut pUC19-DhHIS3-Δcmo1 (to make strain TLS0002), Swal-cut pUC19-DhHIS3-Δcmo1-YCMO1<sub>1536/311</sub> (to make strain TLS0003), Swal-cut pUC19-DhHIS3-Δcmo1-YCMO1<sub>1007/498</sub> (to make strain TLS004) or sterile water (negative control) was added to each sample. Transformation reactions were incubated at −70 °C overnight before heat-shock the following day. Samples were immediately transferred from −70 °C to a 37 °C heating block and incubated for exactly 1 min followed by vigorous agitation (1200 r.p.m.) in a shaker at room temperature for 2 min. An aliquot (1 ml) of transformation buffer 2 [200 mM Bicine-NaOH (pH 8.35), 40 % PEG10000] was added to each of the samples, which were then inverted several times and incubated at 30 °C for 30 min. Cells were collected by centrifugation (16200 g, 1 min), washed twice in 1 ml transformation buffer 3 [10 mM Bicine-NaOH (pH 8.35), 150 mM NaCl] and then resuspended in 3 ml YMPD and incubated at 30 °C for 2–3 h. The cells were then collected once more through centrifugation (1730 g, 3 min), washed twice in 1 ml transformation buffer 3 and then resuspended in 200 μl transformation buffer 3. Transformed cells were spread on MMD agar plates and incubated at 30 °C until colonies appeared (no colonies were ever observed on the control plates). Correct chromosomal integration and the removal of the CMO1 gene was confirmed by PCR of purified genomic DNA using the primers shown in Fig. 1b. The presence or absence of an intact CMO1 gene was determined using primers ScCMO1 control fwd and ScCMO1 control rev, which produce a 1043 bp amplification product in strains CBS 6054, SF1 and TLS0001, but no product in strains TLS0002, TLS0003 and TLS0004 (Fig. 1c). Successful integration of the Δcmo1 constructs was determined using primers pUC control fwd and ScCMO1 control rev, which produce a 709 bp amplification product in the strains TLS0002, TLS0003 and TLS0004, but no product in strains CBS 6054, SF1 and TLS0001 (Fig. 1d).
yeasts (phylum Ascomycota, subphylum Saccharomycotina) and two from the fission yeasts (phylum Ascomycota, subphylum Taphrinomycotina). A conscious effort was made to sample as broadly as possible across the Saccharomycotina, since most genomes currently available belong to either of the two families Debaryomycetaceae and Saccharomycetaceae. Nitrogen utilization was assayed on 12 of the aliphatic amines listed in Fig. 2 and compared to an ammonium chloride reference sample, as well as a non-supplemented control. Growth was monitored every 6 days up to 18 days after inoculation (Fig. S1, available in the online Supplementary Material). The results are summarized in Fig. 3. An absolute OD$_{600}$ value of 0.1 was set as a threshold for significant growth. The majority of yeast in this study did not exceed this limit. One notable exception was Lip. starkeyi, where the optical density of non-supplemented cultures after 18 days was $0.24 \pm 0.01$. The yeast nitrogen base formulation contains several nitrogen-containing vitamins (4-aminobenzoic acid, nicotinic acid, riboflavin, thiamine), which suggested that Lip. starkeyi was able to utilize these compounds as nitrogen sources as well.

Preliminary experiments where Lip. starkeyi was cultivated on higher concentrations of three different nitrogen-containing vitamin salts (2.5 mM thiamine hydrochloride, 10 mM sodium nicotinate and 10 mM sodium 4-aminobenzoate) resulted in weak but significant growth (data not shown).

There were a number of instances where growth on a particular nitrogen source was preceded by an extended lag phase such as Schiz. pombe on ethanolamine, Schef. stipitis and O. arabinofermentans on glycine betaine, Pac. tannophilus on triethylamine, T. caseinolytica on glycine, Yar. lipolytica on ethanolamine and glycine (Fig. S1). Yam. tenuis displayed a very long lag phase on methylvamine, with growth only detectable at the 18 day time point. Although the growth was strong, the cells displayed a severe cell-aggregation phenotype that did not allow for accurate OD$_{600}$ measurement. This result was curious as dimethylamine and trimethylamine are thought to be assimilated through a methylamine intermediate (Fig. 2), yet Yam. tenuis displayed strong growth on both dimethylamine and trimethylamine without any apparent lag phase or aggregation phenotype.

**Fig. 2.** A composite of proposed assimilation pathways for the amines used in this study. Adapted from Zwart et al. (1983).
The majority of yeasts assayed in this study were able to utilize one or more of the primary amines. This was well correlated with the presence of one or more amine oxidase-encoding genes in all genomes except that of *Sacch. cerevisiae* (Fig. S2). Apart from *Sacch. cerevisiae*, *Saito. complicata* was the only other yeast in this study that failed to grow on any of the primary amines assayed, despite the fact that its genome contains a putative amine oxidase (Fig. S2).

*Schiz. pombe* only grew on ethanolamine even though it had previously been shown that heterologous expression of one of its two amine oxidases, Cao1, permitted growth on ethylamine in *Sacch. cerevisiae* (Laliberté & Labbé, 2006).

Several yeasts in this study could not utilize glycine as a nitrogen source, which was quite unexpected. *Sacch. cerevisiae* displayed weak growth on glycine with OD$_{600}$ 0.69 ± 0.01 after 18 days, although this value is significantly lower than previous reports using other strain backgrounds (McNeil et al., 1997; Villas-Boás et al., 2005). There are currently two known enzymic activities for the deamination of glycine (reaction B4 in Fig. 2). The first involves the tetrahydrofolate (THF)-dependent conversion of glycine into ammonia and carbon dioxide by the glycine cleavage complex (EC 2.1.2.10):

\[
glycine + THF + NAD^+ \rightarrow 5,10 - \text{methylene} - \text{THF} + CO_2 + NH_3 + NADH + H^+ \]

Deletion of the T subunit of the glycine cleavage complex (encoded by the *GCV1* gene) has previously been shown to abolish the use of glycine as a nitrogen source in *Sacch. cerevisiae* (McNeil et al., 1997). The glycine cleavage complex is ubiquitous in all yeasts assayed in this study, yet only a subset could utilize glycine as a nitrogen source. The second known glycine deaminating mechanism involves a putative glycine oxidase-like activity (EC 1.4.3.19), which deaminates glycine to form glyoxylate:

\[
\text{Glycine} + \text{O}_{2} + \text{H}_{2} \text{O} \rightarrow \text{Glyoxylate} + \text{H}_{2} \text{O}_2 + \text{H}^+ \]

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glycine $\rightarrow$ \text{glyoxylate} + \text{NH}_3 + \text{H}_2\text{O}

In animal cells this reaction is catalysed by a D-amino acid oxidase (Neims & Hellerman, 1962). Although D-amino acid oxidases are commonly found in most yeast genomes, there was no subset of D-amino acid oxidases that appeared to correlate well with the ability to utilize glycine as a nitrogen source (data not shown). A metabolomic study in Sacch. cerevisiae using $^{13}$C-labelled glycine reported that glycine can be deaminated also into glyoxylate via an as-yet-unidentified glycine deaminase (Villas-Bôas et al., 2005) as the Sacch. cerevisiae genome lacks any D-amino acid oxidase-like genes. An older study of enzymic activities in cell extracts from the budding yeast Ogataea boidinii (syn. Candida boidinii) demonstrated that growth on glycine did not produce any detectable amine oxidase activity, but did produce catalase activity comparable to that of amine-grown cells (Haywood & Large, 1981), which would point towards an oxidase-like enzyme.

The two cytochrome P450-like monoxygenase activities required for the step-wise de-alkylation of tertiary and secondary amines (reactions D2/E1 and D3/E2 in Fig. 2, respectively) still remain to be identified. Currently characterized yeast cytochrome P450 enzymes include C-22 sterol desaturase (ERG5) and N-formyltyrosine oxidase (DIT2), n-alkane monoxygenases, as well as a number of uncharacterized members. However, a bioinformatic survey of yeast cytochrome P450 enzymes (Pfam accession no. PF00067) in the 16 yeasts included in this study did not reveal a distinct subgroup that was exclusive to those species capable of utilizing secondary and tertiary amines as nitrogen sources (data not shown). This does not exclude that these activities are found within these groups. Another possibility is another haem protein that would display similar characteristics as a cytochrome P450 monoxygenase, such as inhibition by carbon monoxide and cyanide (Green & Large, 1983, 1984).

The majority of strains assayed in this study could not utilize sarcosine and N,N-dimethylglycine as nitrogen sources (Fig. 3). Cyb. jadinii and Lip. starkeyi were the only yeasts to display significant growth on N,N-dimethylglycine with OD$_{600}$ 1.60 ± 0.25 and 2.65 ± 0.77 at 18 days after inoculation, respectively. The only yeast in this study to display significant growth on sarcosine was Lod. elongisporus, which reached OD$_{600}$ 7.63 ± 0.41 after 18 days following an initial lag phase (Fig. S1). As no yeast within this study displayed significant growth on both sarcosine and N,N-dimethylglycine, this further suggested that pathway B (Fig. 2) is absent in the Saccharomycotina and perhaps also in Taphrinomycotina. Although several yeasts possess enzymes homologous to mammalian sarcosine dehydrogenase (EC 1.5.8.3), previous studies have suggested that these enzymes instead act as fructosyl amino acid oxidases (EC 1.5.3), also known as amadoriases (Lin & Zheng, 2010).

A potential inhibitory effect of sarcosine was observed in some yeast, whereby growth on sarcosine was lower than the non-supplemented control (Fig. S1). This was most obvious in Lip. starkeyi cultures supplemented with 10 mM sarcosine, where growth was barely detectable with the OD$_{600}$ value for sarcosine only reaching 0.01 ± 0.00 after 18 days compared to 0.24 ± 0.01 for the non-supplemented control. This inhibitory effect of sarcosine on growth was not characterized further in this study. However, preliminary experiments demonstrated an extended lag phase in yeast cultures grown on mixtures of 10 mM sarcosine with an equimolar amount of a readily utilizable nitrogen source, such as ammonium chloride or glutamic acid (data not shown). Sarcosine has previously been shown to use the same amino acid import system as conventional amino acids (Magaña-Schwencke et al., 1973; Dabrowa & Howard, 1981), but the delay in growth on ammonium chloride (which utilizes a different uptake system) would also suggest a regulatory mechanism of inhibition. Experiments are currently under way to further characterize this observation.

In summary, the amine-utilization profiles (Figs 3 and S1) were largely consistent with pathway D, but not pathway B, in Fig. 2. However, pathway C can still not be excluded as an alternative, as all yeasts capable of utilizing glycine betaine and choline as a nitrogen source were also capable of using ethanolamine. In addition, it has been shown that the budding yeast Can. tropicalis is able to use both N-methyllethanolamine and N,N-dimethylethanolamine as the sole nitrogen source (Mori et al., 1988). There were a few notable cases of deviations from the model in Fig. 3, where yeasts were able to utilize one particular amine but unable to utilize one or more of the proposed downstream intermediates. Both Kl. lactis and Kom. pastoris grew weakly on trimethylamine (0.19 ± 0.04 and 0.25 ± 0.01 after 18 days, respectively) even though neither yeast was capable of utilizing dimethylamine. Likewise Schiz. pombe displayed significant growth on diethyamine (OD$_{600}$ 0.13 ± 0.02 after 18 days) even though there was no detectable growth on ethylamine. In addition to Schiz. pombe, both Kl. lactis and Z. rouxii displayed weak but significant growth on diethyamine (OD$_{600}$ 0.43 ± 0.02 and 0.20 ± 0.04 after 18 days, respectively), yet neither yeast was able to utilize dimethylamine although both substrates are thought to be metabolized by the same enzyme (Green & Large, 1984). There are a number of possible explanations for these observations. One possibility is that the unutilized downstream substrate is not recognized as an available nitrogen source by the cell or that it cannot be imported into the cell. Another interesting possibility is enzyme promiscuity, whereby an enzyme in an unrelated metabolic pathway is capable of catalysing one or more steps in the amine assimilatory pathway albeit at lower efficiencies. In some cases, the amine substrate may have compensated for a portion of the nitrogen requirement of the cell and thereby allow some limited growth. This may explain the weak growth observed in Kom. pastoris and Spath. passalidarum on choline (OD$_{600}$ 0.14 ± 0.02 and 0.49 ± 0.13 after 18 days, respectively).
Identification of a putative choline monooxygenase that is essential for growth with choline as the sole nitrogen source

With the amine assimilation data for 16 sequenced yeasts, it might be possible to identify novel genes involved in amine catabolism by correlating the presence of the gene with the ability of a particular species to utilize a certain amine as a nitrogen source. A previous study from this lab (Linder, 2012) identified a putative Rieske [2Fe-2S] cluster-containing monooxygenase in a subset of yeast species (Fig. S3) and it was hypothesized to be involved in choline metabolism by virtue of its homology to plant choline monooxygenases (EC 1.14.15.7). The plant enzyme catalyses the first step in the biosynthesis of the osmoprotectant glycine betaine by oxidation of choline into betaine aldehyde (Rathinasabapathi et al., 1997; reaction A3 in Fig. 2). A betaine aldehyde dehydrogenase (EC 1.2.1.8) then converts betaine aldehyde into glycine betaine (reaction A4 in Fig. 2). Further sequence analysis revealed that the putative yeast monooxygenases were also homologous to the \textit{Pseudomonas aeruginosa} GbcA protein (GenPept accession no. YP_793885, Fig. S3), which catalyses the demethylation of glycine betaine into N,N-dimethylglycine (Wargo et al., 2008; Wargo, 2013; reaction B1 in Fig. 2). Both plant choline monooxygenases and the \textit{Pse. aeruginosa} GbcA protein belong to the Rieske non-haem iron ring-hydroxylating oxidases, which consists of an N-terminal Rieske [2Fe-2S] domain (Pfam accession no. PF00355) with the consensus sequence Cys-Xxx-His-Xxx$_{15-17}$-Cys-Xxx$_2$-His (where Xxx is any amino acid) followed by a C-terminal aromatic ring hydroxylating domain (Pfam accession no. PF00848). These enzymes play a wide variety of roles in bacteria (Kweon et al., 2008), but the only characterized members of the group in eukaryotes are the plant choline monooxygenases.

The occurrence of the putative choline/glycine betaine monooxygenase-encoding gene in yeasts was compared with their corresponding assimilation profiles to determine whether there was a correlation between the presence of the choline monooxygenase-encoding gene and its ability to utilize either choline or glycine betaine as a nitrogen source. Out of the six yeasts within this study that possessed a putative monooxygenase (\textit{Cyg. jadinii}, \textit{Lod. elongisporus}, \textit{O. arabinofermentans}, \textit{Pac. tannophilus}, \textit{Schef. stipitis}, \textit{Yam. tenuis}), only \textit{Yam. tenuis} was unable to utilize glycine betaine as a nitrogen source (Fig. 3), while all six yeasts were able to utilize choline as a nitrogen source. Conversely, \textit{Lip. starkeyi} was the only yeast in this study that lacked the putative choline monooxygenase-encoding gene but was still capable of utilizing both choline and glycine betaine as nitrogen sources.

The amine-utilization profiles and gene content data strongly suggested that this putative choline monooxygenase-encoding gene (from now on referred to as \textit{CMO1}) is involved in the assimilation of choline and glycine betaine. In order to determine whether \textit{CMO1} plays a role in the assimilation of either choline or glycine betaine, the gene was deleted in \textit{Schef. stipitis}. The \textit{Schef. stipitis} SF1 strain, which lacks a functional non-homologous end-joining pathway, was used for this experiment (Maassen et al., 2008). The putative \textit{Schef. stipitis} \textit{CMO1} gene is currently annotated as two smaller genes (PICST$_{58359}$ and PICST$_{59125}$) situated closely in tandem, which are separated by a short stretch of 45 bp. However, a putative full-length \textit{CMO1} gene in the \textit{Schef. stipitis} genome is also possible as PICST$_{58359}$ and PICST$_{59125}$ and their intergenic region together form a single-exon, contiguous sequence (GenBank accession no. NC_009044, residues 77845–79200) as the 5' gene, PICST$_{58359}$, lacks a stop codon. In addition, full-length single-exon variants of the \textit{CMO1} gene could be found in the genomes of several yeasts, including \textit{Candida orthopsilosis} (Entrez GeneID no. 14540816), \textit{Can. tropicalis} (Entrez GeneID no. 8298286), \textit{Clavispora lusitaniae} (Entrez GeneID no. 8495662), \textit{Cyb. jadinii} (GenBank accession no. DG000065, residues 964768–966132), \textit{Lod. elongisporus} (Entrez GeneID no. 5231483), \textit{Meyeromyza guilliermondii} (Entrez GeneID no. 5127462) and \textit{Millerozyma farinosa} (Entrez GeneID no. 14520895), which strongly suggests that the current annotation of \textit{Schef. stipitis} \textit{CMO1} as two separate genes is a gene prediction artefact.

The full-length \textit{CMO1} gene was removed by homologous replacement with a deletion construct containing the \textit{D. hansenii} HIS3 gene (Fig. 1b). An isogenic control was generated by transforming the \textit{Schef. stipitis} SF1 strain with the wild-type \textit{HIS3} gene from \textit{Schef. stipitis} CBS 6054, thereby regenerating the native \textit{HIS3} gene. Surprisingly, neither \textit{Schef. stipitis} strain was capable of growth on glycine betaine as the sole nitrogen source, in contrast to the wild-type CBS 6054 strain (data not shown). The parent \textit{Schef. stipitis} SF1 strain was originally derived from \textit{Schef. stipitis} CBS 5773 (Hagedorn, 1990; Maassen et al., 2008), which raises the possibility that there are strain-specific differences in the ability to utilize glycine betaine as a nitrogen source in \textit{Schef. stipitis}. The \textit{Δcmo1} strain (TLSS002) and \textit{CMO1} control strain (TLSS001) displayed nearly identical growth kinetics on ammonium chloride, methylamine, dimethylamine, trimethylamine, ethylamine, diethylamine, ethanolamine and glycine (Fig. 4). However, the \textit{Δcmo1} strain displayed extremely weak growth on choline as the sole nitrogen source, with an optical density at 600 nm of only 0.17±0.04 after 18 days. This was in stark contrast to the \textit{CMO1} control strain, which reached an optical density of 10.13±0.52 after 18 days. Although the \textit{Δcmo1} strain grew extremely weakly on choline, the optical density on choline after 18 days was still detectably higher than if grown without any added nitrogen source (0.043±0.00 after 18 days). This could suggest that there is an alternative pathway for choline catabolism, but could also be due to the direct uptake of choline by \textit{Schef. stipitis} to satisfy its nitrogen requirements for the biosynthesis of phosphatidylcholine, and thereby freeing up the remaining trace amounts of available (non-choline) nitrogen in the medium for the synthesis of amino acids, nucleotides and other nitrogen-containing metabolites.
To ensure that this phenotype was not the result of a simultaneous random mutation elsewhere in the Schef. stipitis genome, the transformation was repeated three times and a total of 20 individual transformants each of the Δcmo1 genotype and the CMO1 control genotype were screened for the ability to utilize choline as a nitrogen source. All 20 Δcmo1 transformants consistently displayed extremely weak growth on choline compared to the CMO1 control strains. The absence of the intact CMO1 gene, as well as the correct integration of the deletion cassette at the CMO1 locus, was confirmed by PCR (see Methods) in all 20 Δcmo1 transformants. It was therefore concluded that the CMO1 gene is essential for the effective utilization of choline as a nitrogen source in Schef. stipitis.

Next it was investigated whether a homologous CMO1 gene from another species could substitute for the endogenous CMO1 gene in Schef. stipitis by gene replacement. The homologous gene from Yam. tenuis (YtCMO1) was selected to replace the Schef. stipitis CMO1 gene since Yam. tenuis also belongs to the Debaryomycetaceae and therefore uses the alternative genetic code whereby the codon CUG is also belongs to the Debaryomycetaceae and therefore uses the codon CUG is translated as serine rather than leucine (Kawaguchi et al., 1989), which is particular to this family. The YtCMO1 coding sequence (corresponding to the GenPept protein accession no. EGV62306), along with 5’ and 3’ flanking sequences, was inserted into the Psfl site downstream of the D. hansenii HIS3 gene within the pUC19-DhHIS3-Δcmo1 construct (Fig. 1b). Upon integration of the construct, the endogenous Schef. stipitis CMO1 gene is replaced by a DhHIS3-YtCMO1 cassette (Fig. 5a).

There is presently no information on how the CMO1 gene is regulated by cis-acting genomic elements. Therefore, two variants of the YtCMO1 gene, which contained different lengths of flanking sequence upstream and downstream of the coding sequence, were used to replace the endogenous Schef. stipitis CMO1 gene. The shorter form (term YtCMO1556/311) included 656 bp upstream and 311 bp of the YtCMO1 coding sequence, while the longer form (YtCMO14007/498) included 1007 bp upstream and 498 bp downstream. Correct integration of both Δcmo1::DhHIS3-YtCMO1 constructs and the loss of the endogenous CMO1 gene was confirmed by PCR of genomic DNA as before (Fig. 1c, d). Both CMO1-substituted strains displayed strong growth when cultured on nitrogen-limited medium supplemented with choline as the sole nitrogen source (Fig. 5b), which demonstrated that the Yam. tenuis CMO1 gene could function in Schef. stipitis and substitute for the endogenous CMO1 gene. However, the growth dynamics were slightly different from the CMO1 control strain, with both YtCMO1-containing strains having a slightly higher OD_{600} after 6 days (P<0.01) followed by a slightly lower OD_{600} after 12 days (P<0.05). After 18 days there were no statistically significant differences in OD_{600} between the CMO1 control strain and the two YtCMO1-containing strains. These minor differences at the 6 and 12 day time points may be due to differences in the regulation of the Yam. tenuis CMO1 gene compared to the Schef. stipitis gene.

Fig. 4. CMO1 is required for utilization of choline as the sole nitrogen source. Schef. stipitis strains TLSS001 (CMO1 wild-type control) and TLSS002 (Δcmo1) were cultured in 3 ml NLD medium supplemented with 10 mM of the indicated nitrogen source (initial OD_{600} 0.005). Samples were incubated in a shaker set at 30 °C, 200 r.p.m., and OD_{600} was measured after 6, 12 and 18 days. Growth assays were performed in triplicate with error bars indicating one SD. The statistical significance between the strains at the specific time points was evaluated using Student’s t-test (*, P<0.05; **, P<0.01). The P value for the choline assay has not been indicated on the corresponding graph, but was below 10^{-6} for all three time points.
investigated in this study, previous studies (Zwart et al., 1997) have shown that the enzymic activity of the Cmo1 protein was not retained an ancestral pathway that was subsequently lost in the remaining subphylum.

The fact that the CBS 5773-derived Schef. stipitis strains and Yam. tenuis all failed to grow on glycine betaine would suggest that the CMO1 gene is not directly involved in the catabolism of glycine betaine, but would be required for the catabolism of the downstream choline intermediate. This would explain the still significant correlation between the presence of CMO1 gene and the ability to utilize glycine betaine as a nitrogen source. Lip. starkeyi was the only yeast species in this study without a CMO1-homologue that was still capable of utilizing both choline and glycine betaine as nitrogen sources. As the family Lipomycetaceae forms a basal branch of the subphylum Saccharomycotina, it is possible that it either diverged before the acquisition of the CMO1 gene and independently acquired another set of enzymes for the catabolism of quaternary amines or retained an ancestral pathway that was subsequently lost in the remaining subphylum.

Although the enzymic activity of the Cmo1 protein was not investigated in this study, previous studies (Zwart et al., 1983; Mori et al., 1988), along with the amine-utilization profiles in Figs 3 and 4, suggest that the most likely placement of the yeast Cmo1 protein is the cleavage of choline into trimethylamine and glycol aldehyde (Fig. 2, reaction D1), with the glycol aldehyde subsequently converted into ethylene glycol. With such a promising candidate gene in hand, it will now be possible to directly fuse an affinity tag to the Schef. stipitis CMO1 gene, which upon purification will enable both biochemical characterization of the enzyme as well as identification of any associated factors. The plant enzyme is a homodimer or trimer and appears to lack other subunits (Rathinasabapathi et al., 1997), while bacterial Cmo1 homologues are often found in multi-protein complexes (Kweon et al., 2008). The Pse. aeruginosa Cmo1 homologue GcbA is associated with the protein GcbB (Wargo et al., 2008), which in turn is homologous to yeast old yellow enzyme-type NADPH dehydrogenases (Pfam accession no. PF00724, EC 1.6.99.1).

**Choline monooxygenase-like Rieske proteins are found throughout the eukaryotic domain**

Rieske non-haem iron ring-hydroxylating oxygenases have been shown to be involved in a great number of metabolic pathways in bacteria (Kweon et al., 2008). In eukaryotic cells, however, this is only the second reported function of this class of enzymes after plant choline monooxygenases. Rieske-type choline monooxygenases appear to be ubiquitous among the green plants (Fig. S3), including more basal lineages such as lycophytes, mosses and green algae. Notably only two currently sequenced metazoan genomes contain CMO1 homologues – three genes in the lancelet Branchiostoma floridae (phylum Chordata) and two genes in the acorn worm Sarcoglossus kowalevskii (phylum Hemichordata).

Among fungi, apart from the yeast species listed in this study, CMO1 homologues are common among the filamentous ascomycetes (subphylum Pezizomycotina), but only found sporadically outside the ascomycetes. Species belonging to the informal group Leotiomyceta within the subphylum Pezizomycotina tend to have 2 or 3 CMO1 homologues, but as many as 8 significant hits were found in the genome of Aspergillus oryzae strain RIB40 and 11 in Nectria haematococca strain mpVI 77-13-4. This would suggest that CMO1 homologues in the Pezizomycotina may have functions unrelated to choline metabolism. This is supported by the fact that the A. fumigatus choA choline oxidase-encoding gene is essential for growth on choline (Lambou et al., 2013) even though the A. fumigatus AF293 genome also contains four CMO1 homologues (GenPept accession nos XP_747446, XP_748437, XP_747685 and XP_747684).

**Concluding remarks**

This study has identified a gene that encodes a putative choline monooxygenase (Fig. S3) and was shown to be essential for the utilization of choline as the sole nitrogen source by Schef. stipitis (Fig. 4). It should be noted that several of the species that possess the CMO1 gene are known insect endosymbionts, as well as commensals and

![Diagram](https://example.com/diagram.png)
opportunistic pathogens associated with different kinds of animals. Perhaps the close association with the host organism provides these yeasts with an opportunity to utilize choline or choline derivatives (phospholipids, acetylcholine, choline o-phosphate or choline o-sulfate) originating from the host organism. It raises a question about how essential is the ability to utilize choline and glycine betaine for the colonization of the host. It also remains to be determined whether some yeasts actively synthesize glycine betaine in response to environmental stresses such as osmotic stress.

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