Characterization of Two Mutant Strains of *Saccharomyces cerevisiae* Deficient in Coproporphyrinogen III Oxidase Activity

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Two new haem-deficient mutants of *Saccharomyces cerevisiae* were isolated on the basis of their catalase deficiency. Mutant H11 accumulated and excreted coproporphyrin III and was completely deficient in haem; the cell-free extract had no coproporphyrinogen oxidase activity. Mutant H12 accumulated uroporphyrin to coproporphyrin III and excreted coproporphyrin III, and contained a small amount of haem; the cell-free extract had a residual coproporphyrinogen oxidase activity. The two mutations were allelic and the mutant phenotypes were under the control of a single, recessive nuclear gene.

INTRODUCTION

Coproporphyrinogen III oxidase (EC 1.3.3.3), an enzyme of the haem biosynthetic pathway, catalyses the oxidative decarboxylation of the 2- and 4-propionate side-chains of coprogen III to vinyl groups to form protogen IX (Granick & Beale, 1978; Tait, 1978; Elder & Evans, 1978; Elder *et al.*, 1978; Grandchamp *et al.*, 1978) – see Methods for definitions of coprogen, protogen etc. The mammalian enzyme has an absolute requirement for molecular oxygen as hydrogen acceptor. However, micro-organisms are known which make proto and haem when grown under anaerobiosis. For *Rhodopseudomonas spheroides* (Tait, 1972), *Micrococcus denitrificans* (Tait, 1973) and the yeast *Saccharomyces cerevisiae* (Poulson & Polglase, 1974), conditions have been described which allow the reactions to proceed in the absence of oxygen but in the presence of methionine, ATP and NAD(P)⁺. In extracts of *R. spheroides* the anaerobic reaction requires at least two proteins while the aerobic one is catalysed by a single protein (Tait, 1972). In yeast both aerobic and anaerobic coprogen oxidase activities are apparently catalysed by the same enzyme which has been partially purified (Poulson & Polglase, 1974).

Studies with mutants deficient in coprogen oxidase activity may lead to an understanding of the mechanism of action of this enzyme and of the regulatory mechanisms controlling its activity and synthesis. Analysis of the haem pathway in such mutants, characterized by the inability to form protoporphyrin and haem, may also give some indication of the regulation of the whole pathway. In addition, the results obtained with these mutants might lead to a better understanding of the mechanism of human coproporphyria, an hereditary disease due to a partial deficiency in coprogen oxidase (Meyer & Schmid, 1978).

We have previously reported a catalase-deficient mutant of *Saccharomyces cerevisiae* (cat9), accumulating 5-COOH porphyrin, which was assumed to have a defect in coprogen oxidase (Labbe-Bois *et al.*, 1977). Gollub *et al.* (1977) isolated a mutant (GL6) accumulating uro and copro *in vitro* which was thought to be deficient in this enzyme. Sugimura *et al.* (1966) described a temperature-sensitive mutant lacking all cytochromes and accumulating
copro III; however, the coproogen oxidase activity of this mutant was normal as compared with the wild-type strain (Miyake & Sugimura, 1968). We describe in the present report the biochemical and genetic analysis of two new mutants of *S. cerevisiae* deficient in coproogen oxidase activity.

**METHODS**

*Abbreviations.* ALA -- 5-aminolaevulinic acid; PBG -- porphobilinogen; urogen, coprogen and protogen -- uroporphyrinogen, coproporphyrinogen and protoporphyrinogen; uro, copro and proto -- uroporphyrin (8-COOH), coproporphyrin (4-COOH) and protoporphyrin (2-COOH); 3-, 5-, 6- and 7-COOH porphyrins -- tri-, penta-, hexa- and hepta-carboxylic porphyrins.

*Strains.* The strains of *Saccharomyces cerevisiae* used in this study are listed in Table 1. The parent strain was SP4 [rho^0] derived from the strain M/S2-1 (Bilinski et al., 1980). The mutants H11 and H12 were isolated as catalase-deficient (Pacholeczka et al., 1974). Standard yeast genetic techniques were used for genetic analysis of the mutants (Sherman et al., 1971).

*Growth conditions.* The growth medium contained (g l^-1): glucose, 20; yeast extract (Difco), 20; Bactopeptone (Difco), 10; Tween 80, 5; ergosterol, 0.020. The cells were grown at 30 °C with vigorous magnetic stirring as reported previously (Labbe-Bois et al., 1977). At the beginning of the last generation of the exponential phase of growth, the cells were harvested, washed and broken to obtain the cell-free extract (Labbe-Bois et al., 1977).

*Cytochrome and haem contents.* The spectrophotometric analysis of the cytochrome and tetrapyrrolic pigment content was performed on whole cells at liquid nitrogen temperature as previously reported (Labbe-Bois et al., 1977; Rytka et al., 1978). The cytochromes were estimated quantitatively from the reduced (with endogenous substrates or with Na$_2$S$_2$O$_4$) minus oxidized (with 0.2 or 0.02 M-I$_2$) difference spectra of cell suspensions (20 to 30 mg dry wt ml$^{-1}$) recorded at room temperature with a Unicam SP 1800 spectrophotometer; the absorption coefficients given by Williams (1964) were used. The CO-difference spectra of reduced yeast cell suspensions were obtained after bubbling CO for 1 min in the measuring cuvette.

Haem content of whole cells was determined as follows: to 5 ml cell suspension (20 to 30 mg dry wt ml$^{-1}$ in 0-1 M-potassium phosphate buffer, pH 7-2) were added 0-75 ml 1 M-NaOH and 0-5 ml pyridine; the difference spectrum of the reduced minus oxidized pyridine haemochrome was recorded and an absorption coefficient of 20-71 mmol$^{-1}$ cm$^{-1}$ was taken for the difference in absorption between 557 and 541 nm (Falk, 1964).

*ALA, PBG and porphyrin contents.* The intracellular contents of ALA, PBG and porphyrins were measured during the growth cycle as reported by Labbe-Bois & Volland (1977). Excreted porphyrins were measured according to the method of Labbe-Bois et al. (1977). They were also extracted from the cell-free culture medium with talc, esterified and identified as described by Brouillet et al. (1975). Isomers I and III of coproporphyrin were identified by the method of Jensen (1963).

*Enzyme assays.* Published procedures (Labbe-Bois & Volland, 1977) were used to measure, in the cell-free extracts, the activities of ALA synthase (EC 2.3.1.37), PBG synthase (EC 4.2.1.24) and the rate of the overall porphyrin synthase from PBG, with the following modifications: (i) the extracts were not dialysed and the assays were done with 4 to 6 mg protein ml$^{-1}$; (ii) the activity of ALA synthase was measured in the presence of succinate plus glycine and added succinohydrolase purified from yeast and with 8 mM-o-phenanthroline instead of EDTA (F. Felix & C. Volland, unpublished). The coproogen III oxidase activity was measured aerobically at 30 °C with cell-free extract containing 0-1 to 0-5 mg protein and 2 µM[^14]Ccoproogen III, using the radiochemical test of Grandchamp & Nordmann (1977). All activities were expressed as nmol product formed h$^{-1}$ (mg protein)$^{-1}$.

*Errors.* All determinations, i.e. intracellular contents of metabolites, excretion of porphyrins and enzyme assays (measured with an error of 5 to 10%), were carried out with the same culture and the whole experiment was done in triplicate.

*Chemicals.* [^14]CCopro III was a gift from Dr B. Grandchamp; ALA was from Fluka; PBG and porphyrin esters from Sigma; CoASH, ATP, pyridoxal phosphate from Boehringer. All other reagents were of analytical grade (Prolabo).

**RESULTS**

*Phenotypes of mutants H11 and H12.*

*Mutant H11.* Mutant H11 [rho^0] grew more slowly than its parent strain (Table 2), but the same cell yield was obtained in the stationary phase of growth. The low-temperature spectra of whole cells (Fig. 1) showed the absence of c and b-like cytochromes, but the presence of pigments absorbing at 412, 535 and 573 nm, a spectrum suggestive of Zn-porphyrins (Chaix & Labbe, 1965; Pretlow & Sherman, 1967; Sugimura et al., 1966; Labbe-Bois et al., 1977).
Table 1. Strains of Saccharomyces cerevisiae used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP4</td>
<td>α leu1 arg4</td>
<td>Bilinski et al. (1980)</td>
</tr>
<tr>
<td>SP20</td>
<td>α leu1 ade1</td>
<td>Bilinski et al. (1980)</td>
</tr>
<tr>
<td>DT30</td>
<td>α his4 ho8</td>
<td>From G. Fink</td>
</tr>
<tr>
<td>H11</td>
<td>α leu1 arg4 hemG1-1 [rho^o]</td>
<td>This work</td>
</tr>
<tr>
<td>H11-1D</td>
<td>α leu1 ade1 hemG1-1 [rho^+]</td>
<td>This work</td>
</tr>
<tr>
<td>H12</td>
<td>α leu1 arg4 hemG1-2 [rho^+]</td>
<td>This work</td>
</tr>
<tr>
<td>H12-4A</td>
<td>α leu1 arg4 hemG1-2 [rho^o]</td>
<td>This work</td>
</tr>
<tr>
<td>cat9</td>
<td>α leu1 ade1 [rho^o]</td>
<td>Labbe-Bois et al. (1977)</td>
</tr>
<tr>
<td>M/S2-1</td>
<td>α leu1 ade1 hem [rho^o]</td>
<td>Labbe-Bois et al. (1977)</td>
</tr>
</tbody>
</table>

Table 2. Content of haem and haem precursors in the parent strain SP4 and mutants H11 and H12

Measurements were made with cells in the last generation of the exponential phase of growth and, in parentheses, with cells in the stationary phase of growth (5 to 10 h after the end of exponential growth). The ranges of values obtained in three separate experiments are given.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Generation time (min)</th>
<th>Intracellular ALA [μmol (g dry wt)^{-1}]</th>
<th>Porphyrins [nmol (g dry wt)^{-1}]</th>
<th>Haem content [nmol (g dry wt)^{-1}]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Intracellular</td>
<td>Excreted</td>
</tr>
<tr>
<td>SP4</td>
<td>105</td>
<td>0.5-0.6 (0-0.6)</td>
<td>1.5-3 (≤1)</td>
<td>0</td>
</tr>
<tr>
<td>H11</td>
<td>210</td>
<td>2.3-2.6 (4-5)</td>
<td>27-33 (5-5.5)</td>
<td>90-110 (65-75)</td>
</tr>
<tr>
<td>H12</td>
<td>150</td>
<td>1.0-1.1 (2)</td>
<td>4-5 (2-2.5)</td>
<td>35-55 (30-40)</td>
</tr>
</tbody>
</table>

Fig. 1. Low-temperature absorption spectra of whole cells of the parent strain SP4 [rho^o] and mutants H11 [rho^o] and H12 [rho^o]. Curves correspond to cells harvested (a) during the last generation of exponential growth and (b) after 5 h of stationary phase. Spectra were recorded as described by Labbe-Bois et al. (1977) using 40 mg dry wt cells. Reduction was achieved by endogenous substrates.
The chromatographic analysis of these porphyrins extracted from the cells revealed that copro III was the main component (more than 95%) with traces of 5- to 8-COOH porphyrins; a 3-COOH porphyrin was hardly detectable and proto was never seen. The total porphyrin content increased during growth to reach a value of about 30 nmol (g dry wt)$^{-1}$ at the end of the exponential phase. But, for the most part, the porphyrins synthesized by the cells were excreted into the culture medium as Zn-copro III (about 85%) and Zn-5-COOH porphyrin (Table 2). The results presented in Table 2 also show a high intracellular pool of ALA which was constant during growth and increased after the end of exponential growth, when the synthesis of porphyrins had apparently stopped (see also Fig. 1). PBG has never been found in the cells.

The difference spectra (reduced minus oxidized) of the whole cells and of the pyridine haemochrome, and the CO-difference spectrum (reduced + CO minus reduced) of the intact cells showed the absence of haem and haemoproteins in this strain. It should be noted that the smallest amount of haem detectable with this method was 1 nmol (g dry wt)$^{-1}$.

The 503 nm absorption band observed in the parent strain and attributed to protoporpho(di)methene was shifted to 501 to 502 nm in the mutant (Fig. 1), suggesting the presence of oxidized forms of linear or cyclized polypyrrylmethane (free or chelated with Zn) intermediates, as previously reported for other haem mutants of yeast (Labbe-Bois et al., 1977).

**Mutant H12.** As shown in Fig. 1 and Table 2, mutant H12 [rho] accumulated and excreted less Zn-porphyrins than mutant H11. The intracellular porphyrins were mainly copro III (60%) and uro (30%) with traces of 5- to 7-COOH porphyrins. Only Zn-copro III was found in the growth medium. The pool of ALA was also smaller than in mutant H11.

The difference spectra of whole cells revealed the presence of small amounts of a cytochrome b-like pigment absorbing at 426, 525 and 558 nm (Fig. 2a), of cytochrome P-450 and of a pigment absorbing at 420 nm in the presence of CO which might represent unassigned 'free' haem, i.e. haem associated with unspecified peptides or proteins (Fig. 2b). The total amount of protohaem, as measured with the pyridine haemochrome assay (Fig. 2c), was about 12 to 14 nmol (g dry wt)$^{-1}$.

**Biochemical analysis of the haem pathway in mutants H11 and H12**

*Enzyme activities measured in vitro.* The specific activities of ALA and PBG synthases and of coprogen III oxidase were measured in cell-free extracts of the parent and mutant strains. The rate of the overall synthesis of porphyrins from PBG was also determined: although the whole enzymic sequence from PBG to proto was measured, analysis of the
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Table 3. Specific activities of some enzymes of the protohaem synthesis pathway measured in cell-free extracts of the parent strain SP4 and mutants H11 and H12

Cell growth and harvest, crude extract preparation and enzyme assays were performed as described in Methods. Results in parentheses are activities estimated in vivo (see text). The ranges of values obtained in three separate experiments are given.

<table>
<thead>
<tr>
<th>Strain</th>
<th>ALA synthase</th>
<th>PBG synthase</th>
<th>Porphyrin synthesis from PBG</th>
<th>Coprogen III oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP4</td>
<td>1.9–2.5</td>
<td>0.4–0.8</td>
<td>0.10–0.11</td>
<td>0.18–0.22</td>
</tr>
<tr>
<td></td>
<td>(2.0–2.4)</td>
<td>(0.5–0.6)</td>
<td>(0.13–0.15)</td>
<td>(0.13–0.15)</td>
</tr>
<tr>
<td>H11</td>
<td>3.2–3.8</td>
<td>0.2–0.6</td>
<td>0.047–0.050</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>(3.1–3.6)</td>
<td>(0.45–0.55)</td>
<td>(0.11–0.14)</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>H12</td>
<td>1.8–3.1</td>
<td>0.3–0.4</td>
<td>0.063–0.070</td>
<td>0.014–0.015</td>
</tr>
<tr>
<td></td>
<td>(1.9–2.3)</td>
<td>(0.3–0.4)</td>
<td>(0.07–0.10)</td>
<td>(0.020)</td>
</tr>
</tbody>
</table>

Nature of the porphyrins could give information about the behaviour of the different intermediate reactions involved in this sequence. The results presented in Table 3 show that the two mutants were deficient in coprogen oxidase. Compared with the parent, this activity was almost nil in strain H11 (<0.1%), while there was a residual activity of about 6 to 8% in the H12 extract. The nature of the porphyrins synthesized from PBG by the different extracts were, respectively: proto (60%), 5-COOH porphyrin (15%), uro (15%) and traces of copro and 3-, 6- and 7-COOH porphyrins for the parent strain; copro III (95%) and traces of 3-, 5- and 6-COOH porphyrins for mutant H11; proto (10%), copro III (40%), 5-COOH porphyrin (15%), 6- and 7-COOH porphyrins (9%), uro (15%) and traces of 3-COOH porphyrin for mutant H12. The lack of proto formation in the cell-free extract of H11, together with the absence of haem in the cells, were consistent with the absence of coprogen oxidase activity. In the case of H12, the presence of a residual coprogen oxidase activity, together with the low proto formation in the extracts, were consistent with the finding of some haem in the intact cells.

The rate of porphyrin synthesis was lower in the two mutants than in the parent (Table 3). The rate of synthesis was constant for at least 3 h in the extracts of the parent and mutant H12. However, in the extract of mutant H11 porphyrin production was linear only for the first 15 min of the assay and then decreased, ceasing after about 1 h. In addition, the extracts of H11 and H12 after dialysis (for 15 h at 0°C against 0.1 M-phosphate buffer pH 7.2) were unable to catalyse any porphyrin formation, whereas the same treatment had no effect on the activity of the parent strain. These results indicate that in the mutants the activity of urogen I synthase was unstable in vitro, particularly in the case of H11.

ALA synthase activity was higher in mutant H11 which completely lacked haem.

Enzyme activities estimated in vivo. Measurements made during growth of (i) the intracellular pools of porphyrin intermediates, (ii) the cytochromes and/or haem contents of the cells and (iii) the amounts of porphyrins excreted (Table 2) allowed the estimation of the in vivo rates of synthesis of these intermediates, based on the known generation time. These rates were expressed as nmol product formed h⁻¹ (mg protein)⁻¹, based on the observation that 300 ± 20 mg protein were recovered in the cell-free extract from 1 g dry wt cells. As can be seen in Table 3 (results in parentheses), the values calculated correlated well with the values of the activities measured in vitro. The fact that the values for ALA synthesis obtained both in vitro and in vivo were identical suggests that the ALA synthase was functioning in the cells at its maximum velocity. The rate of porphyrin synthesis for mutant H11 was two- to three-fold higher when estimated in vivo than when measured in vitro; this may reflect the instability of the urogen I synthase mentioned above.
Wild type [rho⁺]

H11-1D [rho⁺]

H12-4A [rho⁺]

H11-1D [rho⁺] × H12-4A [rho⁺]

Tetrad analysis

A

B

C

D

Fig. 3. Low-temperature whole cell spectra of the different segregants obtained during genetic analysis of mutants H11 and H12. Spectra were recorded using a paste of cells harvested in stationary phase (Rytka et al., 1978). Reduction was achieved by endogenous substrates.

Genetic analysis of mutants H11 and H12

Mutants H11 and H12, derivatives of strain SP4 [rho⁰], were crossed with two standard [rho⁺] strains – the isogenic strain SP20 and the non-isogenic strain DT30 (Table 1). The diploids grew well on non-fermentable carbon sources and showed the normal cytochrome spectra when grown in yeast extract/peptone/glucose medium, indicating that both mutations were recessive. Tetrad analysis of the crosses was carried out. The spore colonies were tested for mating type, auxotrophic markers and ability to grow on non-fermentable carbon sources; their whole cell cytochrome spectra at low temperature were also recorded. In the crosses H11 × DT30 (12 tetrads analysed), H11 × SP20 (8 tetrads analysed), H12 × DT30 (13 tetrads analysed) and H12 × SP20 (8 tetrads analysed), the segregation of mutant to wild-type phenotype was 2⁺:2⁻. Therefore the observed lack of normal cytochrome spectra was due to single nuclear mutations. It should be noted that the phenotypes of the mutants did not differ in the [rho⁰] and [rho⁺] states (Figs 1 and 3). The H11 [rho⁺] strain had no cytochromes, whereas the spectrum of the H12 [rho⁺] segregant showed traces of cytochromes confirming that the H12 mutation was leaky.

Genetic analysis of two mutant segregants, H11-1D (from the cross H11 × SP20) and H12-4A (from the cross H12 × SP20), was performed to determine if the two mutations were allelic. The diploid H11-1D × H12-4A showed the cytochrome spectra characteristic of the H12-4A segregant (Fig. 3). Tetrad analysis of this diploid was done. The spores germinated very poorly and so only four complete tetrads were analysed: all were of the parental ditype (Fig. 3). The spores from incomplete tetrads were also analysed and no wild-type recombinants were found. These results indicated that the two mutations were allelic. They were designated hemGl-1 and hemGl-2, according to the nomenclature proposed previously (Labbe-Bois et al., 1977), where the designation hemG refers to the metabolic block at the level of coprogen III oxidase.
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DISCUSSION

The results reported in this paper show that the two mutants H11 and H12 of \textit{S. cerevisiae} were blocked in coprogen III oxidase activity. The observed accumulation and excretion of copro III and other intermediates by both mutants, together with the deficiency in coprogen oxidase activity as measured in cell-free extracts, were strong evidence that enzyme assays \textit{in vitro} reflected the physiological situation \textit{in vivo}. The mutant cat9, which was thought to be defective in coprogen oxidase activity (Labbe-Bois \textit{et al.}, 1977), has been analysed and a high coprogen oxidase activity could be demonstrated in the cell-free extract: 1.2 to 1.4 nmol proto h\(^{-1}\) (mg protein\(^{-1}\)) compared with 0.2 nmol proto h\(^{-1}\) (mg protein\(^{-1}\)) in the parent strain M/S2-1. Further analysis of this mutant is in progress.

The aerobic coprogen oxidase activity of the wild-type strain SP4 \([\rho^0]\) was lower than has been reported by others using different \([\rho^+]\) wild-type strains of \textit{S. cerevisiae} (Miyake & Sughura, 1968; Poulson & Polglase, 1974). However, the same low activity was also found with strain SP4 in the \([\rho^+]\) state and, in fact, we found that the level of this activity was highly strain-dependent, whatever the \([\rho^0]\) state of the different wild-type strains tested: activities varied from 0.2 to 2 nmol proto h\(^{-1}\) (mg protein\(^{-1}\)) (R. Labbe-Bois, unpublished results). Such a strain dependence has not been observed for the other enzymes of the haem pathway. With regard to the anaerobic coprogen oxidase activity, studies with mutants H11 and H12 may help to elucidate whether or not the same protein catalyses both the aerobic and the anaerobic reactions. Such studies are currently in progress.

The deficiency in coprogen oxidase activity and the absence of detectable haem in mutant H11 led to alterations in the activities of ALA and urogen synthases. That the ALA synthase activity was higher than in the parent strain might be taken as evidence for repression of ALA synthase by the end-product haem, as shown in liver cells (Granick & Beale, 1978). However, considering the high dependence of the level of this activity on the physiological conditions of the yeast cells (Labbe-Bois & Volland, 1977; Labbe-Bois & Labbe, 1978), it is possible that the increase of ALA synthase in H11 was due to derepression caused by haem deficiency or was reflecting a different metabolic state of the mutant cells. The reasons for the instability of the urogen synthase \textit{in vitro} are unclear at present. The arrest of copro formation at the end of growth, together with the increased intracellular ALA content, suggested that the PBG synthase was not functioning \textit{in vivo}, since no PBG or uro formation was observed. The fact that Zn-copro and not coprogen was found probably resulted from non-enzymic oxidation of coprogen and insertion of Zn into copro.

The phenotypes of mutants H11 and H12 showed properties indicative of a defect in a single, recessive nuclear gene. The fact that the mutations were allelic but the mutants differed in their phenotypes indicated that both mutations affected the same locus to different degrees. In H11 the mutation led to a complete block of the function of the gene involved, whereas in H12 the mutation was leaky. Whether the mutation represented lesions in the structural gene of coprogen oxidase or in a regulatory gene involved in the expression of the structural gene is not known.

These mutants should be useful in the study of linkage relationships between the different \textit{hem} loci and in exploring the location of \textit{hemGI}. For, although many haem mutants have been described, little is known about the genetics of haem biosynthesis in yeast. Gollub \textit{et al.} (1977) reported that the mutation in strain GL6, thought to be deficient in coprogen oxidase, was suppressible by nonsense suppressors, and that its locus was apparently not linked to loci controlling \textit{ALA} and urogen synthase activities. However, none of the \textit{hem} genes has been mapped.

A mutant strain of \textit{E. coli} has been described which grew very slowly on non-fermentable carbon sources, accumulated copro III, contained less cytochromes and catalase and possessed one-fifth of the coprogen oxidase activity of the parent strain (Cox & Charles, 1973; R. Cox, unpublished results quoted by McConville & Charles, 1979). It is interesting to
note that an 80% deficiency of the coprogen oxidase activity in *E. coli* and a 92 to 94% deficiency in *S. cerevisiae* led to a significant decrease in haem and/or haemoprotein content, whereas human cells can still synthesize enough haem despite a 98% enzyme deficiency (Grandchamp et al., 1977).

We are grateful to Drs Y. Nordmann and B. Grandchamp for their help with the coprogen oxidase assays which were carried out in Dr Nordmann's laboratory. Acknowledgment is made to Mrs T. Laska for her excellent technical assistance.

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**REFERENCES**


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