JOURNAL OF BACTERIOLOGY, Dec. 1975, p. 1205-1212

Vol. 124, No. 3
Printed in U.S.A.

Uroporphyrin-Accumulating Mutant of *Escherichia coli* K-12

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Received for publication 8 July 1975

An uroporphyrin III-accumulating mutant of *Escherichia coli* K-12 was isolated by neomycin. The mutant, designated SASQ85, was catalase deficient and formed dwarf colonies on usual media. Comparative extraction by cyclohexanone and ethyl acetate showed the superiority of the former for the extraction of the uroporphyrin accumulated by the mutant. Cell-free extracts of SASQ85 were able to convert 5-aminolevulinic acid and porphobilinogen to uroporphyrinogen, but not to copro- or protoporphyrinogen. Under the same conditions cell-free extracts of the parent strain converted 5-aminolevulinic to uroporphyringen, coproporphyrinogen, and protoporphyrinogen. The conversion of porphobilinogen to uroporphyrinogen by cell-free extracts of the mutant was inhibited 98 and 95%, respectively, by *p*-chloromercuribenzoate and *p*-chloromercuriphenyl-sulfonate, indicating the presence of uroporphyrinogen synthetase activity in the extracts. Spontaneous transformation of porphobilinogen to uroporphyrin was not detectable under the experimental conditions used [4 h at 37 C in tris(hydroxymethyl)aminomethane-potassium phosphate buffer, pH 8.2]. The results indicate a deficient uroporphyrinogen decarboxylase activity of SASQ85 which is thus the first uroporphyrinogen decarboxylase-deficient mutant isolated in *E. coli* K-12. Mapping of the corresponding locus by P1-mediated transduction revealed the frequent joint transduction of *hemE* and *thiA* markers (frequency of co-transduction, 41 to 44%). The results of the genetic analysis suggest the gene order *rif, hemE, thiA, metA;* however, they do not totally exclude the gene order *rif, thiA, hemE, metA*.

Mapping of the *hem* genes of *Escherichia coli* K-12 and *Salmonella typhimurium* LT2 is much less advanced then the genetic studies of the other markers of these two organisms. This is mainly due to lack of hemin uptake by these bacteria, which makes it difficult to study their Hem$^-$ mutants. Ivanovics and Koczka (14) were the first to report the absence of adsorption of mesohematin by the *Enterobacteriaceae*; lack of hemin uptake in *E. coli* K-12 was later reported by one of us after observing that growth of HemA$^-$ mutants of this organism was not improved by hemin (33).

Heme-deficient mutants of bacteria are partially resistant to aminoglycoside antibiotics, which were often used for their isolation (1, 2, 16, 29, 30, 31, 37). The mechanisms of this resistance is not yet well established, and a diminished uptake of the drug has been suggested (30). The resistance to aminoglycoside antibiotics seems to be a common property of several types of respiratory mutants of bacteria, for menaquinone-deficient mutants of *Staphylococcus aureus* are also resistant to them (32).

Beljanski and Beljanski (2) isolated the first heme-deficient mutant of *E. coli* using streptomycin selection. Several years later we isolated new heme-deficient mutants of *E. coli* K-12 (19, 33) and mapped the first *hem* locus in this organism. A 5-aminolevulinic acid (5-ALA)-requiring mutant was described by Wulff (39) in the same organism, and a preliminary map location was shown for the affected gene. Subsequently, we mapped the *hemA* locus in *E. coli* K-12 (31) and found a location different from that reported by Wulff. Thus, the two mutants differ from each other, and the mutant of Wulff might be similar to that recently described by Powell et al. (26).

Mapping of *hemA* and *hemB* genes in *Salmonella typhimurium* LT2 showed that both have similar location to the corresponding genes in *E. coli* K-12 (30); this seems also to be true for at least a third *hem* gene in the two organisms (A. Săsărman et al., unpublished data). A relatively exhaustive mapping of *hem* genes in *S. aureus* showed that in this organism the *hem* loci were clustered in a single region of the chromosome (37). A similar finding was also reported in *Bacillus subtilis*, at least for its three known *hem* genes (3).

Important progress in mapping the *hem* genes

of *E. coli* K-12 was recently made by Powell et al. (26). Using the method of Cox and Charles (5), Powell et al. obtained new types of heme-deficient mutant and mapped the corresponding genes. None of the mutants described by Powell et al. (26) was deficient in uroporphyrinogen decarboxylase or in uroporphyrinogen III cosynthetase; such mutants would accumulate uroporphyrin III in the first case and uroporphyrin I and coproporphyrin I in the second (see Fig. 3 and Discussion).

To identify the *hem* genes not yet mapped in *E. coli* K-12, new heme-deficient mutants were selected by neomycin in the present study. A first uroporphyrin-accumulating mutant was eliminated, being for an unknown reason recombination deficient (A. Săsărman and S. Sonea, Abstr. 1st Intersect. Congr. Int. Assoc. Microbiol. Soc. 1974, Tokyo, p. 10). The results obtained with a second uroporphyrin-accumulating mutant which lacks uroporphyrinogen decarboxylase activity are now reported. The genetic studies with this mutant allowed us to map the corresponding locus (*hemE* locus) on the chromosome of *E. coli* K-12.

MATERIALS AND METHODS

Bacterial strains. The various derivatives of *E. coli* K-12 used to obtain heme-deficient mutants and for genetic studies are listed in Table 1.

Media. The basic medium used for the isolation of heme-deficient mutants and for the growth of bacterial strains was brain heart infusion agar (Difco). This medium was supplemented with 50 μg of 5-ALA and 20 μg of neomycin/ml for the selection of the mutants or with 50 μg of 5-ALA/ml for some of the cultures used for the extraction of porphyrins. Brain heart infusion (Difco) was used for growing bacteria for the enzymatic studies; for Hem^- mutants the medium was supplemented with 0.2% glucose and 0.5% yeast extract (Difco) and, in some cases, with 0.2% pyruvic acid.

For the selection of Hem^+ transductants, brain heart infusion agar was used, on which the growth of Hem^- mutants was too weak to be prejudicial to the growth of the former. The other transductants, as well as the analysis of the recombinants, were performed on Simmons agar base (Difco) supplemented with 0.4% glucose and the required growth factors.

Chemicals. Organic solvents used for the extraction and identification of porphyrins were reagent or USP grade except for kerosene. Peroxides were removed from diethyl ether as described by Perrin et al. (24). Absolute dry methanol was prepared as recommended by Falk (9). Chloroform was washed with water and dried (9).

Porphyrin methyl esters and porphyrin precursors were obtained from Sigma Chemicals, Co., St. Louis, Mo., 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. MacDonald. Biuret reagent was obtained from Hycel Inc., Houston, Tex., and the human protein standard was obtained from Dade, Miami, Fla. Neomycin sulfate was obtained from Sigma Chemicals Co.

Selection of heme-deficient mutants. Heme-deficient mutants were selected by neomycin as described previously (30). To detect porphyrin accumulation by the mutants, the medium was supplemented with 50 μg of 5-ALA per ml. Under these conditions, heme-deficient mutants, with the exception of $HemA^-$ mutants, form dwarf colonies which become visible after an incubation of 2 to 3 days; the fluorescence of porphyrin-accumulating dwarf colonies is not visible before day 4 or 5.

Extraction and separation of porphyrins. Bacteria were grown for 3 days at 37 C in Roux bottles and 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 initially by the ethyl acetate-acetic acid method (9), but subsequently the ether-cyclohexanone method (7, 18) was adapted by us for the extraction of porphyrins from bacteria. In this second method, coproporphyrin and protoporphyrin are first extracted by acid ether, until no further fluorescence passes into the ether phase. The ether extract is then washed twice with 0.1 volume of 3% sodium acetate, and the washes are added to the aqueous phase. Coproporphyrin and protoporphyrin are extracted from the ether by 0.1 N HCl and 3 N HCl, respectively. The aqueous phase is then acidified to pH 1.6 with 3 N HCl, and uroporphyrin is extracted by cyclohexanone. Finally, an equal volume of diethyl ether is added to the cyclohexanone phase, and uroporphyrin is extracted by 1.5 N HCl.

The homogeneity of each porphyrin fraction was assayed by ascending paper chromatography on Whatman no. 1 paper in the solvent system 2,6-lutidine–water (5:3, vol/vol) and in ammonia atmosphere (8). The yield of porphyrins was calculated using the molar extinction coefficients for free porphyrins with the corrections recommended by Rimington (28) and Porra and Falk (25) and was expressed in nanomoles per gram (dry weight) of bacteria.

Identification of uroporphyrin isomers I and III. Free porphyrins were esterified by the methanol-sulfuric acid method (9, 11). Isomers I and III of uroporphyrin methyl esters were identified by the method of Falk and Benson (10) using Chromagram cellulose thin-layer plates (Eastman), as recommended by Gajdos-Török (12).

Porphyrin synthesis in cell-free extracts of bacteria. Bacteria were grown overnight in semianaerobic or anaerobic conditions and disintegrated in an X-Press model X-5 disintegrator (Biotec Inc., Rockville, Md.) in a frozen state. The preparation was then suspended in 1 to 2 volumes of tris(hydroxymethyl)aminomethane or potassium phosphate buffer (pH 8.0 to 8.2) and centrifuged for 20 min at 14,000 × *g*. 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 (20); the

TABLE 1. *E. coli K-12 strains used*

Strain	Genotype	Origin	Supplied by:
SASQ85	*hemE85, argH1, purF1, metE46, xyl-7, rel-1?, sup-16, sup-48?*	Hfr AB1931	
Hfr AB1931	*argH1, purF1, metE46, xyl-7, rel-1?, sup-16, sup-48?*; Hfr		E. Adelberg
PA505-1-5	*metA90, argH1, proA44, pps-4, aceA4, thiA*$^-$,[a] *sup-?*,[a] *str-9*, λ^-; F^-	H. Kornberg strain	CGSC (4869) B. Bachmann
Hfr Cavalli	*metB1, rel-1*; Hfr		W. Hayes
Y-53	*thr-1, leu-6, thi-1, lac-1*; F^-	J. Lederberg strain	CGSC (4429) B. Bachmann
C600 rif^r [b]	*thr-1, leu-6, thi-1, lac-1, supE44, tonA21, rif*^{r}, λ^-; F^- [b]	Y. Chabbert strain	Y. Chabbert
SAS251	*argH1, purF1, metE46, thi-1, xyl-7, rel-1?, sup-16, sup-48?, rif*^{r}	Transductants P1 (C600rif^r) × SASQ85; Sel: *hemE*$^+$	
SAS252	*metA90 argH1, aceA4, thiA*$^-$, *purF1, xyl-7, rel-1, sup-16, sup-48?*	Transductant: P1 (CGSC4429) × SASPNC; Sel: *metE*$^+$	
SASPNC	*metA90, argH1, aceA4, thiA*$^-$, *purF1, metE46, xyl-7, rel-1? sup-16, sup-48?*	Transductant: P1 (Pa505-1-5) × SASQ85; Sel: *hemE*$^+$	

[a] Discovered during the mapping of *hemE* locus.
[b] Carries an R factor.

protein content in various assay mixtures varied from 20 to 45 mg.

Porphyrin synthesis in the cell-free extracts was assayed by two methods. In the first, porphyrin synthesis from 5-ALA was assayed as described by Jacobs et al. (15), without determining the individual steps in synthesis. Incubation was for 4 h at 37 C in 0.05 M tris(hydroxymethyl)aminomethane buffer (pH 8.0) with shaking.

In the second method, only the conversion of porphobilinogen to uroporphyrinogen was assayed, as described by Bogorad (4); *p*-chloromercuribenzoate and *p*-chloromercuriphenyl-sulfonate were used as inhibitors. Incubation was for 4 h at 37 C in 0.125 M tris(hydroxymethyl)aminomethane-potassium phosphate buffer (pH 8.2) without shaking.

Extraction of the porphyrins synthesized by the cell-free extracts were performed by the ether-cyclohexanone method. Porphyrinogens were converted to porphyrins by shaking with 0.2 volume of 0.01% iodine solution for 20 min in the dark.

Assay of PBG in cell-free extracts of the heme-deficient mutant. The PBG porphobilinogen method is an adaption of the technique recommended for determination in erythrocyte lysates (11). The cell-free extracts were diluted ½ to ⅓ with distilled water and then freed from interfering substances by the addition of 0.2 ml of 0.06 M iodacetamide to 3 ml of diluted extract. After 1 to 2 min, 1 ml of 20% trichloroacetic acid was added to the mixture. The sample was then shaken vigorously and centrifuged at 10,000 rpm for 10 min. PBG was assayed in the supernatant by the method of Mauzerall and Granick (23).

Assay of catalase activity. Bacteria were grown for 48 h at 37 C and washed twice with buffered saline (pH 7.0). They were then suspended to an optical density of 60 (about 15 mg [dry weight] of bacteria per ml), and catalase activity was assayed by the method of Herbert and Pinsent (13).

Mapping of the hemE locus by P1-mediated transduction. Transduction by phage P1 was performed by the method of Lennox (21). In the case of the heme-deficient mutant, the method was slightly modified to detect very rare transductants. For the same reason, the phage lysates were irradiated by an optimal dose of ultraviolet light (38).

RESULTS

Isolation of heme-deficient mutants by neomycin. Among the 28 independent dwarf

colony-forming mutants selected by neomycin, 14 accumulated various porphyrins. The presence of 5-ALA in the selection medium greatly facilitated the detection of porphyrin-accumulating mutants. One of the mutants, designated as SASQ85, accumulated great quantities of uroporphyrin. Since a preliminary transduction with this strain gave positive results, this mutant was retained for further studies.

Comparison of the ethyl acetate and cyclohexanone methods for extraction of uroporphyrin from bacteria. The results of a comparative extraction by ethyl acetate and cyclohexanone are presented in Fig. 1 (the diethyl ether extraction step was eliminated in this case, as the mutant SASQ85 accumulated only uroporphyrin). It is evident that the cyclohexanone method extracted more uroporphyrin in two steps than the ethyl acetate method did in eight. The low efficiency of the ethyl acetate method was not due to the inactivation of porphyrins, since one additional extraction by cyclohexanone (Fig. 1C) was able to remove more uroporphyrin then the eight previous extractions by ethyl acetate (Fig. 1A and B). However, the difference in the total yield between the combined extractions with ethyl acetate and cyclohexanone (Fig. 1A, B, and C) and extraction with cyclohexanone (Fig. 1D) might be due to inactivation, since the former required almost 3 days as compared to only 3 h for the latter.

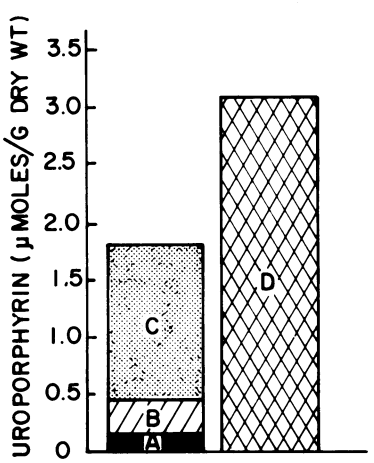

FIG. 1. *Ethyl acetate versus cyclohexanone extraction of the uroporphyrin accumulated by SASQ85. (A) Two extractions by ethyl acetate (127 nmol/g [dry weight]; (B) six additional extractions by ethyl acetate (330 nmol/g [dry weight]); (C) one additional extraction by cyclohexanone (1,376 nmol/g [dry weight]); (D) two extractions by cyclohexanone alone (3,127 nmol/g [dry weight]).*

Accumulation of porphyrins by the mutant SASQ85 and its parent strain. When grown with 5-ALA both SASQ85 and its parent strains accumulated great quantities of porphyrins. As expected, the mutant accumulated only uroporphyrin (6,206 nmol/g [dry weight]), whereas the parent strain accumulated uroporphyrin, coproporphyrin, and protoporphyrin (346, 434, and 175 nmol/g [dry weight], respectively). On the other hand, when grown without 5-ALA, the mutant continued to accumulate uroporphyrin (3,016 nmol/g [dry weight]), whereas the parental culture accumulated only traces of porphyrins.

Determination of uroporphyrin isomers. The uroporphyrin isomers accumulated by the mutant SASQ85 were determined after esterification by the method of Falk and Benson (10). This method cannot resolve mixtures of the isomers I and III when the minor component represents less than 10%. The results of these experiments are recorded in Fig. 2 and show the predominant or exclusive presence of isomer III. This is in accordance with the supposed deficiency of the mutant in uroporphyrinogen decarboxylase (Fig. 3).

Porphyrin synthesis by cell-free extracts. Porphyrin synthesis by cell-free extracts was assayed in the presence of 5-ALA. As shown in Table 2, cell-free extracts of the parent strain converted 5-ALA into uro-, copro-, and protoporphyrin, whereas those of the mutant produced only uroporphyrin. Although the mutant was grown under semianaerobic conditions, the cell-free extract contained some uroporphyrin, which could not be avoided even by growing the mutant in anaerobiosis in media supplemented with 0.2% pyruvic acid (11). Net synthesis of uroporphyrin by the cell-free extracts of the mutant was, therefore, calculated by substracting "endogenous" uroporphyrin from total porphyrin yield (Table 2).

Uroporphyrinogen synthetase activity of cell-free extracts of mutant SASQ85. Uropor-

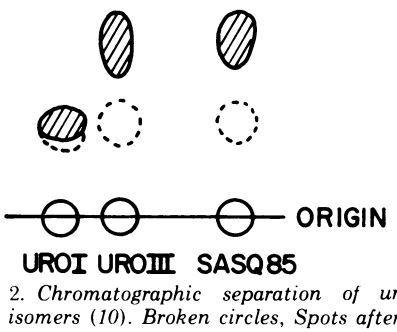

FIG. 2. *Chromatographic separation of uroporphyrin isomers (10). Broken circles, Spots after preliminary development; hatched circles, spots after final development.*

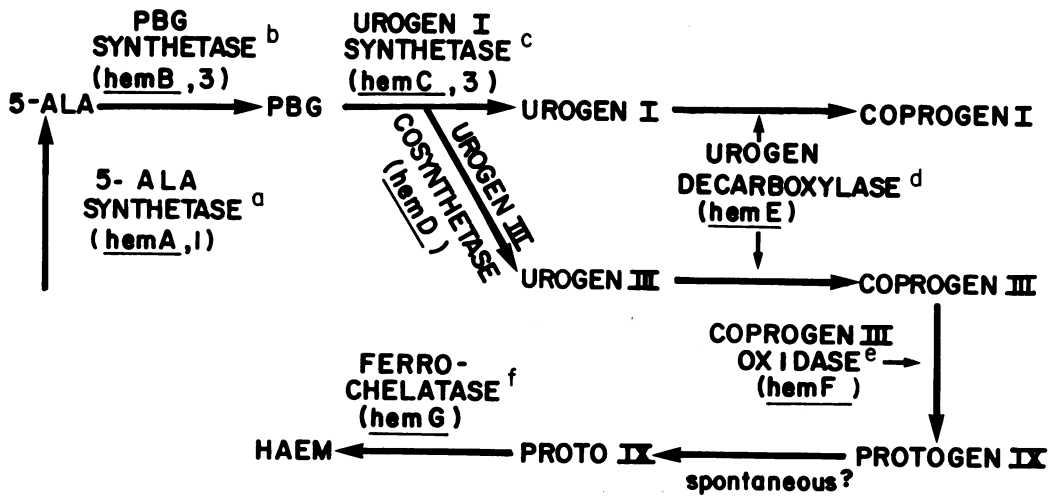

FIG. 3. *Enzymes of the heme-biosynthetic pathway (11, 19, 35) and the proposed designation of some of the corresponding genes. EC numbers of the enzymes are: (a) 2.3.1.37; (b) 4.2.1.24; (c) 4.3.1.8; (d) 4.1.1.37; (e) 1.3.3.3; (f) 4.99.1.1. UROGEN, Uroporphyrinogen; COPROGEN, coproporphyrinogen; PROTOGEN, protoporphyrinogen; PROTO; protoporphyrin.*

TABLE 2. *Porphyrin-synthesis in cell-free extracts of bacteria*[a]

Strain	5-ALA added (nmol)	o-Phenanthroline (M)	Porphyrins (nmol)			Net synthesis of porphyrins (nmol)
			Uro-porphyrin	Copro-porphyrin	Proto-porphyrin	
SASQ85	597	1.25×10^{-4}	69	0	0	41[b]
SASQ85	0	0	28	0	0	
AB1931	597	2.5×10^{-4}	18	36	13	67[c]
AB1931	0	0	0	0	0	

[a] Incubation was for 4 h at 37 C in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0); "enzyme": 45 mg (SASQ85) and 22 mg (AB1931).
[b] Porphyrin synthesis from added 5-ALA (line 1 less line 2).
[c] Total porphyrins.

phyrinogen synthetase activity was assayed in the presence of PBG as described by Bogorad (4). Conversion of PBG to uroporphyrin by cell-free extracts of the mutant was almost completely inhibited by PCMB and PCMS (Table 3). These results indicate that the mutant is capable of converting PBG to uroporphyrinogen enzymatically. No spontaneous transformation of PBG to uroporphyrinogen was detected under the experimental conditions used. Therefore it can be concluded that most, if not all, of the uroporphyrin accumulated by the mutant was produced enzymatically.

Catalase activity. The catalase activity of the mutant and parental strains are illustrated in Fig. 4. As expected, the mutant SASQ85 was catalase negative, whereas the parent strain AB1931 showed normal catalase activity.

Mapping of the hemE locus. Mapping of the *hemE* locus was performed by P1-mediated transduction after observing, in a preliminary experiment, that the former was co-transducible with *thiA* (36). Co-transduction frequencies of the *hemE* locus with various markers in this region are recorded in Table 4. From these data it is evident that the *hemE* locus is closer to *thiA* than to any other analyzed marker. As *thiA* is about halfway between *rif* and *metA*, and *hemE* seems to be closer to *rif* than to *metA*, the results suggest the gene order *rif*, *hemE*, *thiA*, *metA* (see below).

DISCUSSION

Nomenclature of heme-deficient mutants. The symbol *hem* has been proposed by Anderson and Ivanovics (1) to designate the genes responsible for heme synthesis in bacteria. This symbol fulfills the criteria formulated by Demerec et al. (6) for the nomenclature of bacterial genes and has since been used by a

TABLE 3. *Conversion of PBG to uroporphyrin in cell-free extracts of SASQ85*[a]

Sample	PBG added (nmol)	PCMB (M)	PCMS (M)	Incubation (h)	PBG consumed (nmol)	Uroporphyrin (nmol)		Inhibition (%)
						Found	Net synthesis	
1	385			4	200	65	39[b]	
2	385	1×10^{-4}		4		23	1[c]	98%
3	385		1×10^{-4}	4		24	2[c]	95%
4				0		22		
5				4		26	4[c]	

[a] Incubation was at 37 C in 0.125 M tris(hydroxymethyl)aminomethane-potassium phosphate buffer, pH 8.2 (4); incubation in buffer at pH 7.6 (17) did not give significantly better results. N.B. PBG incubated for 4 h at 37 C in the same buffer (pH 8.2) did not show any inactivation. PCMB, *p*-chloromercuribenzoate; PCMS, *p*-chloromercuriphenyl-sulfonate.

[b] Uroporphyrin formed from added PBG (line 1 less line 5).

[c] As compared to the zero time control (line 4).

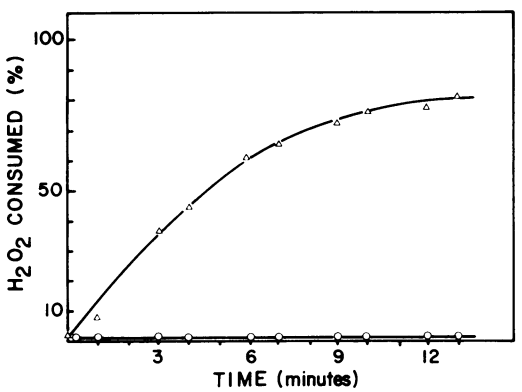

FIG. 4. *Catalase activity of strains SASQ85 (O) and AB1931 (Δ) assayed by the method of Herbert and Pinsent (13).*

number of authors to designated *hem* genes in *E. coli* (31, 33), *S. typhimurium* (30), and *B. subtilis* (3). Recently, Powell et al. (26) used the symbol *pop* for designating the same genes in *E. coli* K-12, although this symbol has already been used for designating a "mutant which contain abnormally high concentrations of porphyrins or metalloporphyrins" (27). Thus, the new use of this symbol would be confusing. Moreover, it should be stressed that in this case the problem is not to find a new symbol for the *hem* genes but to justify the rejection of the present symbol. As long as no sufficient reason is given for this rejection, there is no valid justification for replacing the symbol *hem*.

In a preliminary report (A. Săsărman and S. Sonea, Abstr. 1st Intersect. Congr. Int. Assoc. Microbiol. Soc. 1974, Tokyo, p. 10), the locus responsible for uroporphyrinogen decarboxylase was described as locus *hemC*, taking into account the order of discovery of the *hem* genes in *E. coli* K-12. In the meantime, however, Berek et al. (3) used the symbol *hemC* for the uroporphyrinogen I synthetase gene in *B. subtilis*. To avoid any confusion, we have now replaced our previous symbol *hemC* by *hemE*, which corresponds to the order of the metabolic steps in heme synthesis (Fig. 3).

Extraction of uroporphyrin from the mutant. The extraction of uroporphyrin from biological materials is difficult because of its insolubility in ether. However, uroporphyrin can be extracted from biological materials with ethyl acetate if the pH is adjusted to 3.0 to 3.2 (9, 11). The extraction of uroporphyrin from the urine using ethyl acetate depends on its concentration; at a high concentration uroporphyrin precipitates at pH 3.2 and, consequently, the yield becomes very low (7). This observation probably explains the low efficiency of the extraction with ethyl acetate in the case of SASQ85, which accumulates large amounts of uroporphyrin. When, however, the biological material contains less uroporphyrin, the advantages of the cyclohexanone method seem to be less evident (34).

Parallel extractions of the bacterial pellet and culture supernatant of the mutant SASQ85 revealed that almost all the accumulated uroporphyrin was found in the cells. On the other hand, almost half of the porphyrins accumulated by the parent strain was found in the culture supernatant. This finding confirms the earlier observation of Tien and White (37) using Hem$^-$ mutants of *S. aureus*.

Enzyme deficiency of the mutant. The third step in heme biosynthesis is the conversion of PBG to uroporphyrinogen III under the influence of uroporphyrinogen I synthetase and uroporphyrinogen III cosynthetase (Fig. 3); when uroporphyrinogen III cosynthetase is lacking, only uroporphyrinogen isomer I is formed. Con-

TABLE 4. *Mapping of the hemE locus by P1-mediated transduction*

Donor	Recipient	Selected marker	No. of transductants analyzed	Donor alleles in transductants (%):				
				argH	*rif*	*thiA*	*metA*	*aceA*
PA505-1-5	SASQ85	*hemE*$^+$	210			41.4	11.4	8.1
C600 rif^r	SASQ85	*hemE*$^+$	149	4.0	17.3	42.9		
Y53	SASQ85	*hemE*$^+$	95	4.2		44.2		
Hfr Cavalli	SAS251	*thiA*$^+$	399	24.3	48.4	100		
Hfr Cavalli	SAS252	*metA*$^+$	198	7.6		41.9	100	82.2

version of PBG to uroporphyrinogen can also occur nonenzymatically by heating the PBG sample at various pH values (22); the uroporphyrinogen isomer formed under these conditions depends on the pH of the solution during heating (22). Therefore, the uroporphyrin accumulated by the mutant SASQ85 might have arisen from a nonenzymatic transformation of PBG. However, the results of the present study clearly indicate that the uroporphyrin accumulated by the mutant originates from an enzymatic synthesis, and thus the mutant has a deficient uroporphyrinogen decarboxylase.

Position of the hemE locus. The gene order *rif, hemE, thiA, metA* deduced from the results of the genetic analysis is based, of course, on the assumption of a uniformly diminished homology of the two fragments. Such an assumption is difficult to verify since *hem*$^-$ alleles (excepting *hemA*$^-$) cannot be introduced into *hem*$^+$ recipients, and other markers cannot be selected for in *hem*$^-$ recipients. Therefore the gene order *rif, thiA, hemE, metA* cannot be totally excluded. It must be added that results of analysis of classes of transductants obtained with SASQ85 were compatible with both gene orders. Therefore analysis of new *hemE* mutants is necessary before concluding on the exact order of genes in this region. This is all the more necessary since true three-point test analysis cannot be performed with heme-deficient mutants of *E. coli*, excepting *hemA* mutants (transductions can be performed only from *hem*$^+$ donors to *hem*$^-$ recipients and selection only for *hem*$^+$ allele).

ACKNOWLEDGMENTS

We are grateful to S. F. MacDonald for his generous gift of pure porphyrin samples and for useful discussion. We thank G. Drapeau for his interest in our work and S. Sonea for his continued encouragement.

This investigation was supported by grant MA4997 from the Medical Research Council of Canada to A.S. P.C. is a recipient of a studentship from the Medical Research Council of Canada.

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