Siroheme biosynthesis in *Saccharomyces cerevisiae* requires the products of both the *MET1* and *MET8* genes

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Abstract Siroheme is a uroporphyrinogen III-derivative used by sulfite reductase as a prosthetic group. We investigated in *Saccharomyces cerevisiae* the possible involvement in siroheme biosynthesis of three genes, *MET1*, *MET8* and *MET20*. The *MET1* gene from *S. cerevisiae* was cloned and shown to be the same gene as *MET20*. Sequence similitudes as well as complementation studies indicate that Met1p and Met8p are both involved in siroheme biosynthesis. In addition, we show formally that *S. cerevisiae* does not need vitamin B_{12} for growth.

Key words: Siroheme biosynthesis; Yeast

1. Introduction

Sulfite and nitrite reductases are the enzymatic keys to biosynthetic assimilation of sulfur and nitrogen, respectively. Sulfite reductases and most nitrite reductases employ a siroheme (reduced porphyrin of the isobacteriochlorin class) coupled to an iron-sulfur cluster to perform the reduction of a single atomic center by a six-electron transfer. Sulfite reductase generates sulfide from sulfite for subsequent sulfur amino acid biosynthesis. Similarly, assimilatory reduction of nitrate to ammonia proceeds by the reduction of nitrate to nitrite followed by direct reduction of nitrite to ammonia by the siroheme containing nitrite reductase. Although many heme proteins react with nitrite, only siroheme proteins have been shown to react significantly with sulfite [1,2]. Since the yeast *Saccharomyces cerevisiae* lacks the enzymes necessary for nitrate reduction, siroheme is used exclusively in sulfite reductase in this organism. An *S. cerevisiae* strain defective in the biosynthesis of siroheme would thus appear as requiring an organic sulfur source for growth.

In *S. cerevisiae*, sulfite reductase is composed of two subunits in a $\alpha_2\beta_2$ structure [3,4]. The α subunit is encoded by the *MET10* gene, and the deduced polypeptide, Met10p, contains putative binding sites for NADPH and FAD [5], both known to take part in the electron transfer of sulfite reductase [6]. The *MET5* gene maps to ORF YJR137c and the deduced polypeptide shows significant homology to the *Escherichia coli* sulfite reductase β subunit. In addition, *MET5* has been shown to be transcribed into a 5.5 kb mRNA [7]. Taken together, these results indicate that the *MET5* gene encodes the yeast sulfite reductase β subunit.

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Abbreviations: aa, amino acid(s); kb, kilobasepair(s); ORF, open reading frame; PCR, polymerase chain reaction.

In *E. coli* and *Salmonella typhimurium* siroheme is derived from uroporphyrinogen III through two methylations, an oxidation and a chelation. One enzyme, siroheme synthetase, catalyses all these reactions in both bacterial species and is encoded by the *cysG* gene [8–11].

In *S. cerevisiae*, in addition to Met5p and Met10p, the products of other genes are known to be necessary for the reduction of sulfite. These genes are *MET1*, *MET8* and *MET20* [12,13]. The *MET8* gene has been isolated and sequenced, but no clue to its function was gained from homology searches in data banks [14]. The *MET1* and *MET20* genes are closely linked on chromosome XI [12,15], but no information was available on the function of these genes.

We show here that the *met1* and *met20* mutations are allelic and that the genes *MET1* and *MET8* genes are both involved in siroheme biosynthesis.

2. Materials and methods

2.1. Strains, media and microbiological methods

The *S. cerevisiae* strains used in this work were M3750 (*MATα ura3*), CC469-13 (MATα *ura3 met1*), CC370-8C (*MATα ura3 met20*), and MM8-1 (*MATα ura3 trp1 leu2 met8*). *E. coli* strain DH5α (Gibco BRL) was used for selection and propagation of plasmids. *S. cerevisiae* was grown as described by Sherman et al. [16]. Yeast was transformed according to Schiestl and Gietz [17].

2.2. Recombinant DNA methods

Plasmid DNA was prepared from *E. coli* according to Sambrook et al. [18], or using Qiagen maxiprep columns (Qiagen Inc.). DNA manipulations were performed according to manufacturers of enzymes (Boehringer Mannheim, Promega or New England Biolabs). Polymerase chain reaction (PCR) was performed using Amplitaq polymerase (Perkin Elmer) and according to the manufacturer.

The *S. cerevisiae* genomic library used for the cloning of the *MET1* gene was constructed by inserting the product of a partial *Sau*3A digest of chromosomal DNA from the wild type strain X2180-1A into the *Bam*HI site of the centromeric plasmid pYCp50 [19].

The construction of a *met1* disrupted allele followed the strategy of Rothstein [20]. The *Hin*dIII-*Xho*I fragment of the *MET1* region was inserted in plasmid pUC19. The *Kpn*I-*Kpn*I fragment of the *MET1* gene was removed and replaced by a 1.1 kb fragment bearing the *URA3* gene. The resulting plasmid was digested by *Bam*HI and *Hin*dIII and used to transform strain W303-1A to uracil prototrophy, yielding strain CD144. The disruption was verified by Southern blotting (data not shown).

2.3. Complementation of yeast with the Sa. typhimurium cysG gene

Yeast transformants were selected on synthetic complete medium without uracil. Independent uracil prototrophic colonies were suspended at equal cell density in sterile water and 20 μl of each cell suspension were applied onto solidified synthetic medium containing all necessary nutrients except uracil or uracil and methionine. The petri dishes were photographed after 48 h incubation at 30°C.

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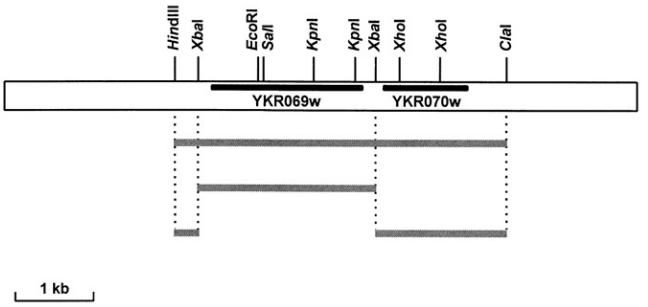

Plasmid	Vector	Complementation	
		met1	*met20*
pM1-1	YCp50	+	+
pM1-3	YCp50	+	+
pM1-JH1	pRS316	+	+
pM1-JH2	YCp50	-	-

Fig. 1. Restriction map of the *S. cerevisiae MET1-MET20* region and plasmids used for complementation of *met1* and *met20* mutants.

3. Results

3.1. Cloning of an S. cerevisiae genomic fragment complementing met1 and met20 mutations

Strain CC469-13 (*met1*) was transformed with the centromeric plasmid based genomic library described in Section 2. Among 14000 Ura$^+$ transformants tested, 5 were found to grow without methionine. Four of them were shown to harbour the same plasmid bearing an 8 kb insert whereas one contained a different plasmid. The plasmid with the 8 kb insert was shown to re-transform strain CC469-13 to methionine prototrophy. The 8 kb insert was subcloned and the sequences required to complement the *met1* mutation of strain CC469-13 (*met1*) were mapped to the *Hin*dIII-*Cla*I fragment (plasmid pM1-3, Fig. 1). Moreover, strain CC370-9C (*met20*) was transformed by plasmid pM1-3 and the resulting transformants were also methionine prototrophs, suggesting that the DNA insert of pM1-3 comprised both genes *MET1* and *MET20*.

3.2. MET1 and MET20 are the same gene

As already pointed out, siroheme is used only by sulfite

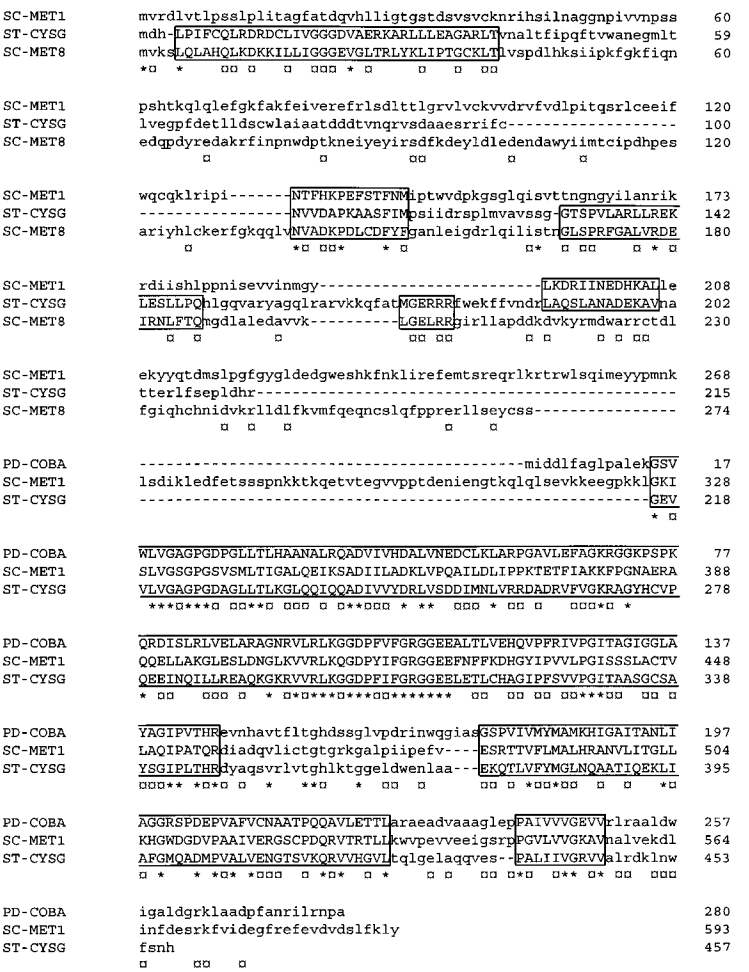

Fig. 2. Sequence homologies between the deduced peptides encoded by *Pseudomonas denitrificans cobA* (PD-COBA), *Saccharomyces cerevisiae MET1* (SC-MET1), *Salmonella typhimurium cysG* (ST-CYSG) and *S. cerevisiae MET8* (SC-MET8), obtained by the Clustal V program [26] and visualized with the Macaw program [27]. Identical amino acid residues on three sequences are indicated by ★, and similitudes between two sequences are denoted by □.

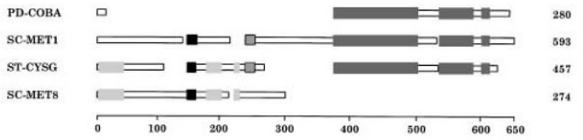

Fig. 3. Schematic representation of the similitudes between the deduced peptides encoded by *Pseudomonas denitrificans cobA* (PD-COBA), *Saccharomyces cerevisiae MET1* (SC-MET1), *Salmonella typhimurium cysG* (ST-CYSG) and *S. cerevisiae MET8* (SC-MET8),

reductase in yeast. A strain impaired in the biosynthesis of siroheme should thus be auxotrophic for methionine, and mutants in the genes *MET1* and *MET20*, mapping to chromosome XI, could thus be impaired in siroheme biosynthesis. With this in mind, we searched for sequence homologies (using the TFASTA computer program of the Genetics Computer Group (GCG) software package), between the *cysG* gene of *Sa. typhimurium* and the deduced aa sequences of open reading frames present on chromosome XI [21]. We found 37% identity in a 238 aa overlap between *cysG* and ORF YKR069w (GenBank accession no. Z28294) in the region of chromosome XI where *MET1* had been mapped [22]. The restriction map of plasmid pM1-3 is similar to that of the DNA region encompassing ORF YKR069w and ORF YKR070w (GenBank accession no. Z28295) (Fig. 1). If *MET1* and *MET20* were two different genes, they would both be contained on plasmid pM1-3. The 2.1 kb *Xba*I fragment of pM1-3, comprising only ORF YKR069w, was inserted into the *Xba*I site of the centromeric yeast shuttle vector pRS316 [23], thus creating plasmid pM1-JH1 (Fig. 1). In addition, plasmid pM1-3 deprived of this *Xba*I fragment was religated and the resulting plasmid, containing only ORF YKR070w, was denoted pM1-JH2 (Fig. 1). The yeast strains CC469-13 (*met1*) and CC370-8C (*met20*) were transformed with plasmids pM1-JH1 and pM1-JH2 and the resulting uracil prototrophic colonies were tested for growth on methionine-deficient medium. Transformants bearing plasmid pM1-JH1 could grow in the absence of methionine, whereas those bearing plasmid pM1-JH2 could not. These results show that the ORF YKR069w contains the information necessary to complement the *met1* and the *met20* mutations. In addition, the 5′-noncoding region of YKR069w contains DNA motifs believed to be implicated in the regulation of expression of the genes of the sulfate assimilation pathway [24,25]. We conclude that *met1* and *met20* are both mutant alleles of the same functional gene, and we will denote this gene *MET1*.

3.3. Structural relationships between Met1p, Met8p and CysG

Employing the Clustal V program [26], the structural relationships between the proteins encoded by the genes *MET1* and *MET8* of *S. cerevisiae* and the *Sa. typhimurium* CysG protein were outlined, and the results are visualized in Fig. 2, using the Macaw program [27]. A 231 aa region of Met1p, stretching from aa 326 to aa 556, shows significant homology (35% identity) to the C-terminal part of CysG. As already pointed out [28], these similarities are shared by the *Pseudomonas denitrificans* CobA protein (uroporphyrinogen methylase) (Fig. 2). Whereas only very little similarity was found between Met1p and the N-terminal parts of CysG, this part of CysG contains larger regions with similarities to Met8p. From the schematic overview in Fig. 3, it is evident that CobA and the C-terminal parts of Met1p and CysG share three boxes of similitudes whereas Met8p exhibits several smaller boxes of similar amino acids with the N-terminal part of CysG (Fig. 3). It has been shown that the C-terminal part of CysG contains the methylase activity but not the chelatase activity of siroheme synthetase [28]. It is thus likely that Met1p is responsible for the uroporphyrinogen III methylase activity. The N-terminal part of Met1p (the first 325 aa residues) show no apparent homology to any known protein sequence.

3.4. Complementation of S. cerevisiae met1 and met8 mutants with Sa. typhimurium cysG

To determine whether the sequence similarities between CysG, Met1p and Met8p have any functional significance, we performed heterologous complementation by the *Sa. typhimurium cysG* gene of *S. cerevisiae met1* and *met8* mutants. The *cysG* gene was amplified by PCR, using primer oligonucleotides complementary to the very ends of the open reading frame. The primers had been tailed with restriction sites so that the amplified fragment contained a *Xba*I site in front of the ATG and an *Eco*RI site after the stop codon. The fragment was inserted into a derivative of the yeast vector pRS316, pPF6, containing the yeast triosephosphate isomerase promoter (TPIp) to be used for transcription of *cysG* [29]. Erroneous nucleotides are known to be incorporated in PCR products, and therefore *cysG* amplified from two independent PCR reactions was inserted into pPF6, creating plasmids pMMC-11 and pMMC-21. *S. cerevisiae met1* and *met8* mutants were transformed with both plasmids as well as with pPF6. The methionine deficiency of the *met1* and the *met8* mutants was complemented by plasmids pMMC-11 (Fig. 4) and pMMC-21 (not shown). It thus seems that the *Sa. typhimurium* enzyme encoded by the *cysG* gene can replace both Met1p and Met8p, suggesting that these proteins are directly involved in siroheme biosynthesis in *S. cerevisiae*.

3.5. Phenotypic study of an S. cerevisiae strain disrupted in the MET1 gene

The results reported above suggest that the *MET1* gene encodes uroporphyrinogen III methylase. In vitamin B_{12}-containing organisms, the vitamin is synthesized from uroporphyrinogen III and the first reaction, catalysed by the methyl-

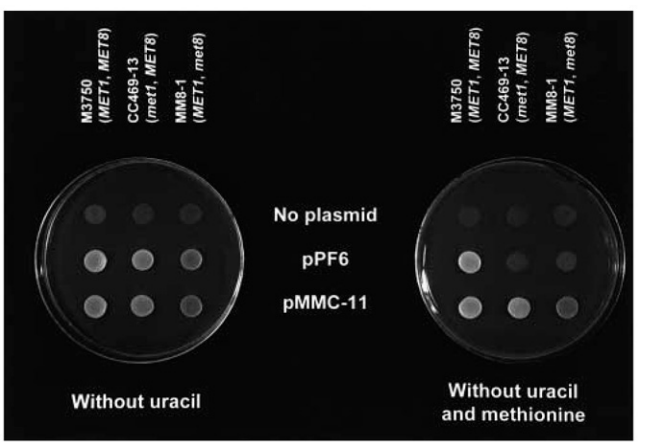

Fig. 4. Complementation of *Saccharomyces cerevisiae met1* and *met8* mutants with the *S. cerevisiae* TPIp (triosephosphate isomerase promoter)/*Salmonella typhimurium cysG* hybrid construct pMMC-11. The *MET1* and *MET8* genotypes are shown for the three *S. cerevisiae* strains used.

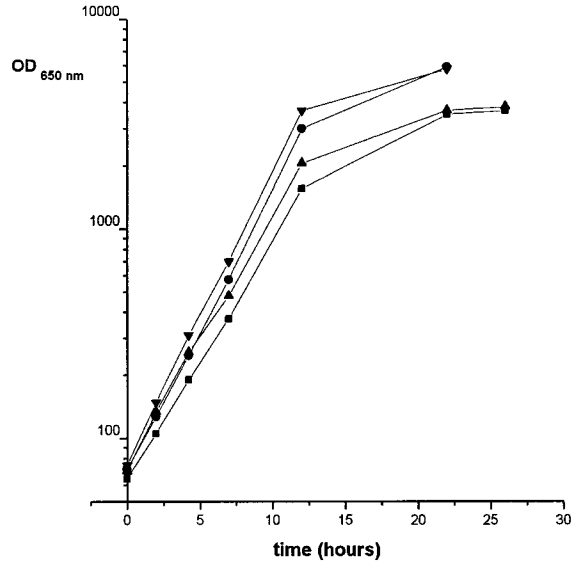

Fig. 5. Growth in B-medium [30] of a *met1* null mutant and of its parental strain. The optical density was measured in a Hitachi 2000 spectrophotometer. Strain W303-1A (parental): growth in B-medium with 0.2 mM DL-homocysteine (■) or with 0.1 mM L-methionine (●). Strain CD144 (*met1::URA3*): growth in B-medium with 0.2 mM DL-homocysteine (▲) or with 0.1 mM L-methionine (▼).

ase, is common to the biosyntheses of vitamin B_{12} and to that of siroheme. If vitamin B_{12} is needed for growth of *S. cerevisiae*, a strain bearing a *met1* null mutation would be dependent on vitamin B_{12} in the growth medium, as such a strain should not be able to synthesize the vitamin itself. Strain CD144 bearing a disruption in the *MET1* gene (see Section 2) was grown with homocysteine or methionine as a sulfur source in the sulfur-less, vitamin B_{12}-less B-medium [30]. This strain grows as well as the parental strain W303-1A (Fig. 5), showing that *S. cerevisiae* is able to grow in the absence of vitamin B_{12}.

4. Discussion

We have shown that *S. cerevisiae* mutants from both complementation groups *met1* and *met20* are complemented by a single yeast gene that we propose to call *MET1*.

As already stated, the enzymatic step catalysed by Met1p is common to the biosyntheses of siroheme and of vitamin B_{12}. If the pathway for vitamin B_{12} exists in yeast, a *met1* mutant should thus be impaired in both biosyntheses. In humans, this vitamin is required in its coenzyme form by enzymes catalysing different metabolically important reactions among which is the regeneration of methionine from homocysteine, catalysed by a vitamin B_{12}-dependent methionine synthase [31]. In yeast, since the pioneering biochemical work of Burton et al. [32], it is admitted that *S. cerevisiae* only has a B_{12}-independent methionine synthetase catalysing the last step of the biosynthesis of methionine. Flavin [33] had pointed out that this is in accord with the fact that yeast was believed to be unable to synthesize adequate amounts of vitamin B_{12} and that media used to grow *S. cerevisiae* do not contain vitamin B_{12}. Here, we used a *met1* null mutant to show formally that yeast does not need vitamin B_{12} for growth.

In *E. coli*, *cysG* encodes a multifunctional protein catalysing the three enzymatic steps that transform uroporphyrinogen III into siroheme, a specific prosthetic group of sulfite reductase. Gene dissection of *cysG* has shown that a truncated protein containing the C-terminal part of CysG (aa 202–247) is able to perform the methylation of uroporphyrinogen III as efficiently as the complete CysG protein [28]. As expected, the plasmid bearing the corresponding truncated *cysG* gene was unable to complement a *cysG* mutation [28]. The N-terminal part of CysG has been suggested to contain the oxidation and chelation activities necessary for siroheme biosynthesis [28,34], partly due to the presence of a putative $NADP^+/NAD^+$ binding motif, GxGxxAxxxAxxxxxxG [28]. Within the part of Met8p showing homology to CysG, there is a somewhat similar motif, namely GxGxxGxxxxxxxxxxG. No such motif could be found in Met1p. If Met8p was responsible for the oxidation of dihydrosirohydrochlorin and/or chelation of the resulting sirohydrochlorin to form siroheme, we would have an explanation why it is essential for sulfite reduction.

We have shown that the *cysG* gene is able to complement both *met1* and *met8* mutants. This, and the sequence similitudes found between Met1p and the C-terminal part of CysG on the one hand and between Met8p and the N-terminal part of CysG on the other, suggest that *MET1* encodes uroporphyrinogen III methylase and that Met8p carries out the oxidation and the chelation activities necessary for siroheme formation. One could thus speculate that the *MET1* and *MET8* genes have emerged from the duplication and further evolution of an ancestral cysG-like gene. However, it is noteworthy that Met1p bears a large region in its N-terminal part which does not share any similitude with any other known protein so that no putative function can be attributed to it. When Met1p is compared to CobA (Fig. 3) it appears that this N-terminal region of Met1p could be dispensable for uroporphyrinogen III methylation. Obviously, more experiments are needed before speculating on the function of this region.

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