Journal of General Microbiology (1979), **113**, 297–303. *Printed in Great Britain*

Mapping of a New *hem* Gene in *Escherichia coli* K12

By A. SĂSĂRMAN, P. CHARTRAND,* M. LAVOIE, D. TARDIF, R. PROSCHEK AND C. LAPOINTE

Department of Microbiology and Immunology, University of Montreal, Montreal, Quebec, Canada

(*Received* 12 *December* 1978)

A new type of haem-deficient mutant was isolated in *Escherichia coli* K12 by neomycin selection. The mutant, designated SASX38, accumulated uroporphyrin, coproporphyrin and protoporphyrin. Since it possessed normal ferrochelatase activity, it was assumed to be deficient in protoporphyrinogen oxidase activity. The gene affected in the mutant was designated *hemG*. Mapping of the *hemG* gene by phage P1-mediated transduction showed that it was located very close to the *chlB* gene (frequency of cotransduction 78·7%), between the *metE* and *rha* markers. This location is distinct from the other known *hem* loci in *E. coli* K12.

INTRODUCTION

The conversion of COPROGEN III to PROTO IX is carried out by two enzymes, the COPROGEN III oxidase and the PROTOGEN IX oxidase (Jackson *et al.*, 1974; Jacobs & Jacobs, 1975; Porra & Falk, 1964; Poulson & Polglase, 1974, 1975; Sano & Granick, 1961; see also Fig. 1). The first is responsible for the conversion of COPROGEN III to PROTOGEN IX, and the second for the oxidation of PROTOGEN IX to PROTO IX. PROTOGEN IX oxidase activity has been detected in yeast (Poulson & Polglase, 1974, 1975) and mammalian mitochondria (Poulson, 1976) and in bacteria (Jacobs & Jacobs, 1975, 1976; Poulson *et al.*, 1976). The enzyme was isolated recently from yeast (Poulson & Polglase, 1975) and mammalian mitochondria (Poulson, 1976). However, no mutants deficient in PROTOGEN IX oxidase activity have been isolated so far.

Haem-deficient mutants have been studied most thoroughly in bacteria. Most of the *hem* genes have already been identified in *Escherichia coli* K12 (Chartrand *et al.*, 1979; Powell *et al.*, 1973; Săsărman *et al.*, 1975; Săsărman *et al.*, 1968*a*, *b*), in *Salmonella typhimurium* (Săsărman & Desrochers, 1976; Săsărman *et al.*, 1970), in *Bacillus subtilis* (Berek *et al.*, 1975) and in *Staphylococcus aureus* (Tien & White, 1968). In an attempt to map the gene of PROTOGEN IX oxidase, a collection of unidentified haem-deficient mutants of *E. coli* K12 were examined. The mutants were selected by neomycin as previously described (Săsărman *et al.*, 1970). One of the unidentified haem-deficient mutants was affected in a gene distinct from the other known *hem* genes, The results of the biochemical and genetic studies of this new haem-deficient mutant of *E. coli* K12 are presented here.

METHODS

Abbreviations. PBG, Porphobilinogen; URO, uroporphyrin; COPRO, coproporphyrin; COPROGEN, coproporphyrinogen; PROTO, protoporphyrin; PROTOGEN, protoporphyrinogen.

Bacterial strains and bacteriophage. The derivatives of *E. coli* K12 are listed in Table 1. The bacteriophage P1*kc* was obtained from Professor P. Fredericq (University of Liege, Belgium).

* Present address: Institute of Virology, University of Glasgow, Glasgow G11 5JR, Scotland.

0022-1287/79/0000-8503 $02.00 © 1979 SGM

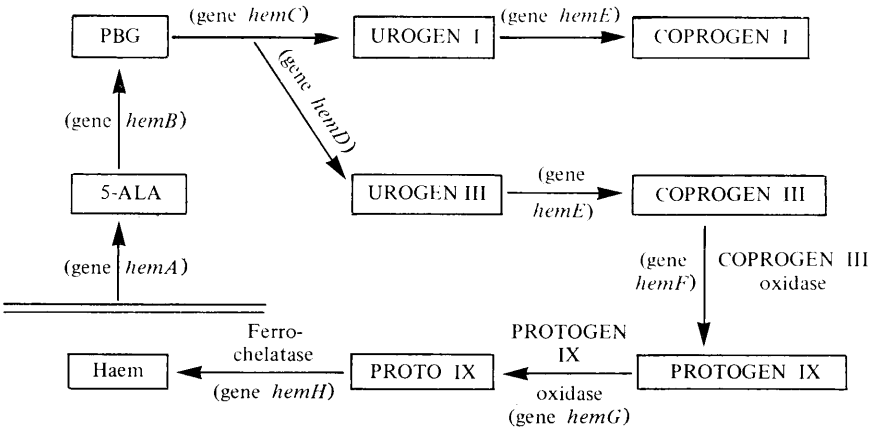

Fig. 1. Biosynthesis of haem: enzymes of the late steps of biosynthesis and proposed nomenclature of *hem* genes of *E. coli* K12 (Jackson *et al.*, 1974; Jacobs & Jacobs, 1975; Lascelles, 1964; Porra & Falk, 1964; Poulson & Polglase, 1974; Poulson & Polglase, 1975; Sano & Granick, 1961; Săsărman & Desrochers, 1976; Tait, 1968). Abbreviations: 5-ALA, 5-aminolaevulinic acid; PBG, porphobilinogen; UROGEN, uroporphyrinogen; COPROGEN, coproporphyrinogen; PROTOGEN, protoporphyrinogen; PROTO, protoporphyrin.

Table 1. *Derivatives of E. coli* K12 *used*

Strain	Genotype*	Source
SASX38	*hemG38* mutation in Hfr Cavalli	This laboratory
Hfr Cavalli	*metB1*, *rel-1*; Hfr	W. Hayes
AB1931	*argH1*, *metE46*, *purF1*, *xyl-7*, *rel-1*(?), *sup-16*, *sup-48*(?); Hfr	E. Adelberg
AT753	*argH1*, *ilv-1*, *metB1*, *thi-1*, *gal-6*, *lacY1*, or *lacZ4*, *rha-1*, *str-8*, *supE44*(?), λ^-; F^-	A. L. Taylor
SAS229	*ilvD75*, *leu-6*, *metE46*, *pro-36*, *thr-1*, *trp-42*, *gal-6*, *lacY1*, *mtl-2*, *str-9*	This laboratory
SAS246	*argH1*, *his-1*, *metE46*, *thr-1*, *thi-1*, *purE43*, *gal-6*, *lacY1*, *xyl-7*, *chlB2*, *str-121*	This laboratory
442-F	*argH1*, *his-1*, *leu-6*, *pro-33*, *thr-1*, *thi-1*, *purE43*, *ara-13*, *gal-6*, *lacY1*, *malA1*, *mtl-2*, *xyl-7*, *chlB2*, *str-121*, *ton-2*, λ^r, λ^-; F^-	B. Bachmann (CGSC); originally described by F. Casse

* For definitions of gene symbols, see Bachmann *et al.* (1976).

Media. Bacteria were grown in brain heart infusion (BHI, Difco) or on brain heart infusion agar (1·5 to 2 %, w/v; Difco). The synthetic medium was Simmons agar base (Difco), supplemented with the required growth factors and 0·4 % (w/v) glucose.

Chemicals. Organic solvents for the extraction and identification of porphyrins were reagent or USP grade. Peroxides were removed from diethyl ether as desribed by Perrin *et al.* (1966). Porphyrin methyl esters and porphyrin precursors were obtained from Sigma and were Sigma grade. Free porphyrins were obtained by hydrolysis of the corresponding methyl esters as described by Falk (1964). Biuret reagent was obtained from Hycel, Houston, Tex., U.S.A., and the human protein standard was obtained from Dade, Miami, Fla., U.S.A. Neomycin sulphate was obtained from Sigma.

Selection of haem-deficient mutants. These were selected by neomycin as described previously (Săsărman *et al.*, 1970).

Extraction and identification of porphyrins in bacterial cultures. Two different types of cultures were used for the extraction of porphyrins: (i) bacteria grown for 72 h at 37 °C on 2 % BHI agar in Roux bottles; (ii) bacteria grown aerobically overnight in BHI at 29 °C. At the end of the incubation period, bacteria were harvested from the first type of culture by washing the agar with saline and then centrifuging. The supernatant and the bacterial pellet were extracted separately. Porphyrins were extracted by the ether–cyclohexanone method (Dresel *et al.*, 1956; Kennedy, 1956), as adapted for bacteria (Săsărman *et al.*, 1975).

On: Wed, 13 Sep 2017 13:46:12

Table 2. *Accumulation of porphyrins in cultures of* SASX38

Growth medium	Incubation conditions	Preparation extracted	Porphyrins [nmol (g dry wt)$^{-1}$]* URO	COPRO	PROTO
BHI agar	37 °C, 72 h	Intact cells	554	1011	trace
BHI	29 °C, 14 h	Supernatant of ultrasonically disrupted cells	972	256	74

* The amount of porphyrins extracted from the supernatant of ultrasonically disrupted cells was normalized to 500 mg protein ml^{-1}.

From the second type of culture, bacteria were harvested by centrifugation and then disrupted in an ultrasonic cell disintegrator (model Biosonik III; Bronwill Scientific, Rochester, N.Y., U.S.A.). The preparation was then centrifuged for 40 min at 14000 *g* in the cold. Porphyrins were extracted from the supernatant by ethyl acetate/acetic acid (3:1, v/v) (Falk, 1964; Sano & Granick, 1961) followed by cyclohexanone, the latter step being identical in both methods.

The yield of porphyrins was calculated using the molar absorption coefficients for free porphyrins, with the corrections recommended by Rimington (1960) and Porra & Falk (1964).

Porphyrin synthesis in bacterial extracts. The cell-free supernatant prepared from disrupted bacteria (see above) was used immediately or stored at −20 °C. The protein content of the bacterial extracts was determined by the biuret method (Layne, 1957); it varied from 20 to 35 mg ml^{-1}. For the determination of *in vitro* porphyrin synthesis, PBG was used as a precursor. The reaction mixture contained 1·0 ml extract in 0·1 M-Tris/HCl buffer (pH 8·2), 3·3 mM-EDTA and 500 nmol PBG, and was incubated aerobically for 4 h in the dark at 29 °C. Porphyrins were extracted by the ether–cyclohexanone method as previously described (Săsărman *et al.*, 1975).

COPROGEN III oxidase activity in bacterial extracts. COPROGEN III was prepared by reducing COPRO III with sodium amalgam (Sano & Granick, 1961). COPROGEN III oxidase activity in bacterial extracts was determined by a modification of the method of Sano & Granick (1961). The reaction mixture contained 1·4 ml extract in 0·1 M-Tris/HCl buffer (pH 7·2), 0·9 % (w/v) KCl, 20 mM-EDTA and 100 nmol COPROGEN III, and was incubated aerobically for 1 h at 38 °C in the dark. Porphyrins were extracted and identified as described by Sano & Granick (1961).

Ferrochelatase activity in bacterial extracts. Ferrochelatase activity was determined as described by Porra & Jones (1963). The reaction mixture contained 1·0 ml extract, 200 nmol potassium phosphate buffer (pH 7·8), 30 nmol reduced glutathione, 1 % (v/v) Tween 80, 400 nmol $FeSO_4$ and 200 nmol PROTO IX, in a final volume of 4·2 ml. It was incubated anaerobically at 37 °C for 2 h. Haem was determined at the end of the incubation period as described by Porra & Jones (1963).

Assay of catalase activity. Catalase activity was determined as described by Herbert & Pinsent (1948).

Mapping of the hemG gene. This was done by phage P1-mediated transduction (Lennox, 1955). The phage lysate was irradiated by an optimal dose of ultraviolet light (Wilson, 1960) in order to increase the low frequency of transduction obtained with haem-deficient recipients.

RESULTS

Biochemical study of the mutant

Strain SASX38 is a spontaneous haem-deficient derivative of *E. coli* K12 Hfr Cavalli, selected by neomycin. Its growth on normal media is slow and results in the formation of dwarf colonies. After several days of incubation, the colonies acquire a red fluorescence in ultraviolet light (365 nm), due to the accumulation of porphyrins.

The extraction and identification of the porphyrins accumulated by SASX38 showed the presence of only URO and COPRO in bacteria incubated for 72 h (Table 2). However, when porphyrins were determined in extracts of 14 h cultures, PROTO was also found, in addition to URO and COPRO. Under the same conditions, the parental strain Hfr Cavalli did not accumulate porphyrins (results not shown). The absence of PROTO from the extracts of bacteria incubated for 72 h (Table 2) may be due to the conversion of PROTO to haem, rather than to its inactivation (see Discussion).

On: Wed, 13 Sep 2017 13:46:12

Table 3. *Enzyme activities in extracts of E. coli* K12 Hfr Cavalli *and* SASX38

Strain	Assay	Substrate (nmol)		Net synthesis of tetrapyrroles (nmol) URO	COPRO	PROTO	Haem
SASX38	Porphyrin synthesis	PBG	(500)*	9	9	4	
	COPROGEN III oxidase	COPROGEN III	(100)			7	
	Ferrochelatase	PROTO IX	(200)				45
Hfr Cavalli	Porphyrin synthesis	PBG	(500)*	34	4	31	
	COPROGEN III oxidase	COPROGEN III	(100)			11·2	
	Ferrochelatase	PROTO IX	(200)				57

* Equivalent to 125 nmol tetrapyrroles.

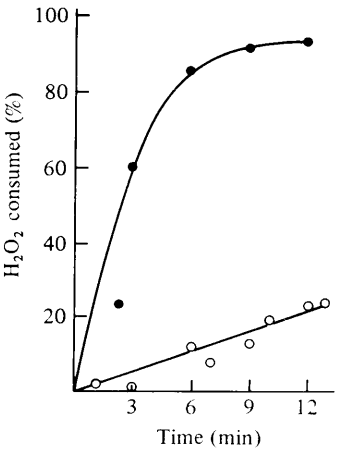

Fig. 2. Catalase activity of *E. coli* K12 strains: ○, SASX38; ●, Hfr Cavalli.

Porphyrin synthesis in extracts of SASX38 was less efficient than in extracts of the parental strain (Table 3). Thus the accumulation of porphyrins cannot be due to a derepression of the early *hem* genes.

COPROGEN III oxidase and ferrochelatase activities in extracts of SASX38 were similar to the corresponding activities of the parental strain. Hence, the accumulation of porphyrins cannot be attributed to a deficiency of the ferrochelatase.

The catalase activity of SASX38 was very low compared with that of the parental strain (Fig. 2). In spite of the normal ferrochelatase activity of the mutant (Table 3), very little haem was synthesized.

Mapping of the hemG gene

The results of the transduction experiments (Table 4) show that *hemG* is located very close to *chlB* (frequency of cotransduction 78·7%), between the *metE* and *rha* markers (Fig. 3). This location corresponds to 85 min on the chromosomal map of *E. coli* K12 (Bachmann *et al.*, 1976). Analysis of classes of transductants did not allow the deduction of the relative order of the *hemG* and *chlB* genes.

On: Wed, 13 Sep 2017 13:46:12

Table 4. *Results of transduction experiments by phage* P1*kc*

Donor	Recipient	Selected marker	No. of transductants analysed	Donor alleles in transductants (%) *metE*	*chlB*	*rha*
SAS246	SASX38	*hemG*$^+$	399	13·5	78·7	
AT753	SASX38	*hemG*$^+$	197			1·5
442-F	AB1931	*metE*$^+$	197	100	15·0	
442-F	SAS229	*metE*$^+$	193	100	10·4	

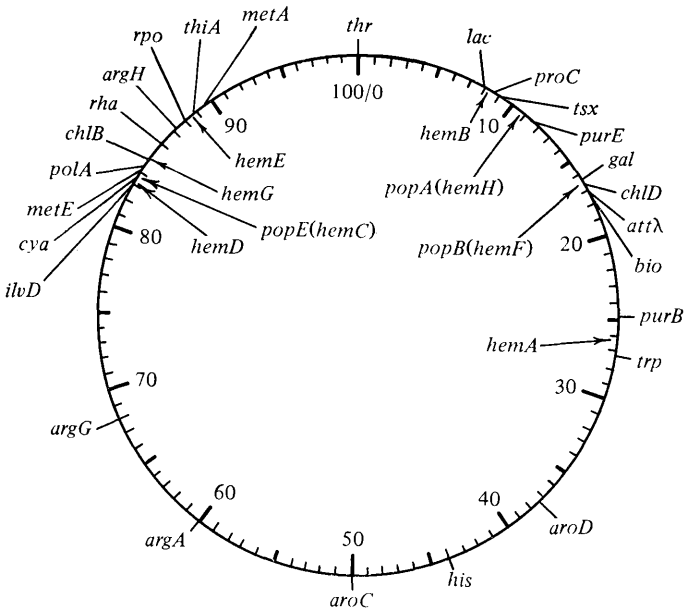

Fig. 3. Location of *hem* genes on the chromosomal map of *E. coli* K12 (Bachmann *et al.*, 1976; Chartrand *et al.*, 1979; Cox & Charles, 1973; Powell *et al.*, 1973; Săsărman *et al.*, 1975; Săsărman *et al.*, 1968*a*; Săsărman *et al.*, 1968*b*); designations proposed for *pop* genes are in parentheses.

DISCUSSION

Hem$^+$ strains do not accumulate porphyrins under normal growth conditions due to a very efficient regulatory mechanism, involving haem as the main effector (Lascelles, 1975). However, porphyrins accumulate in bacteria when the synthesis of haem is deficient. Only the intermediates which precede the mutation accumulate and hence the pattern of accumulation reflects the site of mutation.

The extraction of porphyrins from strain SASX38 showed that it accumulates URO, COPRO and PROTO. This may be due to mutations in two different genes. The better known of the two is the gene of ferrochelatase which is responsible for the insertion of iron into the molecule of PROTO IX to give protohaem. The corresponding gene has already been mapped in *E. coli* K12 (Cox & Charles, 1973; Powell *et al.*, 1973), and its location is different from that found in strain SASX38. Moreover, the ferrochelatase activity of SASX38 is normal which indicates that the accumulation of porphyrins cannot be due to a deficiency of this enzyme. The second gene whose mutation could also lead to the accumulation of PROTO is that of PROTOGEN IX oxidase. This enzyme is responsible for the oxidation of PROTOGEN IX to PROTO IX, and it was isolated only recently (Poulson & Polglase, 1975). PROTOGEN IX oxidase activity has been detected directly in extracts of *E. coli* by Jacobs & Jacobs (1975, 1976). Unfortunately, their technique cannot be applied to mutant

On: Wed, 13 Sep 2017 13:46:12

SASX38 due to the presence of large amounts of porphyrins in the extracts (Table 2). However, indirect evidence strongly supports the assumption that the mutant is deficient in PROTOGEN IX oxidase activity. Indeed, the accumulation of PROTO IX associated with normal ferrochelatase activity is paradoxical, unless the accumulation is that of PROTOGEN IX. Since PROTOGEN IX cannot be utilized by ferrochelatase to produce haem, it will accumulate in cells. During the extraction of porphyrins by organic solvents, PROTOGEN IX is readily oxidized to PROTO IX, which explains the results of the extraction experiments.

Hence, due to the auto-oxidation of PROTOGEN IX during extraction, both a PROTOGEN oxidase-deficient mutant and a ferrochelatase-deficient mutant would accumulate PROTO. However, ferrochelatase activity would be normal in a PROTOGEN oxidase-deficient mutant, but absent in a ferrochelatase-deficient mutant. On this basis, we consider that strain SASX38 is a PROTOGEN IX oxidase-deficient mutant (Fig. 1).

The PROTOGEN IX oxidase deficiency of the mutant also provides an explanation for the disappearance of PROTO in cultures incubated for 72 h (Table 2). In long-term cultures of the mutant, PROTOGEN IX is oxidized spontaneously to PROTO IX, which in turn is converted to haem by ferrochelatase. This haem is probably the source of the small amount of catalase activity found in 48 h cultures of the mutant.

According to the nomenclature proposed earlier (Săsărman & Desrochers, 1976), the gene affected in strain SASX38 was designated *hemG*. The results of the mapping show that *hemG* is located very close to *chlB*, but analysis of classes of transductants did not allow us to determine the relative order of the *hemG* and *chlB* genes. Thus, the real sequence in this segment could be *metE chlB hemG rha* or *metE hemG chlB rha* (Bachmann *et al.*, 1976).

The location of the newly identified *hemG* gene (see Fig. 3) confirms the well known dispersion of *hem* genes on the chromosome of *E. coli* K12 (Cox & Charles, 1973; Powell *et al.*, 1973; Săsărman *et al.*, 1975, 1968*a*, 1968*b*). One exception to this rule could be the contiguity of the *hemD* (Chartrand *et al.*, 1979) and *popE* (Powell *et al.*, 1973) genes, as was suggested for the equivalent genes in *S. typhimurium* (Săsărman & Desrochers, 1976). However, the published data (Cox & Charles, 1973; Powell *et al.*, 1973) do not allow an assessment of the contiguity of these two genes in *E. coli* K12, and further study would be necessary to clarify this point.

We are indebted to Michèle Paris and D. Charbonneau for their valuable contribution to the present work. This investigation was supported by grant MA4997 from the Medical Research Council of Canada to A.S. P.C. was the recipient of a studentship from the Medical Research Council of Canada.

REFERENCES

BACHMANN, B. J., LOW, K. B. & TAYLOR, A. (1976). Recalibrated linkage map of *Escherichia coli* K-12. *Bacteriological Reviews* **40**, 116–167.

BEREK, I., MICZAK, A., KISS, I., IVANOVICS, G. & DURKO, I. (1975). Genetic and biochemical analysis of haemin dependent mutant of *Bacillus subtilis*. *Acta microbiologica Academiae scientiarum hungaricae* **22**, 157–167.

CHARTRAND, P., TARDIF, D. & SĂSĂRMAN, A. (1979). Uroporphyrin and coproporphyrin I-accumulating mutant of *Escherichia coli* K12. *Journal of General Microbiology* **110**, 61–66.

COX, R. & CHARLES, H. P. (1973). Porphyrin-accumulating mutants of *Escherichia coli*. *Journal of Bacteriology* **113**, 122–132.

DRESEL, E. I. B., RIMINGTON, C. & TOOTH, B. E. (1956). Determination of urinary uroporphyrin by a direct extraction method. *Scandinavian Journal of Clinical and Laboratory Investigation* **8**, 73–78.

FALK, J. E. (1964). *Porphyrins and Metallo-porphyrins*. New York. American Elsevier.

HERBERT, D. & PINSENT, J. (1948). Crystalline bacterial catalase. *Biochemical Journal* **43**, 193–202.

JACKSON, A. H., GAMES, D. E., COUCH, P., JACKSON, J. R., BELCHER, R. B. & SMITH, S. G. (1974). Conversion of coproporphyrinogen III to protoporphyrin IX. *Enzyme* **17**, 81–87.

JACOBS, N. J. & JACOBS, J. M. (1975). Fumarate as alternate electron acceptor for the late steps of anaerobic heme synthesis in *E. coli*. *Biochemical and Biophysical Research Communications* **65**, 435–441.

JACOBS, N. J. & JACOBS, J. M. (1976). Nitrate, fumarate, and oxygen as electron acceptors for

On: Wed, 13 Sep 2017 13:46:12

a late step in microbial heme synthesis. *Biochimica et biophysica acta* **449**, 1–9.

KENNEDY, G. Y. (1956). A new method for the extraction of uroporphyrin I from mollusc shells. *Scandinavian Journal of Clinical and Laboratory Investigation* **8**, 79–80.

LASCELLES, J. (1964). *Tetrapyrrole Biosynthesis and its Regulation*. New York: W. A. Benjamin.

LASCELLES, J. (1975). The regulation of heme and chlorophyll synthesis in bacteria. *Annals of the New York Academy of Sciences* **244**, 334–347.

LAYNE, E. (1957). Spectrophotometric and turbidimetric methods for measuring proteins. *Methods in Enzymology* **3**, 447–454.

LENNOX, E. S. (1955). Transduction of linked genetic characters of the host by bacteriophage P1. *Virology* **1**, 190–206.

PERRIN, D. D., ARMAREGO, W. L. F. & PERRIN, D. P. (1966). *Purification of Laboratory Chemicals*. Oxford: Pergamon Press.

PORRA, R. J. & FALK, J. E. (1964). The enzymic conversion of coproporphyrinogen III into protoporphyrin IX. *Biochemical Journal* **90**, 69–75.

PORRA, R. J. & JONES, O. T. G. (1963). Studies on ferrochelatase from a pig liver mitochondria extract. *Biochemical Journal* **87**, 181–185.

POULSON, R. (1976). The enzymic conversion of protoporphyrinogen IX to protoporphyrin IX in mammalian mitochondria. *Journal of Biological Chemistry* **251**, 3730–3733.

POULSON, R. & POLGLASE, W. J. (1974). Site of glucose repression of heme biosynthesis. *FEBS Letters* **40**, 258–260.

POULSON, R. & POLGLASE, W. J. (1975). The enzymic conversion of protoporphyrinogen IX to protoporphyrin IX. Protoporphyrinogen oxidase activity in mitochondrial extracts of *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **250**, 1269–1274.

POULSON, R., WHITLOW, K. J. & POLGLASE, W. J. (1976). Catabolite repression of protoporphyrin IX biosynthesis in *E. coli* K12. *FEBS Letters* **62** 351–353.

POWELL, K. A., COX, R., MCCONVILLE, M. & CHARLES, H. P. (1973). Mutations affecting porphyrin biosynthesis in *Escherichia coli*. *Enzyme* **16**, 65–73.

RIMINGTON, C. (1960). Spectral-absorption coefficients of some porphyrins in the Soret-band region. *Biochemical Journal* **75**, 620–623.

SANO, S. & GRANICK, S. (1961). Mitochondrial coproporphyrinogen oxidase and protoporphyrin formation. *Journal of Biological Chemistry* **236**, 1173–1180.

SĂSĂRMAN, A. & DESROCHERS, M. (1976). Uroporphyrinogen III cosynthase-deficient mutant of *S. typhimurium* LT2. *Journal of Bacteriology* **128**, 717–721.

SĂSĂRMAN, A., SURDEANU, M. & HORODNICEANU, T. (1968*a*). Locus determining the synthesis of Δ-aminolevulinic acid in *Escherichia coli* K-12. *Journal of Bacteriology* **96**, 570–572.

SĂSĂRMAN, A., SURDEANU, M., SZEGLI, G., HORODNICEANU, T., GRECEANU, V. & DUMITRESCU, A. (1968*b*). Hemin-deficient mutants of *Escherichia coli* K-12. *Journal of Bacteriology* **96**, 570–572.

SĂSĂRMAN, A., SANDERSON, K. E., SURDEANU, M. & SONEA, S. (1970). Hemin-deficient mutants of *Salmonella typhimurium*. *Journal of Bacteriology* **102**, 531–536.

SĂSĂRMAN, A., CHARTRAND, P., PROSCHEK, R., DESROCHERS, M., TARDIF, D. & LAPOINTE, C. (1975). Uroporphyrin-accumulating mutant of *E. coli* K-12. *Journal of Bacteriology* **124**, 1205–1212.

TAIT, G. H. (1968). General aspects of haem synthesis. In *Porphyrins and Related Compounds*. Edited by T. W. Goodwin. New York: Academic Press.

TIEN, W. & WHITE, D. C. (1968). Linear sequential arrangement of genes for the biosynthetic pathway of protoheme in *Staphylococcus aureus*. *Proceedings of the National Academy of Sciences of the United States of America* **61**, 1392–1398.

WILSON, D. E. (1960). The effects of ultraviolet light and ionizing radiation on the transduction of *E. coli* by phage P1. *Virology* **11**, 533–546.

On: Wed, 13 Sep 2017 13:46:12