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The Genes Required for Heme Synthesis in *Salmonella typhimurium* Include Those Encoding Alternative Functions for Aerobic and Anaerobic Coproporphyrinogen Oxidation

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Insertion mutagenesis has been used to isolate *Salmonella typhimurium* strains that are blocked in the conversion of 5-aminolevulinic acid (ALA) to heme. These mutants define the steps of the heme biosynthetic pathway after ALA. Insertions were recovered at five unlinked loci: *hemB*, *hemCD*, and *hemE*, which have been mapped previously in *S. typhimurium*, and *hemG* and *hemH*, which have been described only for *Escherichia coli*. No other simple *hem* mutants were found. However, double mutants are described that are auxotrophic for heme during aerobic growth and fail to convert coproporphyrinogen III to protoporphyrinogen IX. These mutant strains are defective in two genes, *hemN* and *hemF*. Single mutants defective only in *hemN* require heme for anaerobic growth on glycerol plus nitrate but not for aerobic growth on glycerol. Mutants defective only in *hemF* have no apparent growth defect. We suggest that these two genes encode alternative forms of coproporphyrinogen oxidase. Anaerobic heme synthesis requires *hemN* function, while either *hemN* or *hemF* is sufficient for aerobic heme synthesis. These phenotypes are consistent with the requirement of a well-characterized class of coproporphyrinogen oxidase for molecular oxygen.

Heme serves in *Salmonella typhimurium* and *Escherichia coli* both for respiration and oxygen defense. Heme b (Fe protoporphyrin IX) and heme d are cofactors for the various cytochromes and also for two catalases encoded by *katE* and *katG* (3, 11, 13, 14, 30). The heme biosynthetic pathway branches to produce two additional products: siroheme and cobalamin (vitamin B_{12}). Siroheme is the cofactor for sulfite and nitrite reductases and is therefore required for synthesis of cysteine during growth on minimal medium containing sulfate (37). Vitamin B_{12} is known to serve as the cofactor for several enzymes in *S. typhimurium* (10, 27, 32), but B_{12}-dependent reactions are not essential for aerobic or anaerobic growth of wild-type *S. typhimurium*.

The biochemistry of heme synthesis is well established and, with the exception of the initial reactions leading to 5-aminolevulinic acid (ALA), the heme pathway is conserved among many different species (16, 34). In different organisms, ALA is made either by a C_5 route from glutamate or by a C_4 route from succinyl coenzyme A and glycine (5). *S. typhimurium* and *E. coli* use the C_5 mechanism (4, 23, 38, 46). The heme pathway can be viewed as having three parts: (i) the tRNA-dependent conversion of glutamate to ALA; (ii) the assembly of eight ALA molecules to give uroporphyrinogen III (the first tetrapyrrole); and (iii) the modification of tetrapyrrole side chains, oxidation, and iron insertion to give heme (Fig. 1). Uroporphyrinogen III lies at the branch point which also leads to siroheme and cobalamin. Recently, substantial gains have been made in our understanding of the heme pathway in enteric bacteria, from both the biochemical and genetic aspects (34). Genes encoding the six enzymes leading to uroporphyrinogen III have been cloned and sequenced (8, 20–23, 28, 35, 36, 39, 40, 56, 64, 65). The four terminal enzymes and their corresponding genes are much less well characterized. However, recently a gene which might be *hemH* was sequenced in *E. coli* (44). The *hem* genes are all unlinked with the exception of the *hemC* and *hemD* genes, which have been suggested to form an operon (36, 56). Virtually nothing is known about the regulation of heme biosynthesis.

In this work, we have isolated and characterized a large number of mutants of *S. typhimurium* blocked in the conversion of ALA to heme. Single mutants defective in heme synthesis define five loci of the pathway: *hemB*, the *hemCD* operon, *hemE*, *hemG*, and *hemH*. We also found an additional locus, apparently required for transport or effective utilization of ALA, which we have named *alu* (for aminolevulinic acid uptake). Notably absent from this group of *hem* mutants are ones defective in the *hemF* gene, which is thought to encode coproporphyrinogen oxidase, required for the conversion of coproporphyrinogen III to protoporphyrinogen IX (Fig. 1) (49). Two types of coproporphyrinogen oxidase have been described previously (16). One enzyme requires oxygen as a substrate. The second is oxygen independent, and its activity accounts for the ability of many bacteria to synthesize heme under anaerobic conditions. We describe the isolation of mutants defective in two genes, *hemF* and *hemN*, which we suggest probably encode oxygen-dependent and -independent forms of coproporphyrinogen oxidase. During aerobic growth, either *hemF* or *hemN* function is sufficient for heme synthesis, while during anaerobic growth *hemN* is essential for heme synthesis, since the *hemF* enzyme requires oxygen for activity.

MATERIALS AND METHODS

Bacteria. *S. typhimurium* strains used in this study and their sources are listed in Table 1. All *S. typhimurium* strains were derived from the wild-type strain LT-2. *S. typhimurium* wild type does not carry the *lac* operon. The basic genetic nomenclature has been described previously (18).

Strain TE1295 (Table 1) was used as the parent strain for

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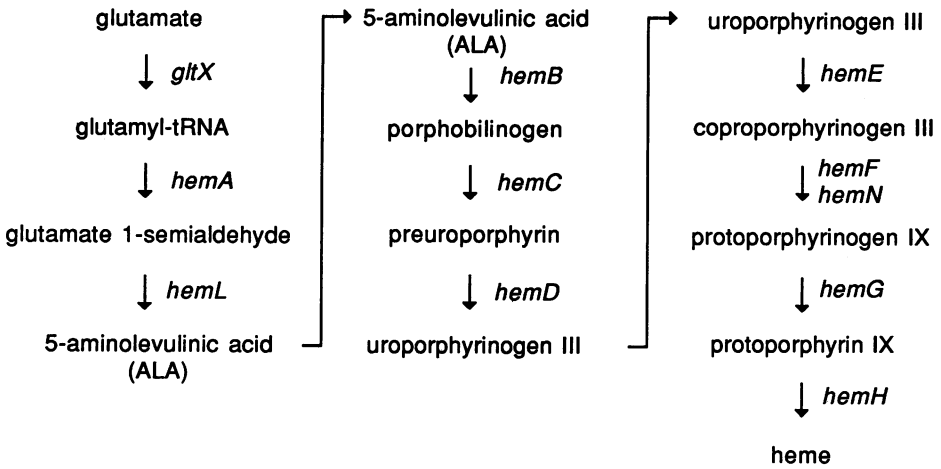

FIG. 1. The heme biosynthetic pathway, consisting of 10 reactions which convert glutamate to heme. Only the names of the intermediates and the genes encoding the enzymes of the pathway are shown. The pathway is divided into three segments marked by the key intermediates ALA and uroporphyrinogen III.

the isolation of most *hem*::Mud-J insertions and for the construction of all reference *hem* strains for testing mutant phenotypes. This strain carries the same genetic markers as strain SA1914 and was constructed from it by backcrossing against LT-2 wild type in several steps.

Media and growth conditions. Nutrient broth (NB; Difco), also containing 0.5% NaCl, and LB broth (43) were used as maximally supplemented media. E and NCE (for no carbon E) media were used as minimal media (6, 66). Carbon sources were added at the following concentrations: glucose or glycerol, 0.2%; lactate, 0.4%; succinate, 0.6%. Difco Bacto Agar was added at a final concentration of 1.5% for solid media.

Supplementation with ALA was at 2 μM in minimal medium and 150 μM in rich medium (25); amino acid and nucleoside supplementation was as described previously (17). Hematin was prepared from hemin (Sigma) as described previously (42) and was used at a final concentration of 20 μg/ml, except where indicated. Antibiotics were added to final concentrations in rich or minimal medium, respectively, as follows: chloramphenicol, 20 or 5 μg/ml; kanamycin sulfate, 50 or 100 μg/ml; tetracycline hydrochloride, 20 or 10 μg/ml. (In some experiments with *env-53*, lower concentrations of chloramphenicol were used.) All cultures were grown at 37°C except that of the temperature-sensitive strain TT10288, used as the donor for Mud-J mutagenesis, which was grown at 30°C (29).

Mutants that grow on Tween 80. The concentrated heme solutions we used were prepared in Tween 80 (42), and the final concentration of Tween 80 in the medium was 0.05%. Several mutants were recovered that could grow on minimal glycerol medium only if Tween 80 was present. Subsequent tests showed that they respond to oleic acid and probably have a defect in the synthesis of unsaturated fatty acids. The mutants were all about 20% linked by P22 transduction to *purF*::Tn*10*. This map position is consistent with a defect in *fabB*, which should be satisfied by oleic acid. The mutants have not been examined further.

Genetic techniques. The high-frequency generalized transducing bacteriophage P22 mutant HT*105/1 int-201* (59) was used for transduction. Phage P22 lysates were prepared as described previously (17).

To maximize the yield of colonies during transduction into strains carrying the *env-53* mutation, transduction mixtures were first plated onto NB plates supplemented with E medium, glucose, heme, and cystine. After the formation of a light lawn (about 6 h at 37°C), plates were replica printed to the same medium containing antibiotic.

Transposon nomenclature. Way et al. have constructed several transposition-defective transposons derived from Tn*10* (67). Their defective Tet^r transposon is referred to here as Tn*10d*-Tet. The construction and use of Tn*10d*-Cam have been described previously (24).

Castilho et al. have constructed several phage Mu-derived transposons that can form *lac* operon fusions and are defective for transposition. One of these, Mu*d* I1734 (Kan^r, *lac*) (9) is used extensively in the present work. For convenience of description, we refer to this Kan^r Mud as Mud-J. Mud-J is missing the Mu A and B genes and lacks Mu transposase.

Mutagenesis by transposon insertion. The methods used for the transposition of Tn*10d*-Tet and Tn*10d*-Cam elements have been described previously (24). Mud-J transposition was carried out by transduction, using phage P22 grown on strain TT10288 as donor (29) and TE1126 or TE1295 as recipient.

Chemical mutagenesis. To perform diethyl sulfate (DES) mutagenesis, cells were grown overnight in E medium with glucose, sedimented and resuspended in E medium, and diluted 1/50 into 5 ml of E medium saturated with DES. After 90 to 120 min at room temperature, samples were diluted 1/50 into E medium with glucose plus heme and cystine and grown to saturation. Aliquots were diluted, plated for single colonies, and then replica printed to identify heme auxotrophs.

Scoring the Cob phenotype. Production of vitamin B_{12} was scored by testing the ability of strains carrying a *metE* mutation to grow anaerobically without added methionine, as described previously (26, 33). The *metE205* mutation was introduced into different *hem* mutants by cotransduction with a linked Tn*10d*-Tet insertion, using strain TE727 as donor. The growth medium used for testing B_{12} production by *metE* suppression was NCE medium containing 0.2% glycerol, 40 mM KNO_3, 10 μg of heme per ml, 0.75 mM cystine, 2 μM ALA, 50 μg of 5,6-dimethylbenzimidazole per ml, and 1 μM $CoCl_2$.

HPLC analysis of tetrapyrroles. Cultures of various *hem*

TABLE 1. Bacterial strains

Strain	Genotype	Source or reference
PP1002	*trpB223 cya*::Tn*10*	P. Postma
SA772	*hemA60*	K. Sanderson (57)
SA1348	*hemC32*	K. Sanderson (55)
SA1914	*hemA60 env-53*	K. Sanderson (31)
SGSC694	*hemB21 met-365 proA24 pyrD197 purB210 trpE4*	K. Sanderson (57)
SGSC695	*hemE1 proC110*	K. Sanderson (19)
SK811	*zif-214*::Tn*10 hisF645*	S. Kustu
TE226-1	*hemL336*::Mud-J(w)	Laboratory collection
TE274	*zae-1863*::Tn*10d*-Tet	Laboratory collection
TE277	*zde-1858*::Tn*10d*-Tet	Laboratory collection
TE727	*zie-1869*::Tn*10d*-Tet *metE205*	Laboratory collection
TE1126	*env-53*	P22.LT-2 × SA1914
TE1295	*hemA60 env-53*	P22.SA1914 × TT11991
TE1300	*hemB21 env-53*	This study
TE1301	*hemC32 env-53*	This study
TE1303	*hemE1 env-53*	This study
TE1628	*hemA60 env-53 hemE464*::Mud-J(w)	This study
TE1629	*hemA60 env-53 hemH465*::Mud-J(b)	This study
TE1630	*hemA60 env-53 alu-1*::Mud-J(b)	This study
TE1631	*hemA60 env-53 hemH467*::Mud-J(w)	This study
TE1633	*hemA60 env-53 alu-2*::Mud-J(w)	This study
TE1636	*hemA60 env-53 hemB472*::Mud-J(b)	This study
TE1637	*hemA60 env-53 hemD473*::Mud-J(w)	This study
TE1640	*hemA60 env-53 hemB475*::Mud-J(w)	This study
TE1647	*hemA60 env-53 hemC482*::Mud-J(w)	This study
TE1648	*hemA60 env-53 hemC483*::Mud-J(b)	This study
TE1662	*hemA60 env-53 hemD497*::Mud-J(b)	This study
TE2031	*zai-6808*::Tn*10d*-Tet *env-53*	This study
TE2033	*zbb-6809*::Tn*10d*-Tet *env-53*	This study
TE2036	*hemN704*::Mud-J(b)	This study
TE2052	*zif-6806*::Tn*10d*-Tet	This study
TE2281	*hsdL6 hsdSA29* ($r_{LT}^-m_{LT}^+r_S^-m_S^+$) *metA22 metE551 ilv-452 trpB2 xyl-404 rpsL120* (Str^r) H1-b H2-e,n,x (Fels2$^-$) *nml srl-203*::Tn*10d*-Cam *recA1*	Laboratory collection
TE2477	*hemA60 env-53 hemE509*::Mud-J(b)	This study
TE2483	*hemA60 env-53 hemG614*::Mud-J(w)	This study
TE2720	*env-53 hemN704*::Mud-J(b)	This study
TE2727	*hemA60 env-53 zie-6805*::Tn*10d*-Tet	This study
TE2849	*env-53 hemN704*::Mud-J(b) *hemF705*	DES.TE2720
TE2850	*env-53 hemN704*::Mud-J(b) *hemF706*	DES.TE2720
TE2894	*zfa-6801*::Tn*10d*-Tet	This study
TE3005	*env-53 hemF707*::Tn*10d*-Tet	This study
TE3006	*env-53 hemN704*::Mud-J(b) *hemF707*::Tn*10d*-Tet	This study
TE3102	*zfa-6803*::Tn*10d*-Cam	(75% linked to *hemF*$^+$)
TE3104	*zie-6804*::Tn*10d*-Cam	(65% linked to *hemN*$^+$)
TE3626	*hemN732*::Tn*10d*-Tet	This study
TE3627	*env-53 hemN732*::Tn*10d*-Tet	This study
TE3681	*zii-6810*::Tn*10d*-Cam	This study
TE3682	*zbb-6811*::Tn*10d*-Cam	This study
TE3842	*env-53 hemN704*::Mud-J(b)*fabB*	DES.TE2720
TE3991	*zeh-6812*::Tn*10d*-Tet	(95% linked to *fabB*$^+$)
TE4479	*hemH465*:Mud-J *env-53*	This study
TE4512	*env-53 hemE1 hemN704*::Mud-J(b) *hemF707*::Tn*10d*-Tet	This study
TE4523	*hemF707*::Tn*10d*-Tet	This study
TN789	*zai-808*::Tn*10*Δ*proC150*	C. Miller
TN1781	*zbb-876*::Tn*10*	C. Miller
TT218	*metE862*::Tn*10*	J. Roth
TT311	*purD1735*::Tn*10*	J. Roth
TT317	*purF1741*::Tn*10*	J. Roth
TT2077	*zid-62*::Tn*10 hisR1223*	J. Roth
TT10288	*hisD9953*::Mud-J *hisA9944*::Mud-1	K. Hughes
TT10508	*cysA1585*::Mud-A(b)	J. Roth
TT10654	*eut-18*::Mud-A(b)	50
TT10681	*eut-46*::Tn*10*	50
TT11715	DEL744 (*cysA1585** Mud-A* *eut-18*)	50
TT11991	*hemA60*	25

Continued on following page

TABLE 1—*Continued*

Strain	Genotype	Source or reference
TT13438	*zfa-3646*::Tn*10*	50
TT13440	*zfa-3648*::Tn*10*	50
TT13670	*hemA640 env-53*	This study
TT13671	*hemA641 env-53*	This study
TT13672	*hemB642 env-53*	This study
TT13673	*hemB643 env-53*	This study
TT13674	*hemCD644 env-53*	This study
TT13675	*hemCD645 env-53*	This study
TT13676	*hemCD646 env-53*	This study
TT13677	*hemCD647 env-53*	This study
TT13678	*hemE648 env-53*	This study
TT13679	*hemE649 env-53*	This study
TT13680	*hemG650 env-53*	This study
TT13681	*hemH651 env-53*	This study
TT13682	*hemH652 env-53*	This study
TT13683	*hemL653 env-53*	This study
TT13684	*hemL654 env-53*	This study

mutants were grown to saturation in minimal glycerol medium containing 5 μg of heme per ml and then diluted 1:20 and grown to saturation in NB medium without heme but containing 150 μM ALA. For the experiments presented here, incubation in the presence of ALA was for 36 h. Porphyrins were prepared and analyzed as described previously (61). Cells were sedimented by centrifugation, and porphyrins in the cell pellet and supernatant fractions were separately prepared and esterified in 5% H_2SO_4 in methanol. Porphyrin methyl esters were extracted into CH_2Cl_2, concentrated, and analyzed by high-performance liquid chromatography (HPLC) on a silica column run in 50% ethyl acetate–50% heptane. Porphyrin methyl ester standards were from Porphyrin Products (Logan, Utah).

RESULTS

Method used to isolate heme auxotrophs. Heme is needed to make cytochromes, which are required for growth on nonfermentable carbon sources. Mutants defective in the early steps of the heme pathway have been recovered as requiring ALA for growth on glycerol (Fig. 1) (25, 57, 58, 68). These mutants (*hemA* and *hemL*) are unable to take up heme and therefore cannot use heme provided in the medium to satisfy their ALA requirement. In previous studies, this problem was solved by using secondary mutations that permit heme to enter cells (31, 41, 42).

Mutants with defects in the later steps of the heme pathway (*hemD* through *hemH*; Fig. 1) accumulate tetrapyrrole intermediates (see below), exhibiting a red fluorescence under UV light, and such strains are apparently photosensitive (44, 68a). Photosensitivity in human patients with porphyria is well known (45) and is attributed to the generation of free radicals by porphyrins. To solve this problem, most experiments were done with strain TE1295 (*hemA60 env-53*), in which the *hemA* mutation prevents flow through the pathway. In this strain, additional *hem* mutations result in failure to convert exogenous ALA to heme. Thus, *hem* mutants were identified as strains that grew on minimal glycerol medium containing heme and cystine but not with either ALA or cystine alone.

Five loci required for heme synthesis from ALA. We isolated a total of 172 Mud insertion mutants that require heme for growth on glycerol. Of these, 93 were authentic Hem$^-$ mutants (Table 2; the remaining 79 mutants are discussed below). The first 43 Hem$^-$ mutants were isolated in a *hemA*$^+$ background (TE1126) and included 10 mutants defective in *hemL*. No *hemA* insertion mutants were recovered in this study or in previous work (22, 25); this can be explained by the presence of an essential gene downstream of *hemA* that renders *hemA* insertion mutants lethal by virtue of their polarity (22). The additional 33 Hem$^-$ mutants isolated in the TE1126 background, and 50 Hem$^-$ mutants isolated in TE1295 (*hemA60 env-53*), are defective in conversion of ALA to heme.

These mutants can be grouped into five loci comprising a total of at least six genes. Our primary means of mutant classification was a cotransduction test (Table 3). We used existing strains carrying Tn*10* insertions that we predicted would be linked to *hem* genes on the basis of the map positions reported for *E. coli hemG* and *hemH* (15, 49, 53) or for *S. typhimurium hemB*, *hemCD*, and *hemE* (19, 51, 54, 55, 57). For known *S. typhimurium hem* loci, linkage was confirmed by using mutant strains isolated previously (Table 1). In addition, for *hemB*, *hemE*, *hemG*, and *hemH*, we isolated a very tightly linked Tn*10d*-Tet or Tn*10d*-Cam insertion (Table 3). For each locus, all mutants showed

TABLE 2. Heme-deficient mutants of *S. typhimurium*[a]

Gene	No. of Mud-J insertion mutants	Representative alleles	No. of mutants induced by DES	Representative alleles
hemA	0		4	640, 641
hemL	10	518, 521	6	653, 654
hemB	15	472, 475	5	642, 643
hemC	6	482, 483	18[b]	644 to 647
hemD	20	473, 497		
hemE	21	464, 509	8	648, 649
hemG	1	614	1	650
hemH	20	465, 467	6	651, 652
Total	93		48	

[a] Mutants were isolated starting from either TE1126 (*env-53*) or TE1295 (*hemA60 env-53*), as described in the text. Two additional genes, *hemF* and *hemN*, are discussed in the text.
[b] Either *hemC* or *hemD*.

TABLE 3. Tn*10* insertions near *S. typhimurium hem* genes

Gene and locus	Map position (min)	Donor strain	Donor genotype	Linkage[a]
hemA	35	TE277	*zde-1858*::Tn*10d*-Tet	70% to *hemA*$^+$
hemB	8	TN789[b]	*zai-808*::Tn*10* Δ*proC150*	10% to *hemB*$^+$
		TE2031	*zai-6808*::Tn*10d*-Tet	95% to *hemB*$^+$
hemCD	83	TT2077	*zid-62*::Tn*10*	75% to *hemCD*$^+$
		PP1002	*cya*::Tn*10*	90% to *hemCD*$^+$
hemE	88	TT311[c]	*purD1735*::Tn*10*	30% to *hemE*$^+$
		TE3681	*zii-6810*::Tn*10d*-Cam	90% to *hemE*$^+$
hemF	50	TE2894	*zfa-6801*::Tn*10d*-Tet	70% to *hemF*$^+$
		TE3102	*zfa-6803*-Tn*10d*-Cam	75% to *hemF*$^+$
hemG	84	SK811	*zif-214*::Tn*10*	30% to *hemG*$^+$
		TE2727	*zie-6805*::Tn*10d*-Tet	50% to *hemG*$^+$
hemH	11	TN1781	*zbb-876*::Tn*10*	ca. 3% to *hemH*$^+$
		TE2033	*zbb-6809*::Tn*10d*-Tet	90% to *hemH*$^+$
		TE3682	*zbb-6811*::Tn*10d*-Cam	40% to *hemH*$^+$
hemL	5	TE274	*zae-1863*::Tn*10d*-Tet	95% to *hemL*$^+$
hemN	85	SK811	*zif-214*::Tn*10*	95% to *hemN*$^+$
		TE3104	*zie-6804*::Tn*10d*-Cam	65% to *hemN*$^+$

[a] Linkage was determined in transductional crosses by using P22 lysates on donor strains carrying a linked Tn*10*, Tn*10d*-Tet, or Tn*10d*-Cam insertion into a *hem* mutant recipient. The linkage recorded is the percentage of Tet^r or Cam^r colonies among transductants first selected as Hem$^+$.

[b] Linkage of this Tn*10* to *hemB*$^+$ was not detected when the donor was *proC*$^+$.

[c] Linkage was about 70% when selecting Tet^r and scoring Hem$^+$.

TABLE 4. Phenotypes of *S. typhimurium hem* mutants

Genotype[a]	Aerobic growth on minimal glycerol agar plus:			Synthesis of vitamin B_{12}[b]	Fluorescence[c]
	ALA	Heme	Heme + cysteine		
hemA	+	−	+	+	+
hemL	+	−	+	+	+
hemA hemB	−	−	+	−	−
hemA hemC	−	−	+	−	−
hemA hemD	−	−	+	−	+
hemA hemE	−	+	+	+	+
hemA hemG	−	+	+	+	+
hemA hemH	−	+	+	+	+

[a] All strains carry the *env-53* allele, which permits heme utilization.

[b] Derivatives carrying a *metE* mutation were tested for suppression of their methionine auxotrophy during anaerobic growth on minimal medium containing ALA, as described in Materials and Methods. Since ALA was provided, the function of the *hemA* and *hemL* genes was not tested in this experiment (25).

[c] Colonies grown on medium containing ALA were tested for red fluorescence upon illumination with long-wave (366 nm) UV light.

similar linkage to the nearby Tn*10*. Four representative insertions for each locus were backcrossed to the TE1295 background, and the phenotypic characterization reported below was carried out on these backcrossed insertion mutants. Allele numbers for two insertions of each type are listed in Table 2.

The collection of *S. typhimurium* mutants described here does not contain any mutants defective in the *hemF* gene (a heme biosynthetic gene described for *E. coli* showing close linkage to *gal* [49]). The collection also lacks mutants analogous to the *sec-130* mutant of *E. coli*, which causes a defect in ALA dehydratase and maps near *ara* (49). In order to extend the range of possible mutants recovered, we repeated our search by mutagenizing strain TE1126 (*env-53*) with the alkylating agent DES. No other *hem* loci were found (Table 2). In other experiments, we have collected an additional 100 DES-induced mutants defective in the second half of the *hem* pathway (between uroporphyrinogen III and heme) without finding any other loci that, when singly mutant, give a Hem$^-$ phenotype.

Growth phenotypes of *hem*::Mud insertion mutants. Mutants representative of each locus were examined for their growth phenotypes. As expected, all *hem* mutants required heme for aerobic growth on minimal medium with nonfermentable carbon sources, including glycerol, lactate, and succinate (Table 4). Aerobic growth on glucose was poor in the absence of heme. The branched heme pathway also leads to vitamin B_{12} and to siroheme, required for cysteine biosynthesis. Mutants defective in *hemB*, *hemC*, and *hemD* were Cys$^-$ during aerobic growth and also failed to make vitamin B_{12} during anaerobic growth, as tested by the suppression of a *metE* mutant (10, 25, 33). Mutants defective in steps of the pathway after uroporphyrinogen III (*hemE*, *hemG*, and *hemH*) were Cys$^+$ during aerobic growth and could make vitamin B_{12} during anaerobic growth.

The *hemC* gene encodes porphobilinogen deaminase, which converts porphobilinogen to preuroporphyrinogen, a linear tetrapyrrole. This compound is cyclized into uroporphyrinogen III, with inversion of one of the pyrrole rings, by action of the uroporphyrinogen III synthase encoded by *hemD* (34). In the absence of the synthase, preuroporphyrinogen spontaneously cyclizes to give a different isomer, uroporphyrinogen I (7). In *E. coli*, the *hemC* and *hemD* genes lie next to each other and are thought to form an operon in which *hemC* is promoter proximal (34, 56). Thus, we expected that *hemD* insertion mutants should still retain *hemC* function and synthesize uroporphyrinogen I from ALA, resulting in a fluorescent red phenotype under UV light. The *hemC* and *hemD* mutants listed separately in Table 2 have been distinguished on this basis.

Precursor accumulation in *hem* mutants. We examined the accumulation of tetrapyrroles in mutants predicted to be defective in the conversion of uroporphyrinogen III to heme. For each mutant class, *hemA*$^+$ derivatives were constructed (manipulations were performed in dim light), and cultures were grown with ALA supplementation to stimulate tetrapyrrole synthesis. Extracts were prepared as described in Materials and Methods, esterified, and analyzed by HPLC on a silica gel column run with 50% ethyl acetate–50% heptane (61). By this method, porphyrinogens are oxidized to the corresponding porphyrins. Figure 2 shows typical column profiles for extracts prepared from different mutants. As expected, *hemE* mutants accumulated uroporphyrin, while *hemG* and *hemH* mutants accumulated all three tetrapyrroles: uroporphyrin, coproporphyrin, and protoporphyrin. On the basis of the appearance of colonies under UV light, porphyrin accumulation did not occur in these mutants (in a *hemA* background) in the absence of ALA. Furthermore, double mutants were constructed that carried both a *hemE* point mutation and a Mud insertion in either *hemG* or *hemH*. These double mutants accumulated only uroporphyrin, showing that *hemE* mutations are epistatic to *hemG* and *hemH*, as expected from the pathway shown in Fig. 1 (68a).

Model for genetic control of coproporphyrinogen oxidase. The studies described above did not recover any mutants that might be defective in coproporphyrinogen oxidase. We reasoned that our failure to obtain these mutants might be due to the existence of multiple enzymes able to catalyze this reaction. Two mechanistically distinct types of copropor-

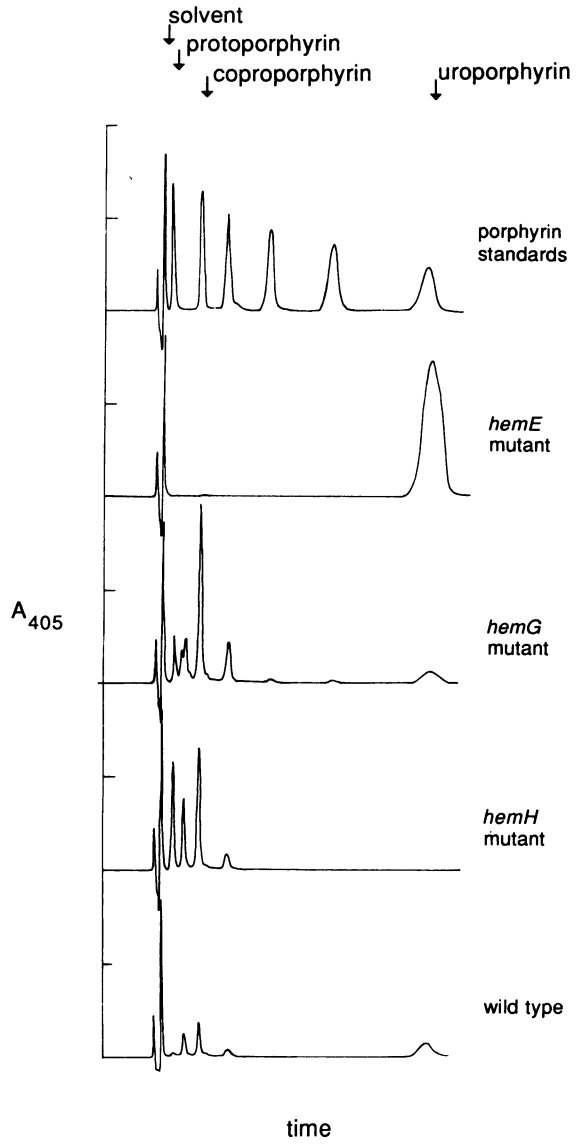

FIG. 2. HPLC analysis of porphyrins accumulated in *hem* mutants. The mutant strains were *hemA*$^+$ derivatives of TE2477 (*hemE env-53*), TE2483 (*hemG env-53*), and TE1629 (*hemH env-53*), and the *hem*$^+$ wild-type strain was TE1126 (*env-53*). The *hemE* sample was prepared from mutant cells, while the other samples contain porphyrins released into the medium. Extracts were prepared, esterified, and analyzed by chromatography on a silica column as described in Materials and Methods (61). Each sample was chromatographed identically, and porphyrins in the eluate were detected by their absorbances at 405 nm. The figure is a tracing of the chart recorder output. The marker contained 100 pmol of each tetrapyrrole standard. The other samples included 1.2% (*hemE*), 2.5% (*hemG*, *hemH*), or 5% (wild type) of the porphyrins obtained from 10 ml of culture.

phyrinogen oxidase have been identified (16, 63). If *S. typhimurium* has both types of enzyme, it would explain our failure to detect mutants defective in this step. If *E. coli* had only one of the activities, it could explain why *hemF* mutants were recovered in this organism. Alternatively, the *E. coli* *hemF* strains might be double mutants defective in both activities.

TABLE 5. Phenotypes of *hemF* and *hemN* mutants

Strain	Hem phenotype	
	Aerobic	Anaerobic
Wild type	+	+
hemF	+	+
hemN	+	−
hemF hemN	−	−

One class of coproporphyrinogen oxidase, which we will call aerobic, is dependent on oxygen (16, 48, 52, 60). Under anaerobic conditions, when the aerobic enzyme cannot function, single mutants defective in the alternative, anaerobic enzyme should have a Hem$^-$ phenotype, giving a requirement for exogenous heme during anaerobic respiration. We designate the gene encoding the aerobic enzyme as *hemF* and the gene encoding the anaerobic enzyme as *hemN*. Mutants defective in *hemF* are predicted to have a wild-type phenotype in a *hemN*$^+$ background, whereas *hemN* mutants are predicted to be Hem$^-$ during anaerobic but not aerobic growth. Mutant phenotypes predicted from these relationships are summarized in Table 5.

An insertion mutant with a Hem$^-$ phenotype that is specific to anaerobic growth. Strain TE2036 (*hemN*::Mud-J) was originally identified as a Mud insertion mutant that makes a fluorescent red colony during aerobic growth on minimal glucose medium. This phenotype can be understood by recognizing that most of the cells in a colony grown aerobically are nevertheless oxygen limited or anaerobic, because they are shielded from oxygen by cells on the colony surface (47, 62). Cells of the *hemN* mutant colony presumably manifest a Hem$^+$ phenotype on its surface but are heme deficient in the anaerobic interior of the colony, resulting in the accumulation of oxidized, fluorescent heme pathway intermediates. Colonies of the *hemN* mutant are not fluorescent during aerobic growth on minimal glycerol medium.

Strain TE2036 (*hemN*::Mud-J) was shown by HPLC analysis to accumulate coproporphyrin, as predicted by the model (see below). Furthermore, strain TE2720 (*hemN*:: Mud-J *env-53*) was found to require heme for anaerobic growth on glycerol nitrate medium but not for aerobic growth on glycerol. A Tn*10d*-Tet insertion linked to *hemN* was isolated, and its map position was determined by the Hfr method for conjugational mapping of Tn*10* insertions (12). The *hemN* gene was mapped to 85 min; it is weakly linked to *hemG*. A cotransduction map of this region is shown in Fig. 3A.

DES mutagenesis to give *hemF* mutants. As indicated in Table 5, double mutants defective in both *hemN* and *hemF* should have an aerobic Hem$^-$ phenotype. Strain TE2720 (*hemN*::Mud-J *env-53*) was mutagenized with DES, and colonies were screened for a heme requirement during aerobic growth on minimal glycerol medium. Since cysteine was not supplied, we expected to recover only mutants defective in the third segment of the heme pathway, between uroporphyrinogen III and heme. Four classes of mutants were expected, including *hemE*, *hemG*, and *hemH*, as well as the additional *hemF* class. Fifty-seven independent Hem$^-$ mutants were isolated (Table 6). Strains carrying a new mutation in *hemE* or *hemH* were recognized by transductional mapping, using donor phage P22 lysates grown on strains carrying Tn*10d*-Tet insertions tightly linked to these genes (Table 2).

Distinguishing *hemG* mutations from the expected *hemF*

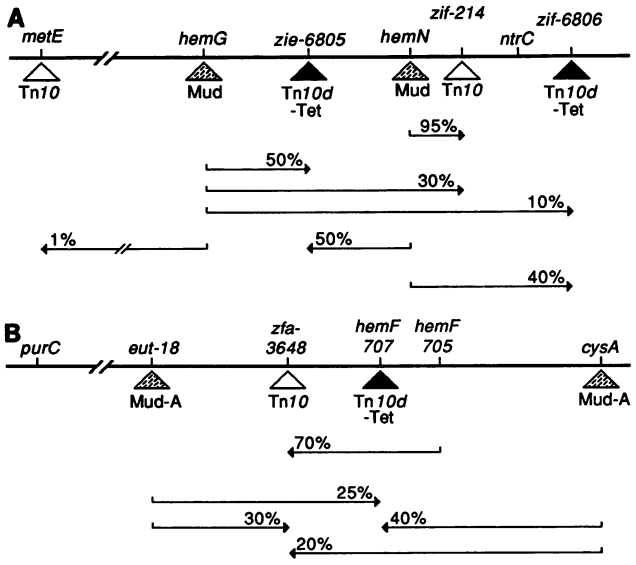

FIG. 3. (A) Genetic linkage map of the *hemN* region. P22 cotransduction frequencies are shown as percentages. The arrowheads point to the marker selected in each cross. (B) Genetic linkage map of the *hemF* region. Two different, closely linked alleles of *hemF* were used in the mapping experiments.

class was complicated by two factors. First, *hemG* is linked to *hemN*. Second, the model predicts that if either *hemF* or *hemN* is functional, the strain will be Hem$^+$. Two different classes of mutants were obtained which could be cotransduced to Hem$^+$ and Tet^r by using phage P22 donor lysates grown on Tn*10* insertions mapping in the *hemG-hemN* region. One class showed a low linkage (15 to 30%) to *zif-214*::Tn*10* and was weakly linked (10%) to *zif-6806*::Tn*10d*-Tet (Fig. 3). This pattern is characteristic of *hemG* mutations.

The second class were *hemF hemN* double mutants. When mutants in this class were transduced with donor P22 phage grown on a strain (SK811) carrying the *zif-214*::Tn*10* insertion, Tet^r and Hem$^+$ were cotransduced with a frequency of 90%. The Hem$^+$ phenotype and loss of the Kan^r phenotype carried by *hemN*::Mud-J were completely linked in such crosses. Furthermore, Tet^r Hem$^+$ transductants were not observed when the donor strain carried both the *zif-214*::Tn*10* and a *hemN*::Mud-J insertion.

Each mutant in the second class contained an additional mutation in the *hemF* gene, lying outside the *hemG-hemN* region, that was required for the Hem$^-$ phenotype. A Tn*10d*-Tet insertion 70% linked to *hemF* was isolated; all *hemF* mutants show nearly the same linkage to this Tn*10d*-Tet. Using the Hfr method (12), we mapped *hemF* to the region near *cysA* at 50 min on the *S. typhimurium* genetic map. A cotransduction map of this region is shown in Fig. 3B. The data indicate that *hemF* lies between *eut* and *cysA*. Strains carrying precise deletions with endpoints in *eut* and *cysA* were isolated by Roof and Roth (50). We have confirmed that their strain TT11715 Δ(*cysA-eut*) lacks *hemF* function.

Either *hemF* or *hemN* is sufficient to confer a Hem$^+$ phenotype. When *hemF*::Tn*10d*-Tet *hemN*::Mud-J (Kan^r) double mutants were transduced by using donor phage grown on the wild-type strain LT-2, about half of the Hem$^+$ transductants were Tet^s Kan^r (*hemF*$^+$) and half were Tet^r Kan^s (*hemN*$^+$). This result is explained by the ability of either *hemF* or *hemN* to confer an aerobic Hem$^+$ phenotype. Single mutants defective in either *hemF* or *hemN* grew on minimal glycerol or minimal lactate medium under aerobic conditions at the same rate as the wild type (Table 7).

DES mutagenesis to give *hemN* mutants. The results described above suggest that *hemF* mutations give a Hem$^-$ phenotype only in a *hemN* mutant background. It is likely that *hemF* encodes the oxygen-dependent coproporphyrinogen oxidase. In order to define all the genes required for the anaerobic reaction, we searched for other mutants with a phenotype like that of *hemN* strains.

Strain TE3005 (*hemF*::Tn*10d*-Tet *env-53*) was mutagenized with DES, and the progeny were screened for a heme requirement. Forty-five mutants were obtained (Table 6). These mutants were tested for the dependence of the Hem$^-$ phenotype on the presence of the *hemF*::Tn*10d*-Tet insertion. Mutants were transduced to Hem$^+$ by using phage P22 grown on the wild-type strain LT-2 and screened for Tet^s Hem$^+$ transductants. Twenty-four mutants which showed an apparent linkage to *hemF* were obtained. The mutants were classified by cotransduction, using a set of Tn*10d*-Cam insertions linked to *hemE*, *hemG*, *hemH*, and *hemN* (Table 1).

All of the candidate *hemN* mutants map in the same region as *hemN*::Mud-J. Six of these are unusual in requiring heme for growth on minimal glycerol medium but not on nutrient agar.

Precursor accumulation in *hemF* and *hemN* mutants. We examined the accumulation of tetrapyrroles in cultures of *hemF* and *hemN* mutants by HPLC analysis. Porphyrin methyl esters were prepared as described in Materials and Methods. In the first experiment (Fig. 4A), we looked at

TABLE 6. Isolation of *hem* mutants starting from a *hemN* or *hemF* strain[a]

Mutant gene	No. of mutants found starting from:	
	hemN strain	*hemF* strain
hemE	39	14
hemF	12	
hemG	1	3
hemH	5	4
hemN		24
Total mutants	57	45

[a] For mutants starting from a *hemN* or *hemF* strain, strain TE2720 (*hemN704*::Mud-J *env-53*) and strain TE3005 (*hemF707*::Tn*10d*-Tet *env-53*), respectively, were mutagenized with DES, and heme auxotrophs were recovered as described in the text. The new mutations were classified by cotransduction analysis, using Tn*10d*-Tet and Tn*10d*-Cam insertions linked to the indicated genes.

TABLE 7. Growth properties of *hemF* and *hemN* mutants

Strain	Genotype	Doubling time (min) in minimal medium with the following sole carbon source:	
		Glycerol	Lactate
LT-2	Wild type	66	103
TE2036	*hemN704*::Mud-J	61	100
TE4523	*hemF707*::Tn*10d*-Tet	69	105

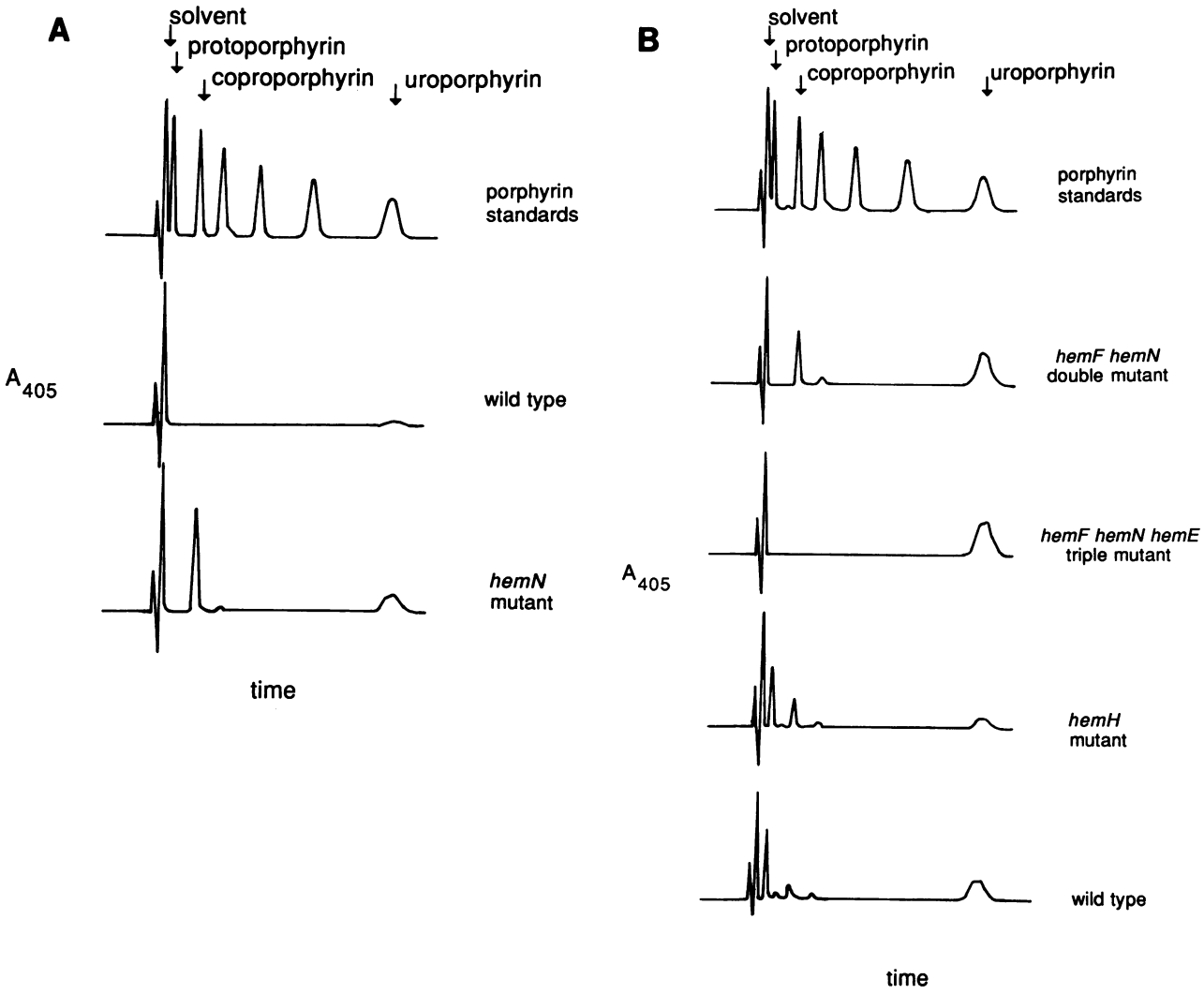

FIG. 4. HPLC analysis of porphyrins accumulated in *hemF* and *hemN* mutants. (A) Strains LT-2 (wild type) and TE2036 (*hemN704*::Mud-J). (B) Strains TE3006 (*hemF hemN env-53*), TE4512 (*hemF hemN hemE env-53*), TE4479 (*hemH env-53*), and TE1126 (*hem*$^+$ *env-53*). The samples in panel A contain porphyrins released into the medium; the samples in panel B were prepared from mutant cells. In each case, qualitatively similar results were obtained with samples prepared both from cells and medium. Extracts were prepared and analyzed as described in Materials and Methods and the legend to Fig. 2. The figure is a tracing of the chart recorder output. Samples included 2.5% (TE3006) or 5% (all other samples) of the porphyrins obtained from 10 ml of culture.

tetrapyrrole accumulation in a *hemN* mutant during anaerobic growth on minimal glucose medium compared with that in the wild-type strain, LT-2. The *hemN* mutant accumulated uroporphyrin and coproporphyrin but not protoporphyrin, consistent with a defect in coproporphyrinogen oxidase (Fig. 1). We also tested for coproporphyrin accumulation in *hemF hemN* double mutants during aerobic growth (Fig. 4B). In this experiment, cultures were grown in the presence of oxygen, starved for heme, and incubated with excess ALA to stimulate production of heme pathway intermediates. The *hemF hemN* double mutant accumulated uroporphyrin and coproporphyrin. A *hemF hemN hemE* triple mutant accumulated only uroporphyrin, consistent with a requirement for *hemE* action to generate coproporphyrin. A *hemH* mutant strain accumulated all three tetrapyrroles: uroporphyrin, coproporphyrin, and protoporphyrin.

***alu* mutants defective in ALA uptake.** As described above, after Mud-J mutagenesis of TE1295 (*hemA60 env-53*), we recovered an additional class containing 79 mutants which mimic the Hem$^-$ phenotype. These strains require heme plus cysteine but are not satisfied by ALA, similar to *hem* mutants with early blocks in the pathway. However, these mutants exhibit their apparent Hem$^-$ phenotype only in a *hemA* mutant background. Unlike authentic *hem* mutants, they do not show a growth defect in a wild-type *hemA*$^+$ background. Furthermore, although the mutants will not grow on minimal medium containing the standard amount of ALA (2 μM), they do grow on medium containing 150 μM ALA. Both of these phenotypes can be explained if the insertion mutants affect a system for transport or uptake of ALA. All 79 insertions in this class map near *sufA* at 78 min, and the locus affected has been designated *alu* (for aminolevulinic acid uptake). Preliminary sequence analysis indicates that the affected genes share amino acid sequence

similarity with *opp* and other binding protein-dependent transport systems (68a).

DISCUSSION

We have used Mud insertion and DES chemical mutagenesis to isolate heme-deficient mutants and thereby define the genes of the heme biosynthetic pathway in *S. typhimurium*. A special genetic background was used (TE1295 *hemA60 env-53*), to allow heme supplementation and prevent the accumulation of toxic intermediates. The search generated 83 *hem*::Mud-J insertion mutants defective in conversion of ALA to heme, together with 10 *hemL* mutants which cannot make ALA. An additional 150 mutants were generated by DES. Relying primarily on cotransduction with linked Tn*10* insertions, we can group the mutants into six loci which include seven known genes (including *hemL*; Table 2). In addition to isolating the *hem* mutants, we also isolated a large number of mutants (*alu*) which are defective in ALA uptake, probably at the level of transport.

The properties of these heme-deficient mutants of *S. typhimurium* conform to what was expected on the basis of the previously known mutants in *S. typhimurium* and its close relative, *E. coli*. We consider first the better-characterized segment of the pathway, between ALA and uroporphyrinogen III. Mutants were assigned to the *hemB* gene and the *hemCD* operon on the basis of map position. As expected, genetic tests showed that these mutants could not make any tetrapyrroles, including siroheme and vitamin B_{12}. We have sequenced the 5′ part of the *S. typhimurium hemB* gene (68a) and find that it is similar to its *E. coli* counterpart (21, 39). The *hemCD* operon has not yet been cloned from *S. typhimurium*, but the insertions assigned to *hemC* and *hemD* are more than 90% linked to *cya*, similar to the *E. coli* mutants. Detailed complementation and recombination analyses have not been carried out, and it remains possible that these loci harbor unknown *hem* genes. However, additional genes are not expected, since each enzyme has a single type of subunit (16, 34).

We did not obtain any Cys^- mutants (blocked between ALA and uroporphyrinogen III) other than these types, and the failure to recover mutants corresponding to the *sec-130* ALA dehydratase-defective mutant of Powell et al. is not understood (49). (The *sec* mutants in these studies were isolated as secondary mutants that eliminated accumulation of a brown pigment, protoporphyrin, in a leaky *hemH* strain.)

The later steps of the pathway convert uroporphyrinogen III into heme. We found three classes of single mutants defective in this part of the pathway. These were characterized as Cys^+ and were still able to make vitamin B_{12}. Each class is fluorescent red when provided with ALA or when the early steps of the pathway are functional. The fluorescent material in *hemE* mutants was identified as uroporphyrin, while that in *hemG* and *hemH* mutants was a mixture of tetrapyrroles, including protoporphyrin. Two striking results were the scarcity of *hemG* mutants and the absence of *hemF* mutants. We do not understand why *hemG* mutants are so rare. The *hemG*::Mud-J insertion we isolated could be backcrossed into TE1295, indicating that the mutation probably does not require a second-site suppressor for viability. Its heme-deficient phenotype is severe enough that other mutants of this type ought to be detectable. We have sequenced the 5′ end of the *S. typhimurium hemH* gene (68a) and find that it is similar to the *E. coli* gene cloned and sequenced by Miyamoto et al. (44) and suggested by them to be *hemH*.

We also described mutants of *S. typhimurium* that define two alternate genes required for heme biosynthesis: *hemF* and *hemN*. The function of either gene is sufficient to allow heme synthesis under aerobic conditions. However, only *hemN* can function during anaerobic growth. Mutants defective in *hemN* accumulate coproporphyrin but not protoporphyrin when grown anaerobically on glucose, conditions that are permissive for heme-deficient mutants. Double mutants defective in both *hemF* and *hemN* accumulate coproporphyrin during heme starvation under aerobic conditions. These results are consistent with the idea that the two genes encode alternative oxygen-dependent and -independent forms of coproporphyrinogen oxidase (16, 48, 52, 60, 63). Extensive searches for more mutants with these properties have recovered only the same types. Complementation tests have not yet been performed, so it remains possible that these loci may contain more than one *hem* gene each. The *hemF* gene lies at 50 min on the genetic map, while *hemN* maps at 85 min.

Charles and coworkers isolated two different mutants of *E. coli* (*sec-20* and *popB7*) that accumulated coproporphyrin and failed to grow on lactate as a carbon and energy source (15, 49). The *sec-20* mutation mapped close to *gal* by cotransduction using phage 363 (88 to 96% linkage [15]). In addition, *sec-20* could be backcrossed to wild type by using linkage to gal^+. Thus, *sec-20* alone conferred a Hem^- phenotype.

Alper and Ames (2) isolated *S. typhimurium* strains bearing deletions of the region on both sides of *gal*, including markers as far away as *nadA* and *chlA*. These strains grew normally when supplemented with nicotinic acid, biotin, and 2,3-dihydroxybenzoic acid and were apparently not defective in heme synthesis. Similar deletions of *E. coli* have been isolated that extend between *gal* and *chlA* (1). The *E. coli* deletion mutants suggest that *sec-20* must lie between *gal* and *nadA*. The *S. typhimurium* deletion mutants suggest that a heme biosynthetic gene does not map near *gal*, so that *S. typhimurium* and *E. coli* must differ in this region. Alternatively, if the original strain used by Charles carried an unrecognized deletion bringing *gal* and *sec-20* close together, these results might be reconciled. Unfortunately, the mutants isolated by Charles are apparently no longer in existence.

We have recently cloned and sequenced the *S. typhimurium hemF* gene, whose predicted product is highly similar in amino acid sequence to the *Saccharomyces cerevisiae* coproporphyrinogen oxidase (68a, 69). Because of this, we suggest that the *hemF* designation properly belongs to the gene encoding coproporphyrinogen oxidase that maps at 50 min in *S. typhimurium* and is described in this work. Sasarman et al. (53a), in imposing a uniform genetic nomenclature on *hem* mutants, replaced the *sec-20*/*popB* designation of Charles with *hemF*. We suggest that the *E. coli* mutants mapping near *gal* should revert to their original designation of *popB*, consistent with their best-described phenotype, prevention of porphyrin overproduction (49).

In this report, we have emphasized the use of Mud-J as a transposon to generate null mutants of the heme pathway. We are currently using these *hem-lac* strains to ask whether the operon fusions they carry are regulated in response to the demand for heme. Our preliminary experiments have not shown induction of β-galactosidase in response to heme starvation, but several factors which may prevent observation of a regulatory effect have yet to be eliminated. In

addition, since heme cannot enter wild-type cells, it may be that changes in physiology rather than concentration of the end product control *hem* gene expression.

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