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Uroporphyrinogen III Cosynthase-Deficient Mutant of *Salmonella typhimurium* LT2

A. SÅSÅRMAN* AND M. DESROCHERS

Department of Microbiology and Immunology, University of Montreal, Montreal, 101, Quebec, Canada

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A new type of heme-deficient mutant of *Salmonella typhimurium* LT2 was isolated using neomycin. The mutant, designated as strain SASY74, accumulated uroporphyrin I and coproporphyrin I. Extracts of the mutant converted 5-aminolevulinic acid to uroporphyrin I. Extracts of the mutant SASY74 and of the uroporphyrinogen synthase-deficient mutant SASY32 complemented each other and converted, when incubated together, 5-aminolevulinic acid to protoporphyrin. This finding excludes the possibility that uroporphyrinogen I synthase in strain SASY74 is deficient in its cosynthase-binding ability. Hence, the most probable explanation for the accumulation of uroporphyrin I and coproporphyrin I by the mutant is the lack of the uroporphyrinogen III cosynthase activity. This mutant is the first isolated in bacteria with such deficiency, and the mutation is analogous, as far as porphyrin synthesis is concerned, to human congenital porphyria. Mapping of the corresponding gene (*hemD*) by conjugation and P22-mediated transduction suggests the following gene order on the chromosome: *ilv. . . . hemC, hemD, cya. . . . metE*. The *hemC* and *hemD* genes are probably adjacent; this is the first case in which two *hem* genes of *Enterobacteriaceae* are contiguous on the chromosomal map.

The conversion of porphobilinogen to uroporphyrinogen III (UROGEN III) is the most controversial step in the biosynthesis of porphyrins (7, 11, 28). This conversion is accomplished by the cooperation of two enzymes, UROGEN I synthase and UROGEN III cosynthase (Fig. 1). The latter was purified by Bogorad (3), and Higuchi and and Bogorad (11), and identified as a heat-labile protein with a molecular weight of about 6.2×10^4 (11). When UROGEN III cosynthase is lacking, the Urogen I synthase can only form UROGEN I (2). However, neither UROGEN I, nor any other products of UROGEN I synthase activity, can serve as a substrate for UROGEN III cosynthase (7, 11, 28). Therefore, the synthesis of UROGEN III, which is the true intermediate in porphyrin biosynthesis, must result from physical interaction of the two enzymes (11). Such an interaction was demonstrated recently by Frydman and Feinstein (8) and by Higuchi and Bogorad (11), but the exact mechanism is not fully understood.

One approach to the study of the mechanism of this interaction is the use of bacterial mutants. Many types of heme-deficient mutants have already been described in bacteria (1, 10, 20, 24, 25); however, none is deficient in UROGEN III cosynthase activity. The isolation of such a mutant of *Salmonella typhimurium* LT2 is reported here. The genetic mapping of this mutation allowed the identification of a new *hem* gene, the *hemD* gene, on the chromosome of *S. typhimurium* LT2.

MATERIALS AND METHODS

Bacterial strains. The various derivatives of *S. typhimurium* LT2 used for biochemical and genetic studies are listed in Table 1.

Media. The basic medium used for growing the bacterial strains was Brain heart infusion (Difco), supplemented, when necessary, with 1.5 to 2% agar (Difco). 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. Absolute dry methanol was prepared as recommended by Falk (5). Diethyl ether was washed before use with several volumes of water. Chloroform was washed with water and dried (5).

Porphyrin methyl esters and porphyrin precursors were obtained from Sigma Chemical Co., St. Louis, Mo., and were Sigma grade except for uroporphyrin I (URO I) octamethyl ester (85% pure). Several samples of pure porphyrin esters were the generous gift of S. F. MacDonald, Division of Biological Sciences, National Research Council of Canada, Ottawa. Neomycin sulfate was obtained from Sigma Chemical Co.

Selection of heme-deficient mutants. Heme-defi-

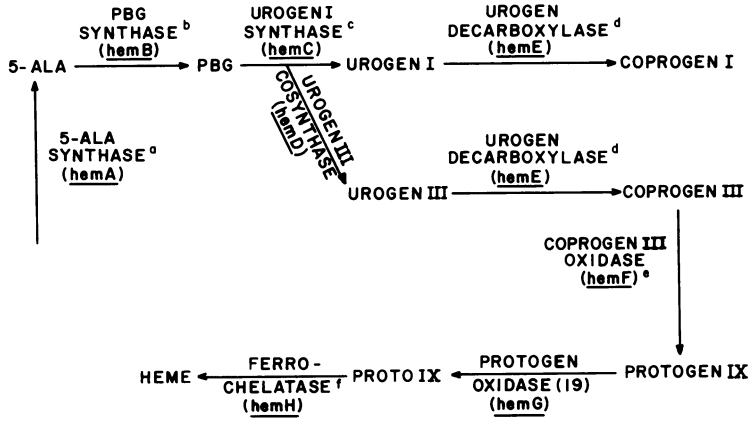

FIG. 1. *Revised heme biosynthetic pathway (15, 19, 29) and the proposed designation of hem genes in microorganisms (1, 24, and present paper). EC numbers of the enzymes are: (a) 2.3.1.37; (b) 4.2.1.24; (c) 4.3.1.8; (d) 4.1.1.37; (e) 1.3.3.3; (f) 4.99.1.1. UROGEN, Uroporphyrinogen; COPROGEN, coproporphyrinogen; PROTOGEN, protoporphyrinogen; PROTO, protoporphyrin. Nb, the function of the gene designated originally as "hemB" in E. coli (27) and S. typhimurium (26) has not been elucidated, and does not correspond to that shown here for a hemB gene.*

TABLE 1. *Salmonella typhimurium LT2 derivatives used*

Strain	Genotype	Source[a]
SASY74	*hemD74* mutation in SAS503	
SAS503	Cya$^+$ revertant of SA1361; supposed *cya*$^+$	
SA1361	*cya*$^-$ *metE*$^-$ *purC7 purI590 proA46 ilvA405 rha-461 fla-506 strA*$^-$	SGSC
SASY32	*hemC32* mutation in LT2	25
SASY44	*hemC44* mutation in LT2	25
SASY52	*hemC52* mutation in LT2	25
SASY53	*hemC53* mutation in LT2	25
LT2	Prototroph	L. Le Minor
SA536 (Hfr K6)	*serA13 rfa-3058;* order of injection of markers: *O-xyl-cysE-ilv . . . malA*	SGSC
SK5	*pyrB16 cya-69*	J. L. Ingraham

[a] L. Le Minor, Institut Pasteur, Paris; SGSC, Salmonella Genetic Stock Centre, University of Calgary, Canada; J. L. Ingraham, University of California, Davis.

cient mutants were selected from strain SAS503 using neomycin as described previously (26); to stimulate porphyrin production by the mutants, 50 μg of 5-aminolevulinic acid (5-ALA) per ml was incorporated into the selection medium (24).

Extraction and separation of porphyrins. Bacteria were grown for 3 days at 37°C in Roux bottles, and were harvested by washing the agar with saline. The cells were collected by centrifugation, and the bacterial pellet and the supernatant were extracted separately.

Extraction of porphyrins was performed by the ether-cyclohexanone method (4, 14), as adapted for bacteria (24). The yield of porphyrins was calculated using the molar extinction coefficients for free porphyrins with the corrections recommended by Rimington (21) and Porra and Falk (18), and was expressed in nanomoles per gram of bacteria (dry weight).

Identification of URO isomers I and III. Free porphyrins were esterified by the methanol-sulfuric acid method (5). Isomers I and III of URO I methyl esters were identified by the method of Falk and Benson (6) using chromagram cellulose thin-layer plates (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N.Y.), as described previously (24).

Porphyrin synthesis in extracts of bacteria. Bacteria were grown in brain heart infusion for several hours and disintegrated in an ultrasonic cell disintegrator (model Biosonik III, Bronwill Scientific Inc., Rochester, N.Y.). The preparation was then centrifuged for 40 min at 24,000 × *g*. Extracts were used immediately or stored at −20°C. The protein content of the extracts was determined by the biuret method (16); the protein content in various extracts varied from 21.3 to 26.5 mg per ml.

Porphyrin synthesis in extracts was assayed by a modification of the method by Jacobs et al. (12). The mixture contained 0.33 mM 5-ALA in 0.1 M tris (hydroxymethyl)aminomethane-hydrochloride (pH 8.2) and 2.5×10^{-4} M *o*-phenanthroline. Incubation was for 4 h at 30°C, with shaking.

Porphyrins synthesized by the extracts were ex-

tracted by the ether-cyclohexanone method, as adapted for bacteria (24).

Assay of porphobilinogen in extracts of bacteria. Porphobilinogen was assayed in extracts of bacteria as described previously (24).

Assay of catalase activity. Catalase activity was assayed as described previously (24).

Mapping of *hemD* locus. Mapping of the *hemD* locus was performed by F factor-mediated conjugation and by transduction with phage P22, according to the methods by Sanderson and Demerec (23) and by Säsärman et al. (26). Due to the leakiness of the *hemD* mutant, a direct selection for the *hemD* allele could not be performed; however, the *hemD79* mutation could be used as a nonselected marker.

RESULTS

Isolation and physiology of the mutant SASY74. Strain SASY74 is a "leaky" heme-deficient mutant that differs from other heme-deficient mutants of *Enterobacteriaceae* by the formation of almost normal colonies after prolonged incubation at 37°C. Despite its "leakiness," which is also confirmed by its catalase activity (see below), the mutant seems to be very stable.

The mutant may be easily differentiated from the wild type due to the accumulation of porphyrins: under ultraviolet light the mutant colonies show a characteristic red fluorescence typical of porphyrins. This fluorescence appears more readily at 37 than at 29°C and probably reflects the difference in the rate of oxidation of porphyrinogens at the two temperatures. The accumulation of porphyrins by the mutant on brain heart infusion agar (Difco) is very high and may reach as much as 1,725 nmol of URO I and 1,038 nmol of coproporphyrin I (COPRO I) per g of dry weight, after 72 h of incubation at 37°C. Under the same conditions, the parental strain SAS503 accumulates only traces of porphyrins. The accumulation of URO I and COPRO I by the mutant strongly suggests the possibility of a deficient UROGEN III cosynthase activity (see Fig. 1).

Porphyrin synthesis in extracts of the mutant. The results of porphyrin synthesis in extracts of the mutant SASY74 are recorded in Table 2. The data show that SASY74 converts 5-ALA only to URO I, although the extract of the mutant contains both URO I and COPRO I ("endogenous" porphyrins). Under the same conditions, the extract of the parental strain forms URO, COPRO, and protoporphyrin (PROTO), and there are no "endogenous" porphyrins in the extract. These results are in agreement with the interpretation that the mutant is deficient in UROGEN III cosynthase.

The results of a mixing experiment show that extracts of the mutants SASY74 and SASY32 (UROGEN I synthase-deficient mutant) incubated together convert 5-ALA to PROTO (Table 2). This finding excludes the possibility that SASY74 might be a particular type of UROGEN I synthase-deficient mutant, since in that case, no complementation would be possible between the two mutants.

Catalase activity of the mutant. As expected for a leaky mutant, catalase activity was present (specific activity: 0.37 μmol of O_2/min per mg [dry weight]) although not as high as in the wild type (specific activity: 1.17 μmol of O_2/min per mg [weight]).

Mapping of the *hemD* locus in *S. typhimurium* LT2. Mapping of the *hemD* locus in *S. typhimurium* LT2 was performed by F factor-mediated conjugation and by transduction with phage P22. The results of mating experiments with Hfr SA536 as donor are presented in Table 3. These results show a high frequency of linkage between the *hemD* gene, and the *ilv* and *metE* genes.

For further mapping, several independent

TABLE 2. *Porphyrin synthesis from 5-ALA in extracts of bacteria*[a]

Extract	Net synthesis of porphyrins[b] (nmol)		
	URO	COPRO	PROTO
SASY74	74.5	0	0
SAS503	47	6	4.5
SASY74 + SASY32	46	9	5.5
SASY32[c]	0	0	0

[a] Incubation was with vigorous shaking for 4 h at 30°C in 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.2) and 2.5×10^{-4} M *o*-phenanthroline; the mixture contained 1.0 ml of various extracts (21.3 to 26.5 mg of protein/ml) and 1 μmol of 5-ALA in a final volume of 3.0 ml.

[b] From added 5-ALA; URO, uroporphyrin; COPRO, coproporphyrin; PROTO, protoporphyrin.

[c] Extract contained porphobilinogen accumulated by the mutant (25); this was excluded when net porphyrin synthesis was determined.

TABLE 3. *Results of mating experiments*

Donor	Recipient	Selected marker	No. of recombinants analyzed	Donor alleles in recombinants (%)			
				ilv	*hemD*	*metE*	*pro*
Hfr SA536	SASY74	*ilv*$^+$	93	100	92	86.6	3.2
		pro$^+$	82	52.4	52.4	56.1	100

hemC mutants (25), and a *cya* mutant, which also map in this region (22, 25), were used. The results of transduction experiments, performed with the help of these mutants, are reported in Table 4. As expected, the data show a close linkage between the *hemD*, *hemC*, and *cya* genes. The data also show that *hemC* gene must be located closer to the *hemD* than to the *cya* gene, since the frequency of linkage of the *hemC* alleles, except *hemC32*, is higher with *hemD74*, than with *cya-69*. The 100% frequency of linkage of *hemD74* allele with the *hemC44* and *hemC53* alleles suggests that *hemC* and *hemD* genes are very close to each other, or even contiguous. Unfortunately, the transduction experiments cannot allow the establishment of a definite gene order in this case, since reciprocal three-point crosses cannot be performed with heme-deficient mutants (24). Therefore, until new data are obtained, a provisional *hemC*, *hemD*, *cya* gene order is suggested, without totally excluding the order *hemD*, *hemC*, *cya*.

DISCUSSION

UROGEN III cosynthase deficiency of the mutant. The accumulation of URO I and COPRO I, by the mutant SASY74, suggests that the site of the deficiency is located at the level of UROGEN III cosynthase activity. As may be deduced from Fig. 1, a UROGEN III cosynthase-deficient mutant would accumulate UROGEN I and COPROGEN I, since the latter cannot be converted further to PROTO IX (only COPROGEN III may represent the substrate for COPROGEN III oxidase).

The physical interaction between UROGEN I synthase and UROGEN III cosynthase required for conversion of porphobilinogen to UROGEN III (11) suggests that a second type of URO I- and COPRO I-accumulating mutant could result from defective cosynthase binding to mutationally altered UROGEN I synthase. Such a mutant would behave like a cosynthase-deficient mutant, but would give a paradoxical biochemical complementation test: it would complement a cosynthase-deficient mutant, but not an UROGEN I synthase-deficient mutant. The possibility of complementation between different subunits of the same molecule was not considered here, since no such subunits were described in the case of these two enzymes (9, 11, 13).

The results of mixing extracts of the mutants SASY74 and SASY32 (UROGEN I synthase-deficient control) show, however, the expected behavior for a cosynthase-deficient mutant. This finding excludes the possibility that the SASY74 mutant might be affected in some binding function of the UROGEN I synthase molecule, and indirectly supports the supposed UROGEN III cosynthase deficiency of the mutant. The study of the UROGEN III cosynthase-deficient mutant may be helpful for a better understanding of the mechanism of congenital porphyria (17).

Mapping of the *hemD* locus in *S. typhimurium* LT2. The mutant SASY74 differs from other heme-deficient mutants of *Enterobacteriaceae* in its ability to grow almost normally on synthetic or rich media. The mutant colonies may be easily distinguished from the wild-type colonies by their fluorescence in ultraviolet light. This feature allows the use of the *hemD74* mutation, in contrast to most other *hem* mutations of *Enterobacteriaceae*, as a non-selected marker in genetic analysis.

The results of the mapping of the *hemD* gene in *S. typhimurium* LT2 show that the *hemD* gene is very close or even contiguous to the *hemC* gene. This is the first case in which two *hem* genes of *Enterobacteriaceae* were found to be adjacent. All the other *hem* genes in *Escherichia coli* K-12 (20, 24, 27) and *S. typhimurium*

TABLE 4. *Percentage of joint transductions of hemD and cya markers with various hemC markers*

Donor	Recipient	Selected marker	No. of transductants analyzed	Donor alleles in transductants (%)		
				hemC	*hemD74*	*cya-69*
SASY74	SASY32	*hemC*$^+$	1,202	100	44-66	
SASY74	SASY52	*hemC*$^+$	100	100	65	
SASY74	SASY44	*hemC*$^+$	100	100	100	
SASY74	SASY53	*hemC*$^+$	100	100	100	
SK5	SASY32	*hemC*$^+$	150	100		71
SK5	SASY52	*hemC*$^+$	127	100		58
SK5	SASY44	*hemC*$^+$	128	100		67
SK5	SASY53	*hemC*$^+$	100	100		96
SASY74	SK5	*cya*$^+$[a]	112		54[b]	100

[a] Selection was for the Mal character.

[b] Frequency obtained with another *hemD* mutant (SASY75) was 92%.

LT2 (25, 26) are located far from each other. The contiguity of the *hemC* and *hemD* genes in *S. typhimurium* LT2 may reflect the close cooperation of the corresponding enzymes in the synthesis of UROGEN III.

The present mapping of the *hemD* gene and the recent mappings of the *hemC* (25) and *hemE* (M. Desrochers and A. Săsărman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, H69, p. 107) genes, reduces to only three the number of the *hem* genes of *S. typhimurium* LT2 that are not yet mapped. These genes are *hemF*, *hemG*, and *hemH* (Fig. 1), and among them only *hemG* is not yet mapped in *E. coli* K-12. This progress in the mapping of *hem* genes of *Enterobacteriaceae* will undoutedly provide a better basis for the understanding of the functioning and control of this biosynthetic pathway.

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