The role of *Saccharomyces cerevisiae* Met1p and Met8p in sirohaem and cobalamin biosynthesis

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MET1 and *MET8* mutants of *Saccharomyces cerevisiae* can be complemented by *Salmonella typhimurium cysG*, indicating that the genes are involved in the transformation of uroporphyrinogen III into sirohaem. In the present study, we have demonstrated complementation of defined *cysG* mutants of *Sal. typhimurium* and *Escherichia coli*, with either *MET1* or *MET8* cloned in tandem with *Pseudomonas denitrificans cobA*. The conclusion drawn from these experiments is that *MET1* encodes the *S*-adenosyl-L-methionine uroporphyrinogen III transmethylase activity, and *MET8* encodes the dehydrogenase and chelatase activities (all three functions are encoded by *Sal. typhimurium* and *E. coli cysG*). *MET8* was further cloned into pET14b to allow expression of the protein with an N-terminal His-tag. After purification, the functions of the His-tagged Met8p were studied *in vitro* by assay with precorrin-2 in the presence of NAD^+ and Co^{2+}. The results demonstrated that Met8p acts as a dehydrogenase and chelatase in the biosynthesis of sirohaem. Moreover, despite the fact that *S. cerevisiae* does not make cobalamins *de novo*, we have shown also that *MET8* is able to complement cobalamin cobaltochelatase mutants and have revealed a subtle difference in the early stages of the anaerobic cobalamin biosynthetic pathways between *Sal. typhimurium* and *Bacillus megaterium*.

Key words: cobalt-sirohydrochlorin, cobyric acid, precorrin-2, sirohydrochlorin.

INTRODUCTION

Sirohaem, the prosthetic group required in the six-electron reduction of both sulphite and nitrite [1], is a modified tetrapyrrole that is structurally related to haem, chlorophyll and cobalamin [2]. All biologically important modified tetrapyrroles share a common biosynthetic pathway up to the synthesis of the first macrocyclic intermediate, uroporphyrinogen III. Sirohaem is biosynthesized from uroporphyrinogen III in four enzyme-mediated steps; two *S*-adenosyl-L-methionine (AdoMet)-dependent transmethylations at positions 2 and 7 (precorrin-2 synthesis), a dehydrogenation (sirohydrochlorin synthesis) and a ferrochelation [3,4] (Figure 1). In *Escherichia coli*, these reactions are undertaken by one multifunctional enzyme, sirohaem synthase (CysG), which is encoded by *cysG* [5]. This 457 amino acid protein can be described in terms of two functional domains; $CysG^A$, the C-terminal catalytic domain (amino acids 202–457), which houses the transmethylase activity, and $CysG^B$, the N-terminal catalytic domain (amino acids 1–201) which undertakes the dehydrogenation and ferrochelation reactions [6]. Unlike $CysG^A$, which displays sequence similarity with other cobalamin biosynthetic transmethylases [6], $CysG^B$ is not similar to any known chelatases or oxidases, such as those involved in cobalamin, haem or chlorophyll biosynthesis [7–10].

In *Saccharomyces cerevisiae*, two genes have been shown recently to be required in the transformation of uroporphyrinogen III into sirohaem, *MET1* and *MET8* [11]. It was found that both *MET1* and *MET8* mutants were unable to reduce sulphite but could be complemented by *Salmonella typhimurium cysG*. The protein encoded by *MET1* is predicted to

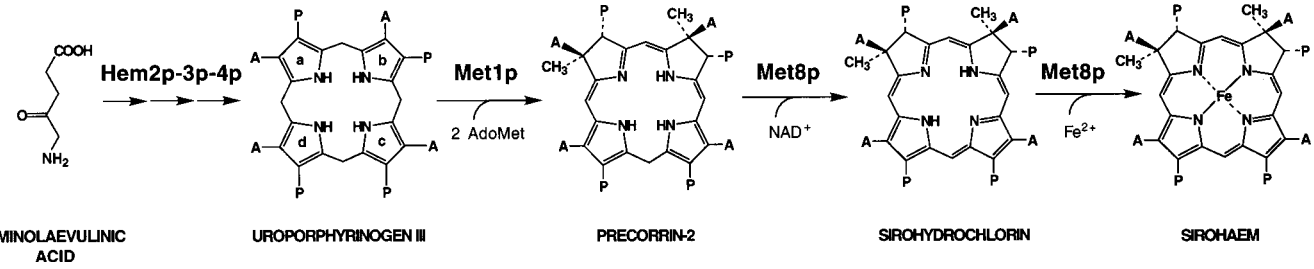

Figure 1 Biosynthesis of sirohaem from ALA

Eight molecules of ALA are required for the synthesis of uroporphyrinogen III, a transformation that requires three enzymes. Sirohaem is generated from uroporphyrinogen III by two AdoMet-dependent C-methylations at positions 2 and 7, an NAD^+-dependent dehydrogenation and ferrochelation.

Abbreviations used: AdoMet, *S*-adenosyl-L-methionine; ALA, 5-aminolaevulinic acid; CobA, uroporphyrinogen transmethylase; CysG, sirohaem synthase; PBG, porphobilinogen.

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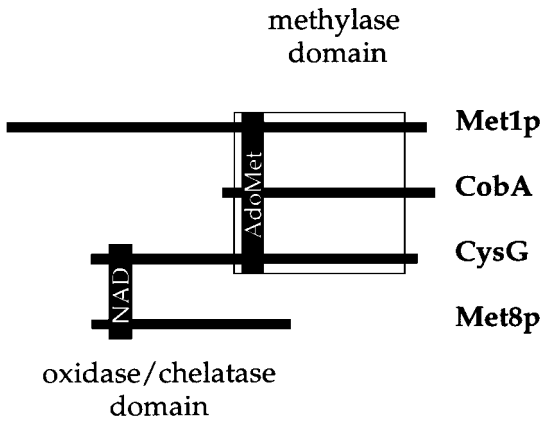

Figure 2 Diagrammatic representation of the similarity between CysG, CobA, Met8p and Met1p

The C-terminal domains of CysG and Met1p have a similar primary structure to the *P. denitrificans* CobA. This similarity is most evident in the putative AdoMet binding site. The only similarity shared between CysG and Met8p is in a 60-amino-acid N-terminus section that probably reflects the NAD^+ binding site.

contain 593 amino acids, which is considerably larger than the 457 amino acid CysG. Sequence comparisons revealed that the C-terminus of Met1p (amino acids 326–556) was similar to the uroporphyrinogen III transmethylase domain of CysG, but no significant similarity was found with the N-terminus of Met1p and any other protein on the databases (Figure 2). The protein encoded by *MET8* is much smaller than Met1p (274 amino acids). There is significant similarity between the NAD^+ binding site of CysG and Met8p, which encompasses the first 60 amino acids of Met8p (Figure 2). The remainder of the protein did not display any significant similarity with any other protein. On the basis of the complementation of *S. cerevisiae MET1* and *MET8* mutants by *cysG* and the sequence similarity, it was suggested that Met1p is responsible for the biosynthesis of precorrin-2, whereas Met8p undertakes both the dehydrogenation and ferrochelation of precorrin-2 into sirohaem. However, one of these latter functions could be undertaken equally by the N-terminus of Met1p. Furthermore, as *S. cerevisiae* does not make cobalamin *de novo*, the implication was that neither Met1p nor Met8p are involved in cobalamin biosynthesis.

In the present work, the overproduction of Met1p and Met8p as recombinant proteins in *E. coli* is reported and, by use of functional complementation and *in vitro* assays, it was concluded that Met1p can only undertake the transmethylation reaction of sirohaem biosynthesis, whereas Met8p performs both the dehydrogenation and ferrochelation reactions. Furthermore, it was shown that Met8p can complement a cobaltochelatase-deficient *E. coli* $CobI^+$ strain, indicating that Met8p, like $CysG^B$, can act as a cobaltochelatase in the anaerobic cobalamin biosynthetic pathway.

EXPERIMENTAL

Chemicals

Most chemicals, reagents, antibiotics, DEAE–Sephacel, phenyl-agarose and the genomic DNA of *S. cerevisiae* were purchased from Sigma Chemical Corporation. Tryptone, yeast extract and agar were purchased from Oxoid. His-bind resin was from Novagen and PD-10 columns were from Pharmacia Biotech. All of the restriction enzymes were purchased from Promega. Oligonucleotide primers were purchased from Genosys or Bioline, *Taq* polymerase and PCR buffers were obtained from Bioline. Porphobilinogen (PBG) was synthesized from 5-aminolaevulinic acid (ALA) using purified 5-ALA dehydratase, as described previously [12].

Bacterial strains, growth conditions and cobyric acid bioassays

All of the strains used in the present study are described in Table 1. Molecular biology protocols were performed as described previously [13]. Strains were routinely grown in Luria-Bertani broth or agar at 37 °C. As necessary, antibiotics were added (100 mg/l for ampicillin and 35 mg/l for chloramphenicol, final concentrations).

For cobalamin production or *in vivo* analysis of accumulated intermediates, strains were grown in minimal medium (0.5 g/l NaCl; 6 g/l Na_2HPO_4; 3 g/l KH_2PO_4; 1 g/l NH_4Cl; 4 g/l glucose; 2 mM $MgSO_4$; 0.1 mM $CaCl_2$) containing 0.1 g/l yeast extract, 1 mg/l $CoCl_2$,$6H_2O$, antibiotics and 50 mg/l cysteine or 50 mg/l methionine, as required. The culture media were inoculated with bacteria grown on Luria-Bertani agar plates and incubated for about 6 h at 37 °C, at which stage 10 mg/l 5-ALA and 0.4 mM isopropyl β-D-thiogalactoside were added and the culture was incubated for a further 18 h at 37 °C. A layer of paraffin oil was placed on the top of the culture to produce anaerobic conditions. The bioassays for cobyric acid synthesis were performed with the indicator strain *Sal. typhimurium* AR3612 (*metE cysG*) as described previously [14].

Plasmids

Details of bacterial strains and plasmids are given in Table 1. *MET1* was cloned after PCR from the genomic DNA of *S. cerevisiae*. The oligonucleotide primers, MET1–RBS (gcg gat cca gga gga att taa aat ggt acg aga ctt agt gac) and MET1–stop (gcg gat cct tag tat aac tta aat aga cta tc) were designed according to the sequence deposited in the database under the accession number z28294. The amplified PCR product was cloned into the vector pTAg and subsequently subcloned into pUC18 and pAR8668 (pKK223.3 modified). The plasmid harbouring *MET1* (pER246) was found to be relatively unstable in JM101 and was transformed into a strain carrying $lacI^q$ (*E. coli* 302Δa/pAR8086).

MET8 was cloned after amplification by PCR from the genomic DNA of *S. cerevisiae*. The oligonucleotide primers, MET8–RBS (gcg tcg acg gag gat aaa aaa tgg tca aatcgc tac agc tag), MET8–ATG (gcc ata tgg tca aat cgc tac agc tag ccc) and MET8–stop (gcc tgc agt caa gac gag cag tac tcg c), and MET8b–stop (gcg gat cct caa gac gag cag tac tcg c) were designed according to the sequence deposited in the database under accession number p15807 (positions 552 to 1376). The PCR product obtained with the primers MET8–RBS and MET8–stop was cloned into pTAg and subsequently subcloned into pER119 (pKK–$cobA^{Pd}$) downstream of the *Pseudomonas denitrificans cobA*. The pKK223–3 plasmid, harbouring *cobA* and *MET8*, was named pER250. A second PCR product obtained with the primers MET8–ATG and MET8b–stop was cloned into the pUAg vector and subcloned into pET14b (pER259).

The *cobA* gene of *P. denitrificans* was amplified from plasmid pCR395 using the primers PdcobA–ATG (cgc gcg cca tat gat cga cga cct ctt tg) and PdcobA–stop (cgg gat cct tat gcc ggg ttc ctg ag). The PCR product was cloned into the *Nde*I–*Bam*HI site of pET14b giving plasmid pER242.

In order to determine if *MET8* could act in cobalamin synthesis with the same function as $cysG^B$, plasmids containing *MET8* and *cobA* were transformed into recombinant *E. coli* strains

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Table 1 Strains and plasmids used in the present study

	Genotype and/or phenotype	Description	Reference
Strains			
302Δa	*E.coli cysG*; Nir^S, Lac^+,		[21]
JM101	*F′ traD36 lacI*^{q} (*lacZ*) *M15 proA*$^+$ *B*$^+$/*supE thia* (*lac-proAB*)		[13]
ER123A	302Δa (pAR8827) (pER119)	Contains all *Sal. typhimurium cbi* genes, except *cbiB*, and *P. denitrificans cobA*	[9]
ER185K^{Δ}A	302Δa (pER126K^Δ)(pER119)	Contains all *Sal. typhimurium cbi* genes, except *cbiB* and with a deletion in *cbiK*; and *P. denitrificans cobA*	[9]
ER243	BL21(DE3)(pLysS) (pER242)	Contains the *P. denitrificans cobA*	Present study
ER246	302Δa(pAR8086) (pER246)	Contains the *S. cerevisiae MET1* gene	Present study
ER251	302Δa(pAR8086) (pER250)	Contains the *P. denitrificans cobA* and *S. cerevisiae MET8*	Present study
ER252	302Δa(pER126K^Δ) (pER250)	Contains all *Sal. typhimurium cbi* genes, except *cbiB* and with a deletion in *cbiK*; *P. denitrificans cobA* and *S. cerevisiae MET8*	Present study
ER255	302Δa (pAR8827) (pER246)	Contains all *Sal. typhimurium cbi* genes, except *cbiB*, and *S. cerevisiae MET1*	Present study
ER256	302Δa(pER126K^Δ) (pER246)	Contains all *Sal. typhimurium cbi* genes, except *cbiB* and with a deletion in *cbiK*; *S. cerevisiae MET1*	Present study
ER262	BL21(DE3)(pLysS) (pER259)	Contains the *S. cerevisiae MET8* under T7 promoter	Present study
ER269	302Δa (pAR8827) (pER250)	Contains all *Sal. typhimurium cbi* genes, except *cbiB*, *P. denitrificans cobA* and *S. cerevisiae MET8*	Present study
ER270	302Δa (pER270)	Contains *B. megaterium cbiW-H*$_{60}$*-X-J-C-D-ET-L-F-G-A-cysG*^{A}*-cbiY-btuR* genes and the *Sal. typhimurium cbiP gene*	Present study
ER271	302Δa (pER270)(pER108)	Contains all *B. megaterium cbi* genes, *Sal. typhimurium cbiP* and *E. coli cysG*	Present study
ER272	302Δa (pER270) (pKKcysG^{G21D})	Contains all *B. megaterium cbi* genes, *Sal. typhimurium cbiP* and *E. coli cysG*^{G21D}	Present study
ER273	302Δa (pER270)(pER119)	Contains all *B. megaterium cbi* genes, *Sal. typhimurium cbiP* and *P. denitrificans cobA*	Present study
ER274	302Δa (pER270)(pER170)	Contains all *B. megaterium cbi* genes, *Sal. typhimurium cbi* and *cbiK*, and *P. denitrificans cobA*	Present study
ER275	302Δa (pER270)(pER250)	Contains all *B. megaterium cbi* genes, *Sal. typhimurium cbiP, P. denitrificans cobA* and *S. cerevisiae MET8*	Present study
ER277	302Δa (pAR8827) (pKKcysG^{G21D})	Contains all *Sal. typhimurium cbi* genes, except *cbiB* and *E. coli cysG*^{G21D}	Present study
ER278	302Δa (pER126K^Δ) (pKKcysG^{G21D})	Contains all *Sal. typhimurium cbi* genes, except *cbiB* and with a deletion in *cbiK*, cobA and *E. coli cysG*^{G21D}	Present study
TMV1		*MET1* gene	Present study
Plasmids			
pAR8086	*E. coli lacI*^{q}	pACYC184 derived	[14]
pAR8531	*Sal. typhimurium cbiP*	pKK223.3 derived	[14]
pAR8668		pKK223.3 modified (only one *Bam*HI site in the MCS)	Present study
pAR8827	*Sal. typhimurium cbiA-C-D-E-T-F-G-H-J-K-L-M-N-Q-O-P*	pACYC184 derived	[14] [14]
pAR8877	*B. megaterium cbiW-H*$_{60}$*-X-J-C-D-ET-L-F-G-A-cysG*^{A}*-cbiY-btuR*	pKK223.3 derived	
pCR395	*P. denitrificans cobA*		[22]
pER108	*E. coli cysG*	pKK223.3 derived	[9]
pER119	*P. denitrificans cobA*	pKK223.3 derived	[9]
pER126K^Δ	*Sal. typhimurium cbiA-C-D-E-T-F-G-H-J-K*^{Δ}*-L-M-N-Q-O-P*	pACYC184 derived, deletion in the *cbiK* gene	[9]
pER170	*P. denitrificans cobA* and *Sal. typhimurium cbiK*	pKK223.3 derived	[9]
pER242	*P. denitrificans cobA*	*Nde*I–*Bam*HI fragment from PCR of the *cobA* gene cloned into pET14b	Present study
pER246	*S. cerevisiae MET1*	*Bam*HI fragment of pTMV1 cloned into pAR8668	Present study
pER250	*P. denitrificans cobA* and *S. cerevisiae MET8*	*Sal*I–*Pst*I fragment from PCR-pTAg of the *MET8* gene cloned into pER119 in 3′ of *cobA*.	Present study
pER259	*S. cerevisiae MET8*	*Nde*I–*Bam*HI fragment from PCR-pUAg of the *MET8* gene cloned into pET14b	Present study
pER270	*B. megaterium cbiW-H*$_{60}$*-X-J-C-D-ET-L-F-G-A-cysG*^{A}*-cbiY-btuR* and the *Sal. typhimurium cbiP*	*Bam*HI–*Sal*I fragment of pAR8877 cloned into pAR8086. The P*tac*–*S.t cbiP* fragment from pAR8531 was inserted into this plasmid at the *Bam*HI site	Present study

Table 1 continued on following page.

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Table 1 (*cont.*)

	Genotype and/or phenotype	Description	Reference
pET14b		His-tag fusion protein vector with T7 promoter	R&D*
pKK223.3		Overexpression vector derived from pBR322 with *tac* promoter	Pharmacia
pKK-cysG^{G21D}	*E. coli cysG^{G21D}*	pKK223.3 derived, site-directed mutant of *cysG* (G21D)	[6]
pTAg		ddT-tailed vector for PCR fragment cloning	R&D*
pTMV1	*S. cerevisiae MET1*	*Bam*HI fragment from PCR cloned into pUC	Present study
pUag		ddU-tailed vector for PCR fragment cloning	R&D*

* R&D Systems Europe Ltd., Abingdon, Oxon OX14 3YS, U.K.

harbouring either the *Sal. typhimurium* or *Bacillus megaterium cobI* operon. The *B. megaterium* operon, *cbiW-H*$_{60}$*-X-J-C-D-ET-L-F-G-A-cysG*^{A}*-cbiY-btuR*, was initially cloned into the vector pAR8086 (pACYC184 carrying *lacI*^{q}) by restriction of plasmid pAR8877 [15] with *Bam*HI and *Sal*I. As the first corrin intermediate that can be detected by bioassay is cobyric acid, the *Sal. typhimurium cbiP* gene under the control of its own *tac* promoter was inserted into the construct in the *Bam*HI restriction site.

Enzyme purification

PBG deaminase and uroporphyrinogen III synthase (cosynthase) were purified as described previously [16,17]. Uroporphyrinogen transmethylase (CobA) and Met8p were purified on a His-bind column using the manufacturer's instructions. Metal-chelate chromatography revealed that Met8p co-purified with chloramphenicol acetyltransferase and, when necessary, the latter was removed from the Met8p preparation by passage through a phenyl-agarose column. Briefly, Met8p, in elution buffer (400 mM imidazole/0.5 M NaCl/20 mM Tris/HCl, pH 7.9), was applied to a phenyl-agarose column (3 ml) equilibrated in the same buffer. The column was washed with five column volumes of elution buffer and Met8p was eluted from the column, whereas chloramphenicol acetyltransferase was retarded on the resin. Purified Met8p was subsequently passed through a PD10 column that had been equilibrated with 50 mM Tris/HCL, pH 7.8 and was stored at -20 °C.

Complementation studies

Two CysG cysteine auxotrophic strains were used for complementation studies; a *Sal. typhimurium cysG metE* strain (AR3612) and *E. coli cysG* 302Δa. A *cysG*$^{-}$ strain is unable to produce sirohaem and is unable to synthesize cysteine. As was demonstrated recently, AR3612 has only a *cysG*^{A} deficiency because *Sal. typhimurium* contains a gene having the same phenotype as the *cysG*^{B} domain (*cbiK*) [9].

Complementation studies were performed by restreaking the strain of interest on minimal medium plates in the absence of or in the presence of cysteine. The effect of cobalt on sirohaem synthesis was also studied by inclusion of 1 mg/l $CoCl_2$ in the medium.

Analysis of accumulated intermediates *in vivo*

The bacterial cultures (100 ml) were grown in minimal medium as described previously [9]. After harvesting the bacteria by centrifugation, the pellets were resuspended in 50 mM Tris/HCl, pH 7.8, and sonicated four times for 30 s at an amplitude of 10 μm (MSE ultrasonic disintegrator). The soluble extract was applied to a DEAE–Sephacel column equilibrated in the same buffer. The column was washed with two column volumes of 0.1 M NaCl and the accumulated compounds were eluted with 50 mM Tris/HCl, pH 7.8, containing 1 M NaCl. Spectra (UV/visible) were obtained using a Hewlett Packard 352 photodiode array spectrophotometer.

In vitro assay of Met8p using a linked assay

The linked assay used for the generation of precorrin-2 consisted of 1 mg CobA, 0.1 mg PBG deaminase, 0.15 mg uroporphyrinogen III synthase, 0.75 mg AdoMet and 0.1 mg PBG. The assay components were mixed in 1 ml of degassed 50 mM Tris/HCl, pH 7.8. To this assay were added 0.1 mg Met8p, 0.5 mg NAD^{+} and $CoCl_2$,$6H_2O$ (42 μM) as required, and the mixture was incubated for a further 30 min. Spectra were recorded as described above.

RESULTS

Cloning and expression of *MET1* and *MET8*

S. cerevisiae MET1 and *MET8* were cloned after the genes had been amplified by PCR using the oligonucleotide primers described in the Experimental section. *MET1* was cloned initially into pTAg but was subsequently cloned into pUC18 and pKK223–3. The 5′ primer that had been used to amplify *MET1* also contained a consensus ribosome binding site to facilitate greater expression of Met1p as a recombinant protein within *E. coli*. Bacteria transformed with plasmids harbouring *MET1* were found to be slightly fluorescent when viewed under UV light, a characteristic observed with all uroporphyrinogen III transmethylase overproducing strains [4,18]. However, the bacteria that harboured plasmids containing *MET1* did not grow very well, having a doubling time twice as a long as comparable bacteria without *MET1*. The growth of the bacteria containing *MET1* improved when they were further transformed with a compatible plasmid harbouring *lacI*^{q}, which was presumably due to the increased regulation of expression of *MET1*, since it was cloned under the control of a *lac* regulatory system. This observation also infers that overproduction of Met1p is toxic to the bacteria for reasons which are not understood although, as high expression of other uroporphyrinogen III transmethylases does not markedly affect growth of *E. coli*, it is likely that the toxicity is mediated by the N-terminus of Met1p. Analysis of the total protein extract of bacteria transformed with plasmids containing *MET1* showed that they did not greatly overproduce the protein, as no protein band with a molecular mass of $\sim$ 65 kDa was observed when total protein extracts of the strains were analysed by SDS/PAGE.

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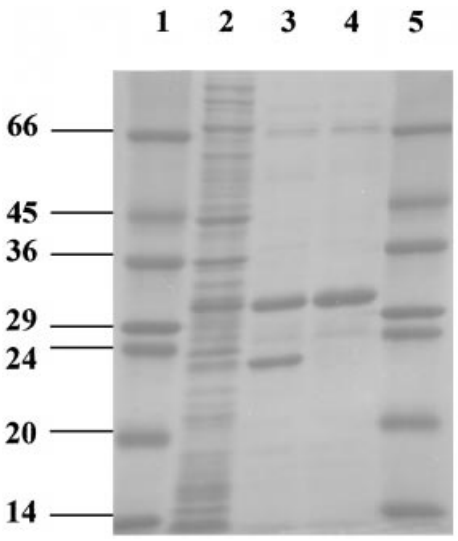

Figure 3 SDS-polyacrylamide gel of purified Met8p

Lanes 1 and 5, molecular-mass markers; lane 2, crude cell extract of ER262, which overproduces the His-tagged Met8p; lane 3, eluate from the His-bind column; lane 4, purified Met8p after passage through a phenyl-agarose column.

Table 2 Complementation of *cysG* strains

Complementation is represented by + whereas lack of complementation is represented by −. Symbols; *, complementation prevented in the presence of exogenous Co^{2+} (1 mg/l); **, complementation unaffected by the presence of exogenous Co^{2+}.

Plasmid	Host strain *Sal. typhimurium* (*metE cysG*)	Host strain *E. coli* 302Δa (*cysG* deletion)
pER246 (*MET1*)	+	−
pER242 (*cobA*)	+	−
pER170 (*cobA* + *cbiK*)	+	+(*)
pER108 (*cysG*)	+	+(**)
pER250 (*cobA* + *MET8*)	+	+(**)

MET8 was also initially cloned into pTAg and was subsequently cloned into pKK223–3 and pET14b. Bacteria transformed with plasmids harbouring *MET8* grew normally in the presence or absence of $lacI^q$ and thus Met8p did not appear to possess the same cytotoxic effect as that observed with Met1p. When *MET8* was cloned into pET14b so that the protein was expressed with an N-terminal poly-histidine tag, a protein with a molecular mass of 32 kDa was observed when cell extracts were analysed by SDS/PAGE, consistent with the expression of Met8p with an N-terminal poly-His extension. The expressed protein was purified by a combination of metal chelate and phenyl-agarose chromatography from crude cell extracts as outlined in the Experimental section. Approx. 5 mg of protein could be obtained from one litre of culture expressing the His-tagged Met8p. A polyacrylamide gel showing the purified protein can be seen in Figure 3.

Complementation of *Sal. typhimurium* and *E. coli cysG* strains

To investigate and differentiate between the roles played by Met1p and Met8p in sirohaem biosynthesis, plasmids containing the genes were used in complementation experiments with previously characterized *Sal. typhimurium* and *E. coli cysG* mutants (Table 2). The plasmid harbouring *MET1* (pER246) was found to complement the cysteine auxotrophy of *Sal. typhimurium* AR3612 (*metE*, *cysG*). Previously we had shown that this strain was, in fact, a $cysG^A$ mutant, as it was defective only in the transmethylase activity of CysG; the $CysG^B$ functions were still active [9,15]. The complementation of AR3612 with *MET1* is consistent with Met1p containing transmethylase activity and the complemented strain is thus able to synthesize precorrin-2 from uroporphyrinogen III. However, the same *MET1*-containing plasmid was unable to complement *E. coli* 302Δa, which was deficient in all CysG activities. This result demonstrates that Met1p is unable to convert precorrin-2 into sirohaem and must be deficient in the dehydrogenation or ferrochelation activities or both.

Although it would have been a natural progression to investigate the effect of *MET1* and *MET8* in tandem, the toxicity effect manifested by the presence of *MET1* made this cloning procedure technically challenging. Thus it was decided to investigate the role of *MET8* by cloning it in tandem with the *P. denitrificans cobA*, a gene which encodes solely uroporphyrinogen III transmethylase [19]. When *E. coli* 302Δa was transformed with pER250 (*cobA* + *MET8*), complementation was observed. This result reveals that Met8p is both a dehydrogenase and a ferrochelatase.

Previously, it had been shown that *E. coli* 302Δa could also be complemented with pER170, a plasmid containing both *P. denitrificans cobA* and *Sal. typhimurium cbiK*. It has been proposed that CbiK acts as a cobaltochelatase in cobalamin biosynthesis and that the ferrochelation reaction mediated by CbiK was a surrogate reaction undertaken in the absence of Co^{2+}. This explains why the complementation of *E. coli* 302Δa with pER170 (*cobA* + *cbiK*) is easily inhibited in the presence of low levels of exogenous Co^{2+} [9]. However, the complementation of 302Δa by pER250 (*cobA* + *MET8*) was not inhibited by exogenous Co^{2+} indicating that Met8p was able to complete the synthesis of sirohaem even in the presence of high concentrations of exogenous Co^{2+} (Table 2). In this respect Met8p behaves like CysG which, likewise, is able to complement *E. coli* 302Δa in the presence of a high exogenous Co^{2+} concentration.

Activity of Met8p *in vivo*

There are many similarities between the UV–visible spectra of sirohydrochlorin and sirohaem, including an absorption maximum at 378 nm, thus making it difficult to differentiate between these compounds. However, there is a very clear difference between the spectra of sirohydrochlorin and cobalt-sirohydrochlorin [9,20], as the latter has absorption maxima at 410 nm and 590 nm. When *E. coli* strains overproducing *cysG* are grown in the presence of Co^{2+} and ALA, the first committed precursor of tetrapyrrole biosynthesis, a large quantity of cobalt-sirohydrochlorin accumulates within the cell. When cell extracts of ER251 (*cobA* and *MET8*) were analysed in a similar manner, a spectrum equivalent to cobalt-sirohydrochlorin was also observed (Figure 4a). In the absence of Co^{2+}, a spectrum consistent with sirohydrochlorin (or sirohaem) was observed (Figure 4b). The spectra are consistent with the ability of Met8p to act as a dehydrogenase and a chelatase, synthesizing sirohydrochlorin and cobalt-sirohydrochlorin from precorrin-2.

Activity of Met8p *in vitro*

To confirm the role of Met8p as a dehydrogenase and chelatase, the protein was purified and used in an assay *in vitro* to monitor

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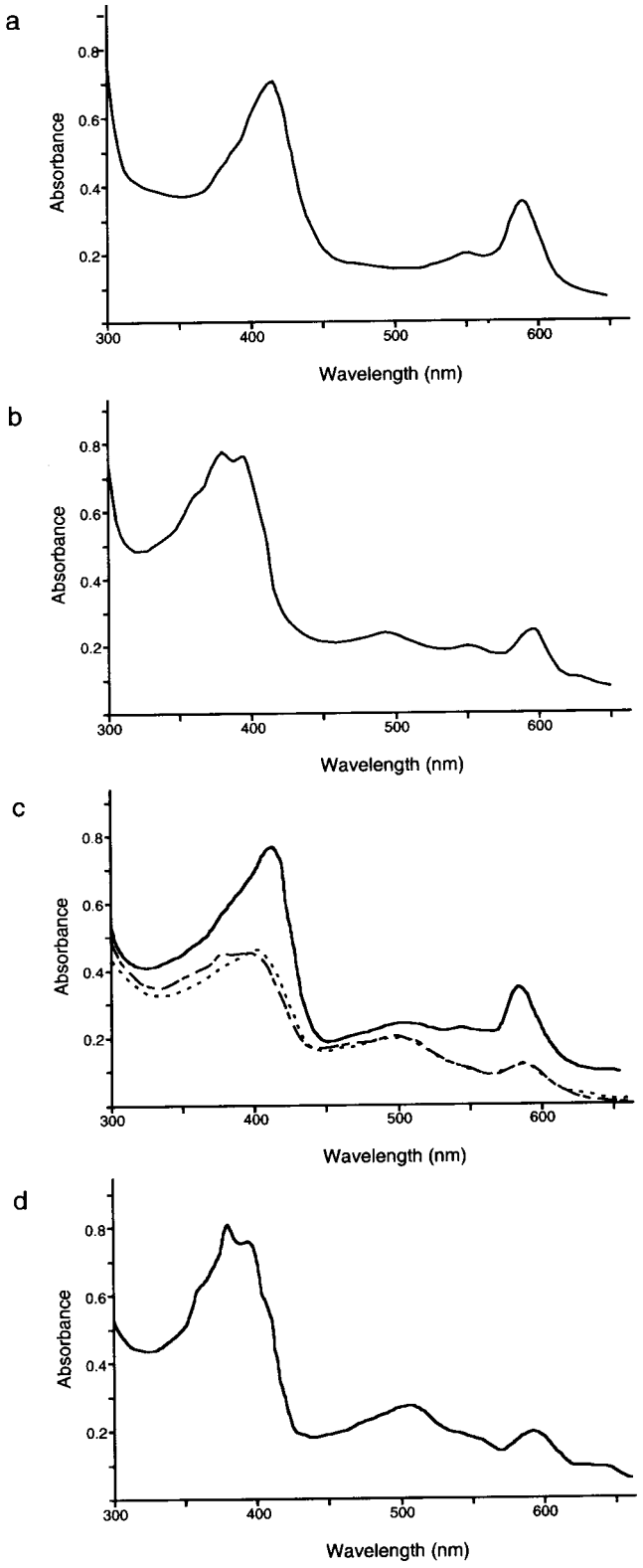

Figure 4 Spectra of pigments accumulated *in vivo* and *in vitro* assays

(**a**) UV–visible spectrum of accumulated pigment isolated from crude cell extracts of ER251, which had been grown in the presence of added ALA and Co^{2+}. The absorption maxima ($\sim$ 410 nm and $\sim$ 590 nm) are equivalent to those of Co^{2+}-sirohydrochlorin. (**b**) UV–visible spectrum of accumulated pigment isolated from crude cell extracts of ER251, which had been grown in the presence of added ALA but with no added Co^{2+}. The spectrum with an absorption maximum at 378 nm is equivalent to that of sirohydrochlorin (or sirohaem). (**c**) UV–visible spectrum obtained from a precorrin-2 assay in the absence of Met8p (broken line), in the presence of Met8p but without NAD^+ (dashed line) and in the presence of Met8p, Co^{2+} and NAD^+ (unbroken line). (**d**) UV–visible spectrum obtained from a precorrin-2 assay in the presence of Met8p and NAD^+ but without Co^{2+}.

the formation of cobalt-sirohydrochlorin. This was achieved by incubating Met8p in a coupled enzyme assay for the synthesis of precorrin-2. Control spectra in the absence of Met8p were also recorded.

The coupled assay with only PBG, PBG deaminase, uroporphyrinogen III synthase, uroporphyrinogen III transmethylase and AdoMet, after 20 min incubation at 37 °C, generated a spectrum which was equivalent to precorrin-2 (Figure 4c). Prolonged incubation of the coupled assay mixture led to spontaneous oxidation of precorrin-2, producing a spectrum with some of the characteristics of sirohydrochlorin, including an absorption maximum at 378 nm. When a similar incubation was undertaken but including Met8p, NAD^+ and Co^{2+}, a spectrum equivalent to that of cobalt-sirohydrochlorin was observed (Figure 4c). This spectrum was very similar to that observed for the crude cell extract of ER251 (strain overproducing CobA + Met8p) (Figure 4a). The omission of Co^{2+} from the incubation mixture gave rise to a spectrum equivalent to sirohydrochlorin (Figure 4d), whereas the omission of NAD^+ gave a spectrum that was consistent with the presence of precorrin-2 (Figure 4c). The conclusion from the *in vitro* assay was that Met8p acts both as an NAD^+-dependent dehydrogenase and a chelatase in sirohaem biosynthesis. Thus Met8p is functionally equivalent to $CysG^B$ with respect to sirohaem synthesis.

Met1p acts as a uroporphyrinogen III transmethylase in the *Sal. typhimurium* CobI pathway

E. coli cysG deletion strain 302Δa, transformed with a plasmid containing the genes encoding the *Sal. typhimurium* CobI enzymes (pAR8827), is capable of converting precorrin-2 into cobyric acid but is unable to make cobyric acid because of the absence of a functional uroporphyrinogen III transmethylase (Table 3). However, when this strain was further transformed with a compatible plasmid containing *MET1* (pER246), cobyric acid was produced. Similar observations were made when the strain was transformed with the *P. denitrificans cobA*. Thus Met1p is able to synthesize precorrin-2, which is chelated with Co^{2+} by CbiK for cobalamin biosynthesis (Scheme 1).

Met8p as a cobaltochelatase in the *Sal. typhimurium* CobI pathway

One of the many interesting features of CysG is that the enzyme can also act as a Co^{2+} chelatase in corrin biosynthesis. This idea was first suggested by Spencer et al. [3], who observed that CysG could act as a Co^{2+} chelatase in synthesizing Co^{2+} sirohydrochlorin. They suggested that cobalt-sirohydrochlorin could be reduced back to Co^{2+}-precorrin-2, which then acted as the substrate for the next cobalamin biosynthetic enzyme CbiL (Scheme 1). However, actual evidence that CysG could act as a Co^{2+} chelatase has been obtained only recently by the demonstration that the presence of the $CysG^B$ domain was essential for cobalamin biosynthesis in *E. coli* strains deleted in *cbiK*. Thus cobalamin biosynthesis is dependent upon either $cysG^B$ or *cbiK* [9].

To investigate the role of Met8p in cobalamin biosynthesis, a plasmid containing the *Sal. typhimurium cobI* genes (pAR8827) was modified by deletion of *cbiK* (producing $pER126K^{\Delta}$) and was transformed into *E. coli* 302Δa. When this host strain was

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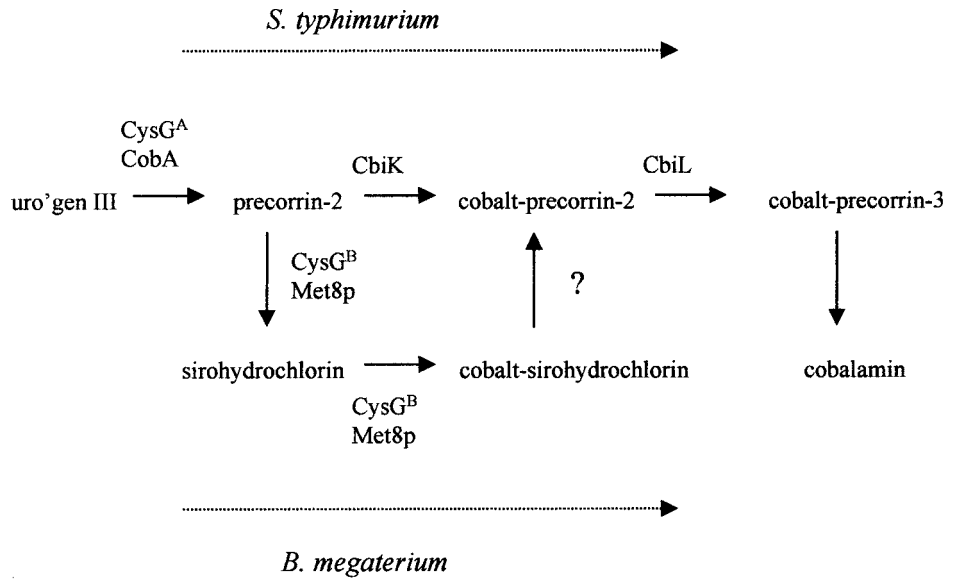
Table 3 Cobyric acid assays

The plasmid pAR8827 contains the *Sal. typhimurium cbiA-C-D-E-T-F-G-H-J-K-L-M-N-Q-O-P* genes. The plasmid pER126K$^{\Delta}$ contains a 54 bp deletion in *cbiK*. The plasmid pER270 contains the *B. megaterium cbiW-H$_{60}$-X-J-C-D-ET-L-F-G-A-cysG^{A}-cbiY-btuR* genes and *Sal. typhimurium cbiP*.

				Cobyric acid (pmol/D_{600})	
cob Operon	Strain	First plasmid	Gene(s) in the second plasmid	Non-inducing conditions	Inducing conditions
Sal. typhimurium	ER255	pAR8827	*MET1*	1	100
	ER123A	pAR8827	*cobA*	90	280
	ER269	pAR8827	*cobA–MET8*	430	235
	ER277	pAR8827	*cysG^{G21D}*	380	380
	ER256	pER126K$^{\Delta}$	*MET1*	0	0
	ER185K$^{\Delta}$A	pER126K$^{\Delta}$	*cobA*	0	0
	ER252	pER126K$^{\Delta}$	*cobA–MET8*	390	290
	ER278	pER126K$^{\Delta}$	*cysG^{G21D}*	250	170
B. megaterium	ER271	pER270	*cysG*	2	110
	ER272	pER270	*cysG^{G21D}*	0	3
	ER273	pER270	*cobA*	0	0
	ER274	pER270	*cobA–cbiK*	0	2
	ER275	pER270	*cobA–MET8*	3	160



Scheme 1 Alternative pathways for cobalamin biosynthesis using the *Sal. typhimurium* and *B. megaterium cobI* operons

uro'gen III, uroporphyrinogen III.

further transformed with a *MET1*-containing plasmid (pER246), it was unable to synthesize cobyric acid because it did not have a functional cobaltochelatase (Table 3). A similar result was obtained when the strain was transformed with a plasmid carrying the *P. denitrificans cobA* (pER119). However, when the strain was transformed with a plasmid containing both *cobA* and *MET8* (pER250), cobyric acid synthesis was initiated. Similarly, transformation of this host strain with plasmids containing either *cysG^{G21D}* (encoding a mutant CysG with no dehydrogenase activity; pKK*cysG^{G21D}*) or *cobA* + *cbiK* (pER170) resulted in the restoration of cobyric synthesis. The conclusion which can be drawn from these results is that, within the *Sal. typhimurium* CobI pathway Met8p can act as a cobaltochelatase but its role as a dehydrogenase is not necessary.

Met8p acts as a precorrin-2 dehydrogenase and cobaltochelatase in the *B. megaterium* CobI pathway

The role of Met8p as a chelatase and dehydrogenase in *B. megaterium* cobalamin synthesis also was investigated. The cloning and sequencing of the *B. megaterium cobI* operon has been described recently [15] and was found to contain many similar *cobI* genes to those found in *Sal. typhimurium*, including *cbiA*, *-C*, *-D*, *-E*, *-F*, *-G*, *-H*, *-J*, *-L* and *-T*, but is lacking *cbiK* and *cbiP*. When the *B. megaterium cobI* operon is cloned into *E. coli* together with the *Sal. typhimurium cbiP* (plasmid pER270), it is able to promote the *de novo* synthesis of cobyric acid. Transformation of the *B. megaterium cobI* operon and *Sal. typhimurium cbiP*-harbouring plasmid (pER270) into the *E. coli cysG*-deletion

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strain 302Δa (ER270; *cysG cobI*$^{+}$) produces a host strain which allows the role of Met8p as a dehydrogenase and chelatase to be investigated. This strain is unable to make cobyric acid as it has no recognizable cobaltochelatase (no equivalent to CysG^{B}, Met8p or CbiK).

As would be expected, transformation of 302Δa/pER270 with a plasmid containing *cysG* gave a strain (ER271) which had the ability to make cobyric acid *de novo* (Table 3). Alternatively, cobyric acid synthesis could also be restored if the strain was transformed with a plasmid containing *cobA* and *MET8* (ER275). Rather surprisingly, though, the addition of a plasmid containing *cobA* and *cbiK* did not endow the host strain with the ability to make cobyric acid. Similarly, transformation of 302Δa/pER270 with a plasmid harbouring *cysG*^{G21D}, encoding a mutant CysG with no measurable dehydrogenase ability, did not lead to any significant production of cobyric acid. The conclusion from these experiments is that cobyric acid synthesis is dependent upon the presence of an enzyme with precorrin-2 dehydrogenase activity. This indicates a subtle variation in the anaerobic cobalamin biosynthetic pathway between *Sal. typhimurium* and *B. megaterium* in that the former is able to produce cobyric acid from precorrin-2, whereas the latter appears to make it from sirohydrochlorin (Scheme 1).

DISCUSSION

The genes, *MET1* and *MET8*, recently identified as being required in *S. cerevisiae* for the transformation of uroporphyrinogen III into sirohaem, have been cloned and expressed in *E. coli*. On the basis of complementation experiments of the *Sal. typhimurium* and *E. coli cysG* strains, AR3612 (*cysG*^{A} minus) and 302Δa (*cysG* deletion), we have concluded that *MET1* encodes an AdoMet-dependent uroporphyrinogen III transmethylase equivalent in function to CysG^{A}, whereas *MET8* encodes a dehydrogenase and chelatase equivalent in function to CysG^{B}. Further experiments, which included the analysis of accumulated pigmented material from a strain overproducing Met8p and CobA and *in vitro* assays, have confirmed that Met8p is an NAD^{+}-dependent dehydrogenase and chelatase. Despite the fact that *S. cerevisiae* does not make cobalamins *de novo*, both *MET1* and *MET8* can complement uroporphyrinogen-III-transmethylase- and cobaltochelatase-deficient cobalamin mutants respectively. In all these respects, the role of Met1p and Met8p is isofunctional with CysG. Previously it had been suggested that the reason for the occurrence of the multifunctional CysG was to facilitate the direct transfer of the labile precorrin-2 from one functional region of the protein to the other [4]. In the case of Met1p and Met8p, precorrin-2 has to diffuse between the separate subunits, indicating that there is no need for a physical association between them.

As a protein, Met1p (593 amino acids) is considerably larger than other uroporphyrinogen III transmethylases, which typically consist of around 280 amino acids. The similarity of uroporphyrinogen III transmethylases to Met1p occurs in the C-terminus. As the N-terminus of Met1p is not required for any of the enzymic transformations of sirohaem biosynthesis, its function remains unknown. It may act in some regulatory fashion to mediate control over the branch-point of tetrapyrrole biosynthesis in *S. cerevisiae*. It would be of interest to know if the N-terminal part of Met1p is essential in *S. cerevisiae*, a point that could be explored by construction of *S. cerevisiae MET*1 mutants.

Although we have shown that Met8p is functionally equivalent to CysG^{B}, there is little sequence similarity between the proteins. Both enzymes contain a putative dinucleotide-binding motif, probably reflecting a common $\beta\alpha\beta$ fold; this similarity covers only the first 60 amino acids of the N-terminus of the proteins. There is thus a distinct possibility that CysG^{B} and Met8p have arisen through convergent evolution.

It is interesting to note that both Met1p and Met8p can function in cobalamin biosynthesis, but as *S. cerevisiae* is unable to synthesize this vitamin, the significance of this result is debatable. The capacity to act in corrin synthesis may be a vestige of an earlier ability that was lost during evolution as the requirement for cobalamin in metabolism was superseded. The ability of other members of the *Ascomycotin*a, and the *Eumycota* in general, to biosynthesize cobalamin is not known.

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REFERENCES

1 Crane, B. R., Siegel, L. M. and Getzoff, E. D. (1995) Science **270**, 59–67
2 Warren, M. J. and Scott, A. I. (1990) Trends Biochem. Sci. **15**, 486–491
3 Spencer, J. B., Stolowich, N. J., Roessner, C. A. and Scott, A. I. (1993) FEBS Lett. **335**, 57–60
4 Warren, M. J., Bolt, E. L., Roessner, C. A., Scott, A. I., Spencer, J. B. and Woodcock, S. C. (1994) Biochem. J. **302**, 837–844
5 Woodcock, S. C., Raux, E., Lanois, A., Levillayer, F., Thermes, C., Rambach, A. and Warren, M. J. (1998) Biochem. J. **330**, 121–129
6 Peakman, T., Crouzet, J., Mayaux, J. F., Busby, S., Mohan, S., Harborne, N., Wootton, J., Nicolson, R. and Cole, J. (1990) Eur. J. Biochem. **191**, 315–323
7 Debussche, L., Couder, M., Thibaut, D., Cameron, B., Crouzet, J. and Blanche, F. (1992) J. Bacteriol. **174**, 7445–7451
8 Ferreira, G. C., Franco, R., Lloyd, S. G., Moura, I., Moura, J. J. G. and Huynh, B. H. (1995) J. Bioenerg. Biomembr. **27**, 221–229
9 Raux, E., Thermes, C., Heathcote, P., Rambach, A. and Warren, M. J. (1997) J. Bacteriol. **179**, 3202–3212
10 Walker, C. J. and Willows, R. D. (1997) Biochem. J. **327**, 321–333
11 Hansen, J., Muldbjerg, M., Cherest, H. and Surdin-Kerjan, Y. (1997) FEBS Lett.. **401**, 20–24
12 Jordan, P. M. and Berry, A. (1981) Biochem. J. **195**, 177–181
13 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
14 Raux, E., Lanois, A., Levillayer, F., Warren, M. J., Brody, E., Rambach, A. and Thermes, C. (1996) J. Bacteriol. **178**, 753–767
15 Raux, E., Lanois, A., Warren, M. J., Rambach, A. and Thermes, C. (1998) Biochem. J. **335**, 159–166
16 Jordan, P. M., Thomas, S. D. and Warren, M. J. (1988) Biochem. J. **254**, 427–435
17 Alwan, A. F., Mgbeje, B. I. and Jordan, P. M. (1989) Biochem. J. **264**, 397–402
18 Roessner, C. A. and Scott, A. I. (1995) Biotechniques **19**, 760–764
19 Blanche, F., Debussche, L., Thibaut, D., Crouzet, J. and Cameron, B. (1989) J. Bacteriol. **171**, 4222–4231
20 Battersby, A. R. and Sheng, Z. C. (1982) J. Chem. Soc. Chem. Commun. 1393–1394
21 Griffiths, L. A. and Cole, J. A. (1987) Arch. Microbiol. **147**, 364–369
22 Roessner, C. A., Spencer, J. B., Ozaki, S., Min, C., Atshaves, B. P., Nayar, P., Anousis, N., Stolowich, N. J., Holderman, M. T. and Scott, A. I. (1995) Protein Expression Purif. **6**, 155–163

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