Uroporphyrin- and Coproporphyrin I-accumulating Mutant of *Escherichia coli* K12

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A new type of haem-deficient mutant was isolated in *Escherichia coli* K12 by neomycin selection. The mutant was deficient in uroporphyrinogen III cosynthase activity as indicated by the accumulation of uroporphyrin I and coproporphyrin I. The mapping of the corresponding *hemD* gene by P1-mediated transduction showed that the new gene was located between *ilv* and *cya*, at min 83 on the chromosomal map of *Escherichia coli* K12.

INTRODUCTION

Congenital erythropoietic porphyria in man is an autosomal recessive disease which is due to a partial deficiency in uroporphyrinogen (UROGEN) III cosynthase activity (Bogorad, 1958; Levin, 1975; Fig. 1). Its clinical manifestations are the result of the formation of large amounts of uroporphyrin (URO) I and coproporphyrin (COPRO) I (Levin, 1975) but the exact mechanism of the disease is difficult to analyse in man. Bacteria represent an ideal model for the study of this deficiency, because the steps in porphyrin synthesis are the same in bacteria and in higher organisms. The corresponding bacterial mutants could also be helpful for the understanding of the mechanism of conversion of porphobilinogen (PBG) to UROGEN III. This step is thought to involve the interaction of UROGEN I synthase and UROGEN III cosynthase and the mechanism of this interaction is highly controversial (Lascelles, 1964; Tait, 1968; Frydman *et al.*, 1975; Higuchi & Bogorad, 1975; Shemin, 1975).

The screening of a large number of haem-deficient mutants of *Escherichia coli* K12 led to the identification of a new type of mutant which accumulated URO I and COPRO I. Such an accumulation would be expected for a mutant deficient in UROGEN III cosynthase activity (Fig. 1) and was confirmed by further biochemical studies. The results of these studies on the mutant and of the genetic mapping of the corresponding *hemD* gene are described in the present report.

METHODS

Bacterial strains. The derivatives of *E. coli* K12 used for biochemical and genetic studies are listed in Table 1.

Media. The basic medium for growing the bacterial strains was brain heart infusion (BHI; Difco) supplemented, when necessary, with 1.5 to 2% (w/v) Bacto-agar (Difco). Simmons agar base (Difco), supplemented with 0.4% (w/v) glucose and the required growth factors, was the synthetic medium used for genetic analysis.

Chemicals. Organic solvents for the extraction and identification of porphyrins were reagent or USP grade. Peroxides were removed from diethyl ether as described by Perrin *et al.* (1966). Haemin, porphyrin methyl esters and porphyrin precursors were obtained from Sigma and were Sigma grade, except for uroporphyrin I octamethyl ester (85% pure). Several samples of pure porphyrin esters were the generous gift of S. F.

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Fig. 1. Simplified scheme of the biosynthesis of haem (Bogorad, 1958; Lascelles, 1964; Tait, 1968; Shemin, 1975). 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*	Origin	Source
SASZ31	<i>hemD31</i> mutation in SAS245	Neomycin selection	This laboratory
SAS245	<i>metE46, chlB2, his-1, argH1, thi-1, xyl-7, str-121</i>	†	This laboratory
Hfr Cavalli	<i>metB1, rel-1</i> ; Hfr PO2A		W. Hayes
SAS343	<i>cya-2, thr-1, leu-6, pro-36, trp-42, ilvD75, lacY1, gal-6, mtl-2, str-9</i> ; F ⁻	P1 LS853 × SAS229	This laboratory
SAS229	<i>thr-1, leu-6, pro-36, trp-42, ilvD75, metE46, lacY1, gal-6, mtl-2, str-9</i> ; F ⁻	†	This laboratory
LS853	<i>cya-2, trpR55, trpA9605, his-85, (λ)⁻</i> ; F ⁻	L. Sol strain	B. Bachmann (CGSC no. 5381)
PA6021	<i>thr-1, leu-6, thi-1, argH1, purE43, pro-36, trp-42, lacY1, xyl-7, mtl-2, ara-13(?)</i> , <i>malA1, gal-6, tonA2(?)</i> , <i>λ^r, str-9, rel-1(?)</i> (λ) ⁻ ; F ⁻		F. Jacob
AB684	<i>ilvD75, purF1, argH1, xyl-7, sup-16(?)</i> (unmapped amber suppressor); Hfr		E. Adelberg

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

† Origin of these strains may be obtained from the author on request.

MacDonald. Biuret reagent was obtained from Hycl, 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. Haem-deficient mutants were isolated by selection with neomycin as described previously (Säsärman *et al.*, 1970).

Extraction and identification of porphyrins from bacterial cultures. Bacteria were grown for 3 d at 37 °C in Roux bottles and harvested by washing the agar with saline. The cells were collected by centrifugation. The bacterial pellet and the supernatant were extracted separately. Porphyrins were extracted by the ether-cyclohexanone method as adapted for bacteria (Säsärman *et al.*, 1975). 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) and was expressed in nmol (g dry wt bacteria)⁻¹.

Identification of isomers of URO and COPRO. Free porphyrins were esterified by the methanol-sulphuric acid method (Falk, 1964). Isomers of URO were identified by the method of Falk & Benson (1953) and of COPRO by the method of Chu *et al.* (1951), using Chromagram cellulose thin-layer plates (Eastman Organic Chemicals Division) as described by Gajdos & Gajdos-Török (1969) and Gajdos-Török (1968).

Synthesis of pyrroles in cell-free extracts of bacteria. Bacteria were grown aerobically at 37 °C to late-stationary phase (18 h) in BHI. After separation by centrifuging, the cells were 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 at 4 °C. Extracts were used immediately or stored at -20 °C. The protein content of the extracts was determined by the biuret method (Layne, 1957); it varied from 20 to 30 mg ml⁻¹.

Pyrrole synthesis in cell-free extracts of bacteria was assayed by two methods. In the first, the assay

Table 2. Porphyrin synthesis in cell extracts

Extracts were assayed in mixtures containing 0.5 μ mol PBG and 3.3 mM-EDTA in 0.1 M-Tris/HCl buffer, pH 8.2, as described in Methods.

Strain	Incubation conditions	Accumulation of porphyrins (nmol)			
		URO	COPRO	PROTO	Total
sasZ31	Aerobic (4 h)	43*	0	0	43
	Anaerobic (16 h)+ aerobic (2 h)	86	trace	0	86
sas245	Aerobic (4 h)	31†	2	4	37
	Anaerobic (16 h)+ aerobic (2 h)	0	39	47	86

* URO I. † URO I+URO III.

mixture, containing 1 ml extract, 0.5 μ mol PBG and 3.3 mM-EDTA in 0.1 M-Tris/HCl buffer, pH 8.2 (Bogorad, 1962), was incubated aerobically at 29 °C for 4 h. In the second, 2 ml extract were added to the same ingredients and incubated for 16 h in anaerobiosis; then a further 1 ml extract was added to the mixture and incubation was continued for another 2 h in aerobiosis at 29 °C ('anaerobic-aerobic' method). Porphyrins synthesized in the various mixtures were extracted by the ether-cyclohexanone method (Säsärman *et al.*, 1975).

Assay of catalase activity. Catalase activity was assayed by the method of Herbert & Pinsent (1948).

Mapping of the hemD locus. Mapping of the *hemD* locus was performed by phage P1-mediated transduction (Lennox, 1955). To increase the frequency of transduction, which is very low with haem-deficient recipients, the phage lysates were irradiated by an optimal dose of ultraviolet light (Wilson, 1960).

RESULTS

Biochemical study of the mutant

Strain SASZ31 originates from *E. coli* K12 SAS245 (Table 1) and was obtained by selection with neomycin as described previously (Säsärman *et al.*, 1970). As expected for a haem-deficient mutant, it formed dwarf colonies on BHI agar (Difco). Prolonged incubation of the mutant on BHI agar led to the accumulation of large amounts of URO [162 nmol (g dry wt)⁻¹] and COPRO [646 nmol (g dry wt)⁻¹]. The isomer of COPRO, as determined by the method of Chu *et al.* (1951), was isomer I, which one would expect to be accumulated in UROGEN III cosynthase deficiency (Fig. 1).

To confirm the supposed deficiency of the mutant, porphyrin synthesis by cell extracts was examined in aerobic and anaerobic ('anaerobic-aerobic' method) conditions (see Methods). In both conditions, the mutant synthesized only URO (URO I) although the yield was better with the second method (Table 2). The absence of COPRO is paradoxical since synthesis *in vivo* gives predominantly COPRO I. Similar results were obtained earlier with an UROGEN III cosynthase-deficient mutant of *Salmonella typhimurium* (Säsärman & Desrochers, 1976). After 4 h aerobic incubation (first method) extracts of the original strain, sas245, synthesized mainly URO; the analysis of the isomers showed a mixture of URO I and URO III (Table 2). When the incubation was prolonged and synthesis took place mainly in anaerobiosis ('anaerobic-aerobic' method), only COPRO and proto-porphyrin (PROTO) were found (Table 2). These results demonstrate the efficiency of the second method since both porphyrins derive from UROGEN III.

The catalase activity of the mutant was negligible when tested in 18 h cultures, but clearly positive in 48 h cultures (Fig. 2). However, even in 48 h cultures, the catalase activity of the mutant did not reach the level of the activity of the original strain. The 'delayed' synthesis of catalase by the mutant would suggest that haem synthesis by a deficient enzyme continues during the stationary phase. In contrast, the catalase activity of the original strain was not influenced by the age of the culture in the limits of 18 to 48 h.

Fig. 2. Catalase activity in cultures of different ages of *E. coli* K12 SASZ31 (18 h, ○; 48 h, ●) and *E. coli* K12 SAS245 (18 h, □; 48 h, ■).

Table 3. Phage P1-mediated transduction experiments

Donor	Recipient	Selected marker	No. of transductants analysed	Donor alleles in transductants (%)		
				<i>ilvD</i>	<i>cya</i>	<i>metE</i>
SAS343	SASZ31	<i>hemD</i> ⁺	483	54.2	54.2	8.1
AB684	SASZ31	<i>hemD</i> ⁺	196	54.1		10.2
Hfr Cavalli	SASZ31	<i>hemD</i> ⁺	100			12
PA6021	SAS343	<i>ilvD</i> ⁺	196	100	26.9	
LS853	SAS229	<i>ilvD</i> ⁺	104	100	13.5	4.8
LS853	SAS229	<i>metE</i> ⁺	104	7.7	26.9	100

Table 4. Analysis of classes of transductants*

Class of transductants	No. of transductants	No. of crossovers if the sequence is:		
		<i>hemD-ilvD-cya</i>	<i>ilvD-hemD-cya</i>	<i>ilvD-cya-hemD</i>
<i>ilvD</i> ⁺ <i>hemD</i> ⁺ <i>cya</i> ⁺	108	2	2	2
<i>ilvD</i> ⁺ <i>hemD</i> ⁺ <i>cya</i>	113	4	2	2
<i>ilvD</i> <i>hemD</i> ⁺ <i>cya</i>	148	2	2	2
<i>ilvD</i> <i>hemD</i> ⁺ <i>cya</i> ⁺	114	2	2	4
Total	483			

* Cross P1/SAS343 × SASZ31 (Table 3).

Mapping the *hemD* gene

The results of P1-mediated transduction experiments are presented in Table 3. The data obtained in the cross P1/SAS343 × SASZ31 suggest that *hemD* is located between *ilv* and *cya*, as the frequencies of associated transduction with both genes were the same (54.2%). The high frequency of cotransduction of *hemD* with *ilv* was confirmed in an independent cross (P1/AB684 × SASZ31, Table 3). The results of reference crosses (Table 3) were generally in agreement with the results expected for the corresponding markers, according to the linkage map of *E. coli* K12 (Bachmann *et al.*, 1976). However, they indicate that *cya* might be closer to *metE* than shown on the published map.

The results of the analysis of classes of transductants (Table 4) confirm the sequence *ilv-hemD-cya*, suggested by the analysis based on linkage frequencies. Hence, both data suggest that *hemD* is located in *E. coli* K12 between *ilv* and *cya* at about min 83 of the chromosomal map.

DISCUSSION

The accumulation of URO and COPRO I by the mutant clearly indicates a deficiency in the conversion of PBG to UROGEN III. This defect may be due to a mutation in the *hemD* gene which codes for UROGEN III cosynthase. A mutation in the *hemC* gene coding for UROGEN I synthase is less probable (Säsärman & Desrochers, 1976) since mixed extracts of the *hemD* mutant and of a *hemC* mutant were able to form PROTO from PBG; the same extracts separately could not synthesize PROTO when incubated with PBG (results not shown).

The identification of the first UROGEN III cosynthase-deficient mutant in *E. coli* K12 provides the opportunity of studying the highly controversial step of UROGEN III synthesis (Lascelles, 1964; Tait, 1968; Frydman *et al.*, 1975; Higuchi & Bogorad, 1975; Shemin, 1975) with a bacterial model. In such a study, a great number of *hemD* and *hemC* mutants as well as their reciprocal complementation should be investigated. The improved method of assay of porphyrin synthesis by cell extracts described in the present report could facilitate a large-scale investigation.

The mapping of the *hemD* gene suggests that it is located between *ilv* and *cya*. This location is far from that of other *hem* genes of *E. coli* K12 with the exception of the *popE* gene mapped by Powell *et al.* (1973). The *popE* gene corresponds to the *hemC* gene in our nomenclature. The *hemD* and *hemC* genes are also closely located in *S. typhimurium* (Säsärman & Desrochers, 1976). However, in *S. typhimurium* both genes are located on the same side of the *cya* gene, whereas in *E. coli* they are separated by *cya*. Since the location of the *popE* gene is provisional (Bachmann *et al.*, 1976; Powell *et al.*, 1973), it is not impossible that the *popE* gene is located between *ilv* and *cya* rather than between *cya* and *metE* as indicated on the published map.

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