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Porphobilinogen-accumulating Mutants of *Salmonella typhimurium* LT2

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SUMMARY

Four independent porphobilinogen-accumulating mutants of *Salmonella typhimurium* LT2 were isolated by selecting for dwarf colony formation on neomycin agar media. Cell-free extracts of the parent strain, but not of the mutants, were able to convert 5-aminolaevulinic acid or porphobilinogen to porphyrins. The results indicated that the mutants were deficient in uroporphyrinogen I synthase (EC. 4.3.1.8) activity: these are the first mutants of this type reported in *S. typhimurium* LT2. Mapping of the *hemC* locus (for uroporphyrinogen I synthase) by F-mediated conjugation and by P22-mediated transduction showed the gene sequence *ilvEDAC–hemC–cya–metE*.

INTRODUCTION

Porphobilinogen (PBG) was isolated by Westall (1952) from a patient with acute porphyria. Cookson & Rimington (1953, 1954) established its chemical structure and Falk, Dresel & Rimington (1953) its role in porphyrin biosynthesis. Being an early precursor of haem (Fig. 1), it often accumulates when porphyrin biosynthesis is affected. Patients with some types of porphyrias have urine that is rich in PBG (Bauer, Ackerman & Toro, 1968; Gajdos & Gajdos-Török, 1969). Bacterial mutants accumulating PBG are also known (Lascelles & Hatch, 1969; Powell *et al.*, 1973; Berek, Miczak & Ivanovics, 1974). However, under normal physiological conditions wild-type bacteria do not accumulate PBG; and so its accumulation in the absence of porphyrin accumulation points to a deficiency in uroporphyrinogen I synthase (EC. 4.3.1.8, Fig. 1). This enzyme is now well characterized (Bogorad, 1958; Jordan & Shemin, 1973).

Mapping of the *hem* genes in *Salmonella typhimurium* is less advanced than that of other genes (Săsărman *et al.*, 1970; Sanderson, 1972). This is mainly due to the slow growth of these haem-deficient mutants, since *S. typhimurium* like other Enterobacteriaceae is not able to take up haemin (Ivanovics & Koczka, 1952; Săsărman *et al.*, 1968). The two *hem* genes that have been mapped in this organism are *hemA* and *hemB* (Săsărman *et al.*, 1970). The *hemA* mutants lack 5-aminolaevulinic acid (5-ALA) synthase, but the basis for haem-deficiency in *hemB* mutants is not yet known.

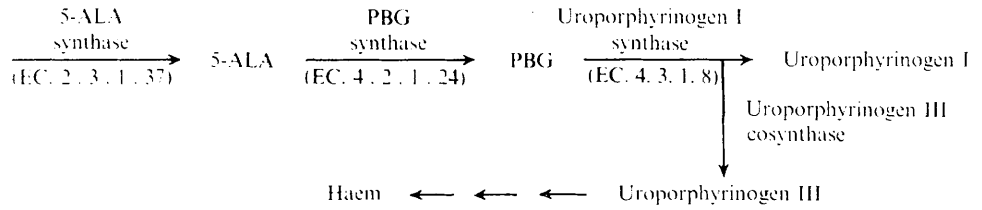

Fig. 1. Early steps in haem biosynthesis (Lascelles, 1964; Tait, 1968; Gajdos & Gajdos-Török, 1969). 5-ALA, 5-aminolaevulinic acid; PBG, porphobilinogen.

To identify *hem* genes not yet mapped in *S. typhimurium* LT2, new haem-deficient mutants were detected among mutants selected for low-level resistance to neomycin. Four of these accumulated PBG and their cell-free extracts failed to convert 5-ALA or PBG to porphyrins; these mutants are in a newly identified gene locus, *hemC*.

METHODS

Bacterial strains. The derivatives of *S. typhimurium* LT2 used for the selection of haem-deficient mutants and for genetic studies are listed in Table 1.

Media. The basic medium used for growing the bacterial strains was brain–heart infusion (BHI, Difco). This was supplemented with 0·2 % glucose and 0·5 % yeast extract (Difco) for growing haem-deficient mutants. Pyrrole accumulation by bacteria was studied with cultures grown on BHI agar, supplemented, when indicated, with 5-aminolaevulinic acid (5-ALA, 50 μg ml^{-1}). Simmons agar base (Difco), supplemented with 0·4 % glucose and the required growth factors, was the synthetic medium used for genetic analysis.

Chemicals. Organic solvents used for the extraction and identification of porphyrins were reagent or USP grade. Peroxides were removed from diethyl ether as described by Perrin, Armarego & Perrin (1966). Porphyrin methyl esters and porphyrin precursors were from Sigma. Biuret reagent was obtained from Hycel Inc. (Houston, Texas, U.S.A.) and the human protein standard from Dade (Miami, Florida, U.S.A.).

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

Accumulation of pyrroles by bacterial cultures. Bacteria were grown for 3 days at 37 °C in Roux bottles and were harvested with saline. The saline was kept in contact with the agar for 30 min in order to extract the PBG from the medium. The cells were separated by centrifuging, and porphyrins were extracted separately from the bacterial pellet and from the supernatant. Porphobilinogen was only assayed in the supernatant.

Pyrrole synthesis in cell-free extracts of bacteria. Bacteria were grown overnight in brain–heart infusion broth without shaking and disintegrated in the frozen state in an X-Press model X-5 cell disintegrator (Biotec Inc., Rockville, Maryland, U.S.A.). The preparation was resuspended in 1 to 2 vols of 0·1 M-tris or 0·1 M-tris–potassium phosphate buffer pH 8·0 to 8·2 (for details see Tables 3 and 4), centrifuged for 30 min at 24000 *g*, and the supernatant retained. These cell-free extracts were used immediately or stored at −20 °C. The protein content of the cell-free extracts was determined by the Biuret method (Layne, 1957); it varied from 10 to 20 mg ml^{-1}. Pyrrole synthesis by cell-free extracts was assayed in the presence of 5-ALA (0·59 μmol) or PBG (0·5 μmol) in 0·1 M-tris or 0·1 M-tris–potassium phosphate buffer pH 8·0 to 8·2 (Bogorad, 1958); reaction was started with substrate and incubation was at 37 °C for 4 h.

Table 1. *Derivatives of Salmonella typhimurium* LT2 *used*

Designation	Genotype	Order of injection of markers (Hfr derivatives)	Source*
SA535 (Hfr K5)	*serA13 rfa-3058*	o–*metG–aroD–purG...his*	SGSC
SA536 (Hfr K6)	*serA13 rfa-3058*	o–*xyl–cysE–ilv...malA*	SGSC
LT2	prototroph		L. Le Minor
SASY32	*hemC32*		This study
SASY44	*hemC44*		This study
SASY52	*hemC52*		This study
SASY53	*hemC53*		This study
SU633	*ilv-401 metE338*		SGSC
	ilvA2		SGSC
	ilvCB		SGSC
	ilvD18		SGSC
	ilvE13		SGSC
DB99	*purC7 purI590 proA46 ilvA405 rfa-461 flaA56 strA metE cya*		D. Berkowitz

* SGSC, Salmonella Genetic Stock Centre, University of Calgary, Canada. L. Le Minor, Institut Pasteur, Paris. D. Berkowitz, University of Pennsylvania, Philadelphia, U.S.A.

Extraction of porphyrins. Porphyrins were extracted separately from the bacterial pellet and from the supernatant by the ether–cyclohexanone method (Dresel, Rimington & Tooth, 1956; Kennedy, 1956; Falk, 1964), as adapted by Săsărman *et al.* (1975). Porphyrinogens formed in cell-free extracts were converted, before extraction, to porphyrins by shaking with 0·2 vols of 0·01 % iodine solution for 20 min in the dark. The homogeneity of each porphyrin fraction was assayed by ascending paper chromatography on Whatman no. 1 paper using the solvent system 2,6-lutidine–water (5:3, v/v) in an ammonia atmosphere (Eriksen, 1958; Falk, 1964). The yield of porphyrins was calculated using the molar extinction coefficients for free porphyrins with the corrections recommended by Rimington (1960) and Porra & Falk (1964), and was expressed in nmol/g dry wt bacteria.

Assay of PBG. Porphobilinogen was assayed as described by Mauzerall & Granick (1956). By using cell-free extracts of bacteria, the sample was free from interfering substances, as recommended by Gajdos & Gajdos-Török (1969) for 5-ALA assay in blood serum. The modified Erhlich reagent was used throughout and yields were expressed in nmol PBG/g dry wt bacteria.

Catalase activity. Bacteria were grown for 48 h at 37 °C and washed twice with buffered saline pH 7·0. The cells were suspended to give an extinction at 600 nm of 60 (approx. 15 mg dry wt bacteria/ml) and catalase activity was assayed as recommended by Herbert & Pinsent (1948).

Mapping of the hemC locus. Mapping of the *hemC* locus was performed by F factor-mediated conjugation and by transduction with phage P22, according to the methods described by Sanderson & Demerec (1965), and by Săsărman *et al.* (1970).

Table 2. *Pyrrole accumulation by* LT2 (*hemC*$^+$) *and by haem-deficient mutants of S. typhimurium*

The mutants and the parent strain were grown on BHI agar, with or without a supplement of 5-aminolaevulinic acid (50 μg ml^{-1}). Porphobilinogen and porphyrins were assayed as described in Methods: concentrations are expressed in nmol/g dry wt.

	Porphobilinogen		Porphyrins	
Strain	BHI agar	BHI agar with 5-ALA	BHI agar	BHI agar with 5-ALA
SASY32	2120	2053	0	0
SASY44	1511	1149	0	0
SASY52	1602	1319	0	0
SASY53	1859	1113	0	0
LT2	0	238	Traces	891*

* Uroporphyrin 510, coproporphyrin 170 and protoporphyrin 211.

Table 3. *Porphyrins formed from PBG in cell-free extracts of bacteria*

Cell-free extracts were prepared as described in Methods. Samples containing either 20 mg protein (extracts from mutants) or 10 mg protein (extracts from wild type) were incubated with porphobilinogen (PBG) and *p*-chloromercuribenzoic acid (PCMB) in 0·1 M-tris buffer pH 8·0 at 37 °C for 4 h. Final volumes were 2 to 3 ml for mutants and 1·5 ml for the wild type.

Strain	Added PBG (μmol)	PCMB (M)	Porphyrins formed (nmol)
SASY32, SASY44, SASY52, SASY53	0·5	0	0
LT2	0·5	0	44
LT2	0·5	10^{-3}	0
LT2	0	0	0

RESULTS

Selection of haem-deficient mutants

Salmonella typhimurium LT2 was plated on to BHI agar containing 25 μg neomycin ml^{-1}, and dwarf colonies were selected. Four of these were haem deficient and were designated SASY32, SASY44, SASY52 and SASY53. These four all gave wild-type recombinants in P22-mediated intercrosses, so they must be of independent origin.

Biochemical studies with the mutants

Accumulation of pyrroles. The mutants accumulated great quantities of PBG, but porphyrins were not detected in their cultures (Table 2). The addition of 5-ALA to the medium did not induce the accumulation of porphyrins by the mutants and did not increase their PBG accumulation. The parent strain, LT2, accumulated pyrroles only in the presence of 5-ALA; the quantity of PBG that it accumulated was less than 25% of that accumulated by the mutant strains under the same conditions.

Synthesis of pyrroles by cell-free extracts of bacteria. Cell-free extracts of the parent strain, but not of the mutants, converted PBG to porphyrins (Table 3). The conversion of PBG to porphyrins by the parent strain was inhibited by *p*-chloromercuribenzoic acid (PCMB), indicating that the reaction was enzyme catalysed. When porphyrin synthesis from 5-ALA

Table 4. *Pyrroles formed from 5-ALA in cell-free extracts of bacteria*

Samples of cell-free extract containing 20 mg protein were incubated at 37 °C for 4 h either with or without 0·59 μmol 5-aminolaevulinic acid (5-ALA) in 0·1 M-tris–potassium phosphate buffer pH 8·2, final volume 2 to 3 ml. Porphobilinogen and porphyrins were assayed as described in Methods.

	PBG formed (nmol)		Porphyrins formed (nmol)	
Strain	with 5-ALA	without 5-ALA	with 5-ALA	without 5-ALA
SASY32	116	0	0	0
SASY44	267	0	0	0
SASY52	220	0	0	0
SASY53	267	0	0	0
LT2	0	0	38	0

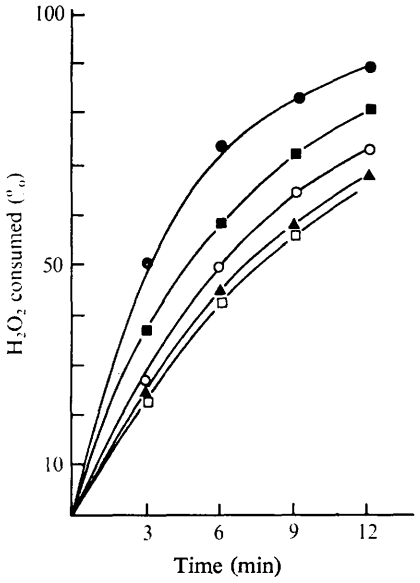

Fig. 2. Catalase activity of *S. typhimurium* LT2 and haem-deficient mutants assayed by the method of Herbert & Pinsent (1948). ●, LT2 (wild type); ○, SASY32; □, SASY44; ▲, SASY52; ■, SASY53.

was assayed (Table 4), the mutants formed PBG but again no porphyrins were detected in their cell-free extracts. Extracts of the parent strain formed porphyrins under the same conditions but not PBG; lack of PBG in these extracts might be due to its degradation, as found in cell suspensions of *Rhodopseudomonas spheroides* (Lascelles, 1956).

The results obtained show a deficiency of uroporphyrinogen I synthase activity in the mutants, which are thus the first mutants of this type reported in *S. typhimurium* LT2.

Catalase activity. The results of catalase determinations are reported in Fig. 2. The four mutants have a surprisingly high catalase activity, approaching that of the parent strain.

Mapping of the hemC locus

The mating experiments performed with two different Hfr strains (Hfr K5 and Hfr K6) resulted in a much higher frequency of *hemC*$^+$ recombinants with Hfr K6. Therefore Hfr K6 (SA536) was used in subsequent interrupted mating experiments; these indicated early transfer of the *hemC* gene by SA536. Since no non-selected markers were available in the respective matings, recombinant analysis could not be performed.

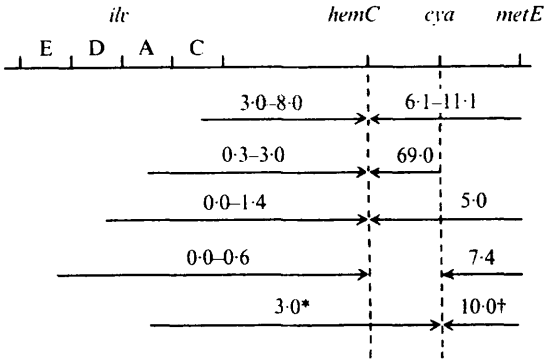

Fig. 3. The percentage of joint transductions of *hemC*, *cya*, *ilv*, and *metE* markers. Data mostly from Table 5 and in text; but * data from J. Gots (personal communication) and † data from Casse *et al.* (1972).

Table 5. *Percentage of joint transductions of different ilv and metE markers with hemC*$^+$

Recipient	Selected marker	Donor alleles in transductants (%) *ilvE13*	*ilvD18*	*ilv-401*	*ilvA2*	*ilvC8*	*metE338*
SASY32	*hemC*$^+$	0 (250)*	0·4 (271)	0·5 (205)	0·3 (304)	3·0 (336)	6·5 (205)
SASY44	*hemC*$^+$	0·4 (236)	0 (217)	0·4 (221)	3·0 (221)	8·0 (259)	9·5 (221)
SASY52	*hemC*$^+$	0·6 (162)	1·4 (118)	2·2 (225)	1·5 (119)	6·0 (203)	11·1 (225)
SASY53	*hemC*$^+$	—	—	0·5 (194)	—	—	6·1 (194)

* The number in brackets indicates the total number of *hemC*$^-$ transductants tested.

P22-mediated joint transduction was tested using strains with mutations in the *ilv* and the *metE* genes as donors (selected marker *hemC*$^+$) since these genes are also donated early by Hfr K6 (Table 1). The results of the transduction experiments reveal that *hemC* alleles are jointly transduced with both *ilv* and *metE* (Table 5). In addition, they indicate the gene order *ilv–hemC–metE* since *ilv* and *hemC* are jointly transduced 0 to 8·0 %, *hemC* and *metE* are jointly transduced 6·1 to 11·1 %, whereas joint transduction cannot be detected between *ilv* and *metE*. In order to locate the position of *hemC* with respect to *cya*, DB99 (*cya*$^-$ *metE*$^-$) was crossed as P22-transduction donor with SASY32 (*hemC4*) and Hem$^+$ recombinants were isolated and tested for unselected markers. Among 78 recombinants, four were Cya$^-$ Met$^-$, 49 were Cya$^-$ Met$^+$, none were Cya$^+$ Met$^-$, and 25 were Cya$^+$ Met$^+$. These data show the following frequencies of joint transduction: *hemC–cya*, 69 %; *hemC–metE*, 5 %; *cya–metE*, 7·4 %. The most probable gene order, based on the crossover pattern, is *hemC–cya–metE*. A summary of the joint transduction data, showing the inferred gene sequence, is given in Fig. 3.

DISCUSSION

In the past, aminoglycoside antibiotics were often used to isolate haem-deficient mutants of bacteria (for details see Săsărman *et al.*, 1975). Being a direct selective procedure, the method has the advantage of enabling the isolation of mutants which occur with a very low frequency. Using the neomycin selection method, we have obtained mutants in the following genes of *S. typhimurium* LT2: *hemA*, *hemB* (Săsărman *et al.*, 1970), *hemC* (this paper) and *hemD* (Săsărman & Sonea, 1975); and in the following genes of *E. coli* K12: *hemA*, *hemB* (Săsărman *et al.*, 1968), *hemD* (Săsărman & Sonea, 1975), and *hemE* (Săsărman *et al.*, 1975). Using a different method (Cox & Charles, 1973), Powell *et al.* (1973) have obtained several new types of haem-deficient mutants, which they designated *pop* mutants (for the nomenclature of haem-deficient mutants see Săsărman *et al.*, 1975). Thus the two methods seem to be complementary, each having advantages for the study of haem-deficient mutants of bacteria.

Several PBG-accumulating mutants have been described in bacteria (Lascelles & Hatch, 1969; Hatch & Lascelles, 1972; Powell *et al.*, 1973; Berek *et al.*, 1974). Hatch & Lascelles (1972) showed that the addition of 5-ALA greatly increased the accumulation of PBG by a mutant of *Rhodopseudomonas spheroides*, but had no effect on its 'revertant'. In this respect our mutants behaved like the 'revertant' of Hatch & Lascelles (1972), since they did not increase the accumulation of PBG in response to the addition of 5-ALA (see Table 2).

The presence of catalase activity in *hemC* mutants of *S. typhimurium* shows that they must have some uroporphyrinogen I synthase activity, although it was too low to be measured by the technique used. The poor growth of the mutants, on the other hand, shows that some other haemoproteins may be lacking. Thus the small amount of haem formed by the mutants would be incorporated preferentially into only some of the haemoproteins.

A porphobilinogen-accumulating mutant of *E. coli* K12 was recently described by Powell *et al.* (1973). The locus affected in the mutant, designated *popE*, was cotransducible with the *ilv* and *metE* genes, but the order of genes was not determined (Powell *et al.*, 1973). Our results show clearly that in *S. typhimurium*, the *hemC* gene maps at 122 min, between the *ilv* and *metE* genes. This is a long way from the other known *hem* genes of this organism, *hemA* gene mapping at 50 min, and *hemB* at 13 min on the linkage map (Săsărman *et al.*, 1970). Knowing the close homology of the chromosomes of the two organisms, it may be supposed that the *hemC* gene in *E. coli* also maps between the *ilv* and *metE* genes.

Our data confirm the orientation of the *ilv* cluster of genes to be as reported earlier (Sanderson, 1972), because *ilvE*, known by transduction data to be at one side of the cluster, gave a low frequency of joint transduction with *hemC* (0 to 0·6%) whereas *ilvC*, known to be at the other side of the cluster, gave a much higher frequency of joint transduction (3·0 to 8·0%). The genes *ilvD* and *ilvA*, known to be in the middle of the cluster, gave intermediate percentages (Fig. 3).

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