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Cloning and Identification of the *hemG* Gene Encoding Protoporphyrinogen Oxidase (PPO) of *Escherichia coli* K-12

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Abstract

Cells of the VSR751 strain, which was previously isolated as a photoresistant revertant of the *visA*-deleted (*hemH*-deleted) strain of *Escherichia coli* K-12, accumulated uroporphyrin (uro), coproporphyrin (copro) and protoporphyrin IX (proto), but did not accumulate as much protoporphyrin as cells of the parental strain (*hemH*-deleted). Therefore, we concluded that strain VSR751 must be defective in protoporphyrinogen oxidase (PPO), the product of the *hemG* gene. By complementation analysis using VSR751, we isolated and identified this gene. The *hemG* gene is located at 86 mim on the *E. coli* chromosome, just upstream of the *rrnA* operon, and is transcribed clockwise in the same direction as the *rrnA* operon. This gene encodes a 181-amino acid protein with a calculated molecular mass of about 21 kDa. Sequence analysis revealed the presence of flavodoxin motif, suggesting that a cofactor of this enzyme is flavin mononucleotide, which is consistent with the previous report that the mammalian PPO had the flavin cofactor.

Key words: *Escherichia coli*; photoresistant revertant; heme biosynthesis; *hemG*; protoporphyrinogen oxidase

1. Introduction

Heme forms the prosthetic group of cytochromes and is required for the function of electron transport chains.[1] Heme also serves as a cofactor for catalase, which protects cells against the toxic effects of active oxygen species.[2] The pathway for the heme synthesis is highly conserved in a wide variety of eubacteria,[3,4] including *E. coli*, and in the chloroplasts of higher plants and algae. In yeasts and in mammalian cells, however, the initial reactions leading to the synthesis of 5-aminolevulinic acid (ALA) are different from those in the former pathway.[4] In *E. coli* and higher plants, ALA is synthesized via the C5 pathway from glutamate,[4] while in yeasts and in mammalian cells the C4 pathway from succinyl coenzyme A and glycin is operative.[3,4] The heme biosynthetic pathway branches to produce two additional products: siroheme and vitamin B12. These compounds play important roles, as cofactors, in the catalytic activities of several enzymes.[5]

We previously reported the isolation of mutants of *E. coli* K-12 that were sensitive to visible light[6] and showed that this photosensitivity, which resulted in death when cells were exposed to visible light, was brought about by a defect in the *visA* (*hemH*) gene that encodes ferrochelatase,[7,8] an enzyme that catalyzes the final step in the heme biosynthetic pathway. A defect in this gene causes the accumulation of protoporphyrin IX,[9] the substrate of ferrochelatase, which is a photosensitizer and produces an active species of oxygen that is harmful to the cell.[10] Such photosensitivity is seen in human patients with porphyria.[11] We isolated photoresistant revertants of a *visA*-deleted strain and found that the second mutations were located in other genes involved in the heme biosynthetic pathway at a step before the reaction catalyzed by ferrochelatase. Thus, we obtained cells with mutations in *hemA*, *hemB* and in the *hemCD* operon.[12] Furthermore, using $hemA^+/hemA^+$ diploid bacterium, we isolated another set of photoresistant mutants and obtained *hemL*, *hemE* and *hemG* mutants.[13]

Genes encoding the enzymes involved in the heme synthetic pathway in *E. coli*, *hemA*, *hemL*, *hemB*, *hemC*, *hemD*, *hemE*, *hemF* and *hemG*, had been mapped[14] and all of them, with the exception of *hemN* and *hemG* gene, have been cloned and sequenced.[6,13,15–22] The *hemN* was found in *Salmonella typhimurium* to encode oxygen-independent coproporphyrinogen oxidase, while *hemF* encodes oxygen-dependent coproporphyrinogen oxidase.[23] The biochemistry of heme pathway seems to be well established, but it was reported recently that the two new genes, *hemM*[24] and *hemK*,[25] are also involved in the heme synthetic pathway. Moreover, virtually nothing is known about the regulation of the C5 pathway.

The *hemG* gene encodes protoporphyrinogen oxidase (PPO).[30] This enzyme catalyzes the six-electron oxida-

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tion of protoporphyrinogen IX (protogen) into protoporphyrin IX (proto IX). In higher eukaryotes, PPO requires molecular oxygen for conversion of protogen IX to proto IX. Prokaryotes including anaerobic bacteria and, in the absence of oxygen, facultative anaerobes must use a different system to oxidize protogen IX. The oxidation is achieved by coupling the oxidation of protogen IX to the respiratory chain and some components that serve as a terminal electron acceptor.[26] This obligate coupling to the respiratory chain complicates the purification of PPO from bacteria. No sequence determination on the *hemG* gene from any organism has been published except in the case of *B. subtilis*, whose *hemY* gene was recently reported to encode PPO.[27] However, this protein from *B. subtilis* has some properties unlike those reported previously for other prokaryotic or eukaryotic PPOs.[26]

Thus, in this study, to gain a better understanding of the nature of PPO, we used a *hemG* mutant isolated as a photoresistant revertant of a *visA* (*hemH*)-deleted strain and characterized the *hemG* gene of *E. coli*.

2. Materials and Methods

2.1. Bacteria, phage clones and growth conditions

The bacterial strains used were derivatives of *E. coli* K-12. VS200 (Δ*visA*) and VSR751 (Δ*visA hemG*) were isolated in the previous study; VS200 was a deletion mutant of the *visA* (*hemH*) gene and was photosensitive, and VSR751 was one of the photoresistant mutants isolated from VS200 by mutagenesis.[12,13] VSR800 was a λxxg1 lysogen of VSR751. λgxx1, λgec7 and λgec5 were constructed and are described in the text. 2×YT medium[43] was used as the basic medium and incubations were performed at 37°C in the dark (with test tubes wrapped in aluminum foil) under aerobic condition, unless otherwise noted. For plotting the growth curve and extracting accumulated porphyrins, overnight culture from a single colony was diluted to OD_{600}=0.02 and the culture was incubated at 37°C.

2.2. DNA techniques

General DNA techniques including DNA preparation and DNA cloning were carried out as described by Sambrook et al.[43] Restriction enzymes and DNA modifying enzymes were purchased from Takara Shuzo Co. (Kyoto, Japan) and Toyobo Co. (Osaka, Japan). The primers 5′-TGGTTGCTGATAACTTTACC-3′ and 5′-GGGGTGTGCATAATACGCCT-3′ were purchased from Biologica (Nagoya, Japan).

2.3. Complementation test

Mutants defective in the heme biosynthetic pathway could not make cytochromes and lacked respiratory ability. Therefore, even in rich medium, they grew very poorly. Since mutants used were derivative of a *visA* (*hemH*)-deleted strain, the complementation test was performed by cross-streaking the mutants with a mixture of λ*visA*[6] and each of Kohara's λ clones, [28] and by observing the restoration of a normal growth rate after this double infection.

2.4. Extraction and identification of accumulated porphyrins in mutant bacteria

The methods used for extraction and identification of accumulated porphyrins in mutant bacteria were based on those of Cox and Charles.[44] At the end of incubation, bacteria were harvested by washing with saline and centrifugation. After a mixture of ethyl acetate and glacial acetic acid (3:1 v/v) was added, bacteria were disrupted in an ultrasonicator (Bioruptor; Cosmobio Co., Tokyo, Japan). The bacterial debris were removed by centrifugation and the supernatant solution in ethyl acetate was washed twice with distilled water to remove any residual acetic acid. The solution of porphyrins in ethyl acetate was then shaken with an equal volume of 3 M HCl. All porphyrins were transferred from the ethyl acetate to the HCl solution and were separated from almost all the other ethyl acetate-soluble bacterial compounds. The separation of the different porphyrins in the solution in 3 M HCl was based on their different partitioning coefficients between ether and acidified aqueous solutions. Protoporphyrin and coproporphyrin were transferred to an ether solution by adjusting the pH of the HCl solution to pH 4 and extracting with diethyl ester while uroporphyrin was retained in the HCl solution. The coproporphyrin and protoporphyrin were then extracted from the diethyl ester with 0.3 M and 1.2 M HCl, respectively. The absorbance spectra of porphyrins in acid solution was recorded from 350 to 450 nm with spectrophotometer (model UV-160A; Shimadzu Co., Kyoto, Japan). Absorbance was determined by drawing a horizontal line between the troughs on either side of the peak around 400 nm and measuring the absorbance from the line to the peak. This absorbance was then converted to the concentration of porphyrins using previously reported molecular extinction coefficients.[45]

3. Results and Discussion

3.1. Identification of the hemG mutant

We previously isolated a large number of photoresistant revertants of *E. coli* from photosensitive mutants (Δ*hemH*). The revertants were unable to grow on a nonfermentable carbon source and accumulated much less proto IX than the parental strain.[6] Therefore, they were assumed to have mutations in other heme genes at a step prior to the reaction catalyzed by ferrochelatase. Since the correlation between phage clones in Kohara's

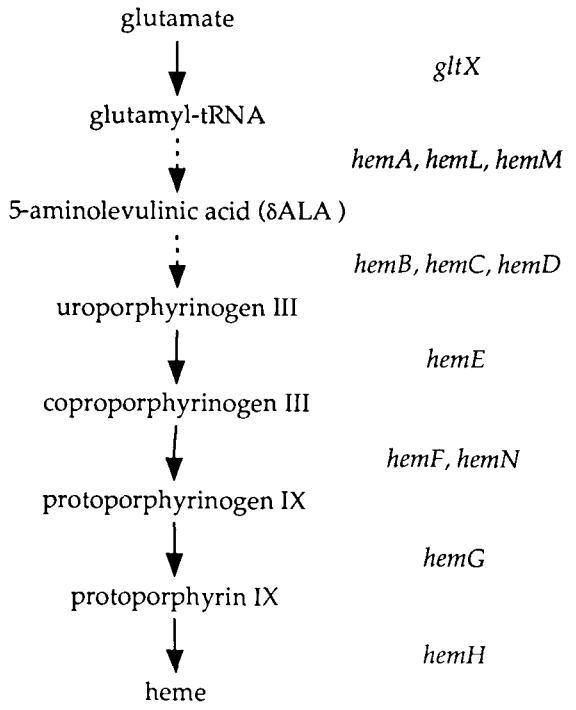

Figure 1. The heme biosynthetic pathway (C5 pathway). The intermediates and the genes encoding the enzymes catalyzing each reaction are shown.

Table 1. Accumulation of porphyrins in cells of various mutant strains.

Strain	Incubation time[a]	OD_{600}[b]	Accumulation of porphyrins[c]	
			coproprophyrin	protoporphyrin
VSR751	36	0.279	6.88	1.67
VSR800	14	0.367	5.06	2.54
VS200	14	0.331	8.73	43.6

[a] Hours. [b] The number of cells was estimated from the following equation: 0.32 (OD600)=2×10^8 cells. [c] 10^{-11} mol/cell.

library[28] and heme genes was known, complementation tests allowed classification of the mutants into six groups, each of which was represented by mutations in *hemA*, *hemL*, *hemB*, *hemCD*, *hemE* and *hemG*.[12,13]

Among the photoresistant revertans, we chose VSR751 as a mutant in *hemG* on the basis of its phenotype and the map position of the complementary Kohara clone.

To obtain more direct evidence that the VSR751 strain is indeed a *hemG* mutant, we examined the intermediate in heme biosynthesis that accumulated in VSR751. As shown in Table 1, VSR751 cells accumulated copro and proto IX. In another experiment, when we used the culture medium together with bacterial cells as a sample, we detected the accumulation of uroporphyrin (uro) in addition to copro and proto IX. According to the pathway for the heme biosynthesis as illustrated in Fig. 1, the *hemG* mutant should accumulate protogen IX, coproporphyrinogen III (corprogen) and uroporphyrinogen III (urogen). However, the accumulated porphyrinogen is readily oxidized to form protoporphyrin (proto IX, copro and uro) during extraction. Thus, the accumulation of proto could be explained by a deficiency in either the *hemG* or *hemH* genes. In this case, accumulation of proto IX in VSR751 was not due to the fact that *hemH* was deleted, because no proto was accumulated under our condition in *hemA*, *hemB*, *hemCD* and *hemE* mutants, in which *hemH* had been deleted, and because the phenotype of VSR751 was not leaky, unlike that of *hemL* mutant,[16,29] in a complementation test. The gene, other than *hemH*, whose mutation could lead to the accumulation of these three porphyrins should be *hemG*, which encodes PPO. The properties of the mutants were consistent with those described for the mutant strain SASX38,[30] which was originally isolated as a *hemG* mutant of *E. coli*.

3.2. Isolation of the hemG gene by complementation

Mutants defective in the heme biosynthetic pathway could not synthesize cytochromes and lacked respiratory ability. Therefore, even in a rich medium, they grew very poorly. Alternatively, their poor growth might have been a consequence of certain compounds introduced into the external medium by the heme mutant bacteria themselves, as previously demonstrated by Alder et al.[31] Since the mutants used in this study were derivatives of a *visA* (*hemH*)-deleted strain, a complementation test was performed by cross-streaking the mutants against a mixture of λ*visA*[6] and each of the Kohara clones and monitoring the restoration of a normal growth rate upon this double infection.

Clones #548 and #549 from Kohara's library were found to be able to complement VSR751 (Fig. 2). From this complementation test, we defined the open reading frame (ORF) for the *hemG* gene as summarized in Fig. 3. For construction of the λgec7 and λgec5, 7-kb and 5-kb *Eco*RI fragments from Kohara clone #549 were inserted into the *Eco*RI site of λgt11.[32] λgbm6 had a 6-kb *Bam*HI fragment in the *Bam*HI site of Charon 28.[33] Phage clones λgec7, λgbm6, and λgec5 all failed to complement the defect in VSR751 cells, while #549 gave a positive result. We obtained the sequence data for this region[34] from the DNA data bank of Japan and found ORF181, the whole sequence of which was not included in any of the phage clones. Therefore, we attempted to amplify only ORF181 and its adjacent region by polymerase chain reaction[35] using, as primers, the oligo deoxyribonucleotides 5′-TGGTTGCTGATAACTTTACC-3′ and 5′-GGGGTGTGCATAATACGCCT-3′. These primers were based on the sequence of ORF181, as shown in

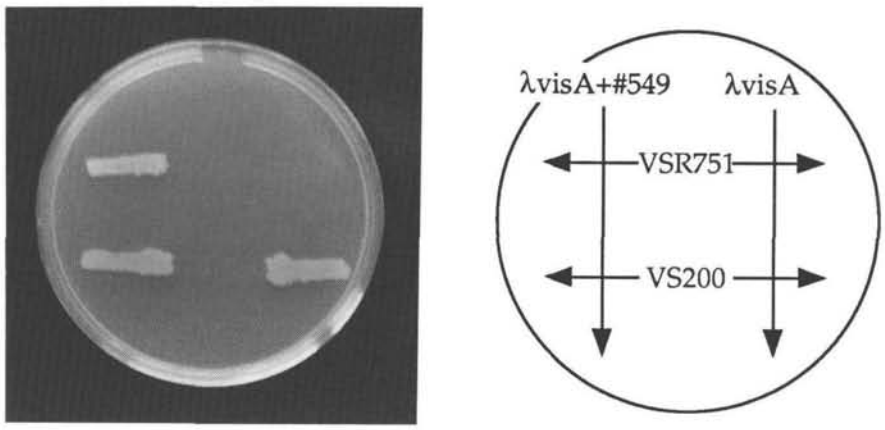

Figure 2. Complementation analysis of the *hemG* mutant VSR751 with Kohara's clone #549. Lysates of λ phage were streaked vertically on an LB plate, and then cultures of each strain were cross-streaked horizontally over the lysate. The limited growth ability of VS200 (Δ*hemH*)[6] was complemented when it was cross-streaked over λ*visA*,[6] while VSR751 showed restored growth only when it was cross-streaked over a mixture of λ*visA* and Kohara's clone #549,[28] an indication that the gene that could complement a second mutation in VSR751 was included in clone #549.

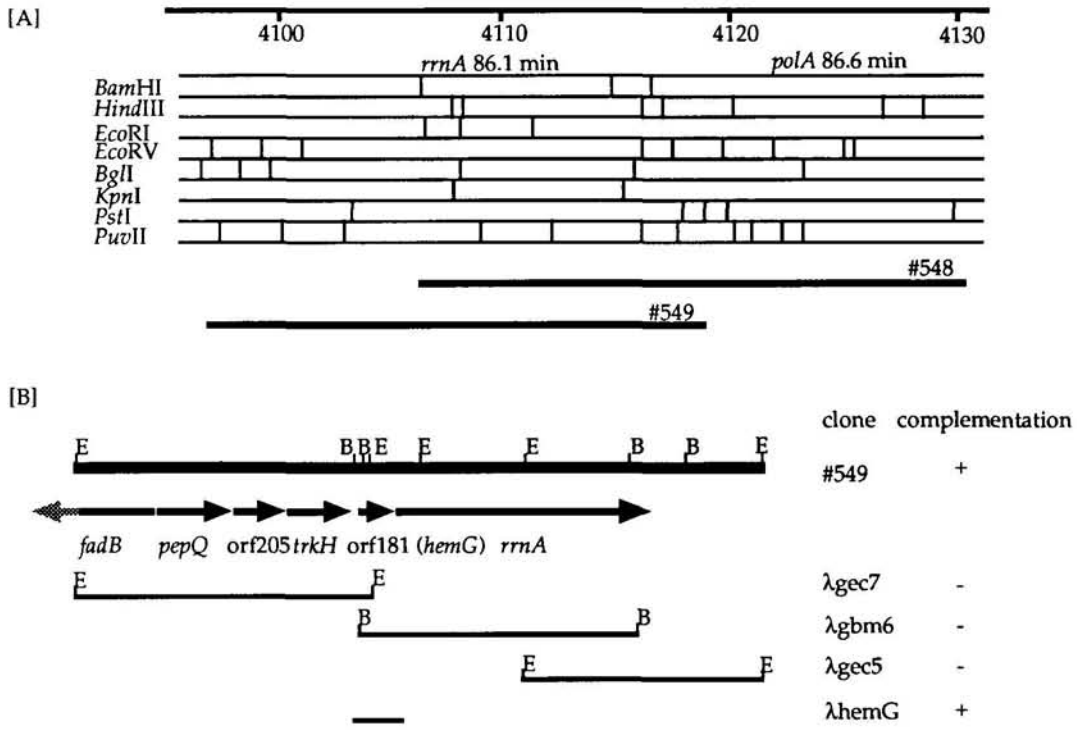

Figure 3. [A] Physical and restriction maps of Kohara's clones #549 and #548[28] on the *E. coli* chromosome. These clones were able to complement the defect in VSR751. [B] Genes included in clone #549 are represented by arrows below clone #549. Restriction sites are indicated above it. The insert DNAs in the phage clone are indicated below the genes. Of these clones, only λ*hemG*, in which ORF181 that had been amplified by PCR was cloned, could complement the defect in VSR751. Abbreviations: E, *Eco*RI; B, *Bam*HI.

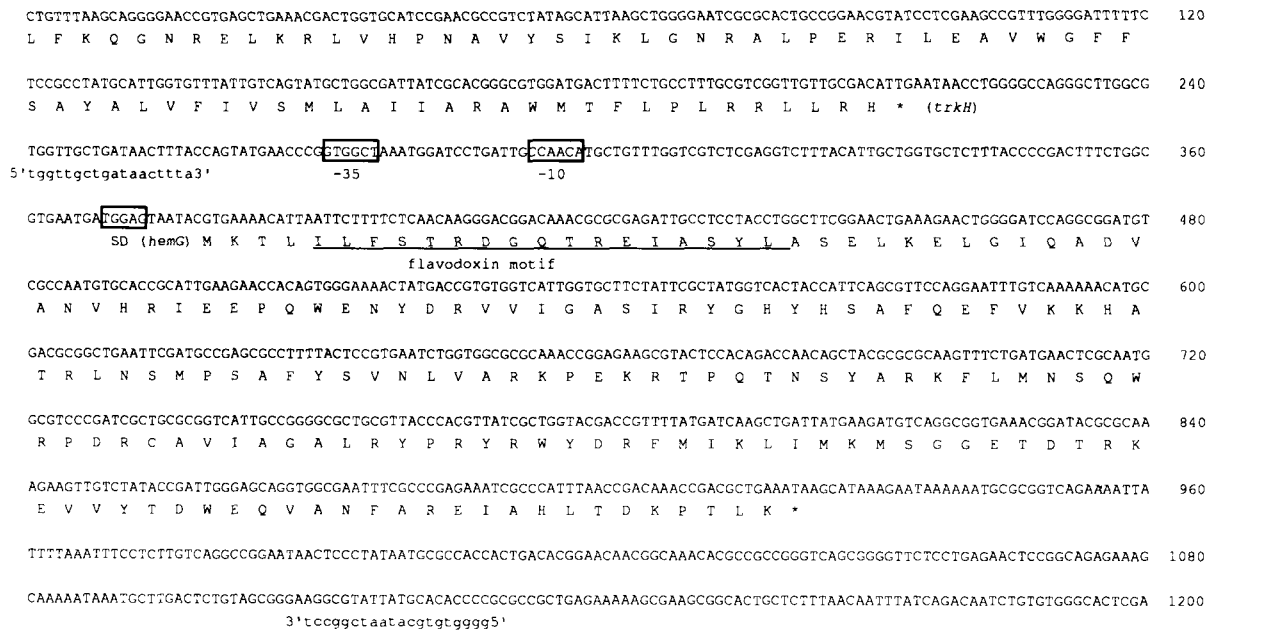

Figure 4. Nucleotide sequence around the *hemG* gene[34] and the deduced amino acid sequence. A potential promoter and Shine-Dalgarno (SD) sequence are boxed. Sequence anadysis was performed with the GENETYX program (SDC Software Development, Tokyo, Japan). The primers used for PCR are shown below the DNA sequence. The flavodoxin motif [LIV]-[LIVFY]-[FY]-x-[ST]-x(2)-[AG]-x-T-x(3)-A-x(2)-[LIV] 37 is underlined.

Fig. 4. Phage clone λgxx1, in which the appropriately purified PCR product had been inserted into a *Pma*CI site of λi^{21}nin5,[36] were able to complement the defect in VSR751 cells.

ORF181 is located at 86 min on the linkage map of the *E. coli* chromosome, just between the *rrnA* operon and the *trkH* gene, and should be transcribed clockwise in the same direction as the *rrnA* operon. This gene starts with a GTG codon (Fig. 4) and encodes a protein of 181 amino acids with a calculated molecular mass of about 21 kDa. No significant sequence similarity to any known proteins was found using the predicted amino acid sequence as the key sequence, FASTA program, and the DDBJ database. However, a motif search using the PROSITE database[37] revealed that ORF181 includes a flavodoxin motif[38] in the N-terminal region. Other enzymes with this motif also contain it in the N-terminal region. This result is consistent with the report that either FAD or FMN is a cofactor of the two mammalian PPOs.[39,40]

Recently, it was reported that the *hemY* gene of *B. subtilis*[27] encoded PPO. The amino acid sequence of *hemG* of *E. coli* did not exhibit any similarity to that encoded by *hemY* of *B. subtilis*. This result is not surprising because some properties of PPO of *B. subtilis* are different from those of PPO of *E. coli*. In particular, the former utilizes molecular oxygen as a terminal electron acceptor for the oxidation of protogen IX, while the latter does not.

3.3. *Accumulated porphyrins in the hemG mutant and in the hemG mutant complemented with ORF181*

To confirm that the ORF181 is *hemG* gene and encodes PPO, we measured the change in levels of accumulated porphyrins in the cells when VSR751 cells were lysogenized by λ*hemG* (VSR751G) which contained a fragment that included only ORF181. As shown in Fig. 5, the growth of VSR751 was poorer than that of V200 (Δ*hemH*, the parental strain of VSR751) or VSR751G cells. We incubated VSR751 cells for 36 h before they reached the early stationary phase, while only 14 h were required for V200 and VSR751G cells to attain the maximum cell density. Then we extracted porphyrins accumulated in each strain. VSR751G accumulated more proto IX and less copro than the parental strain VSR751 (Table 1), an indication of complementation by ORF181. However, the amount of the accumulated proto IX in VSR751G was not so much when compared with that in VS200. There are two possibilities that might explain this observation.

The first possibility is that the level of expression of ORF181 (=*hemG*) inserted in the phage vector was low. It has been proposed that the region around the *hemG* gene is bent.[34] When ORF181 was cloned with a limited adjacent region on the phage vector, its expression might

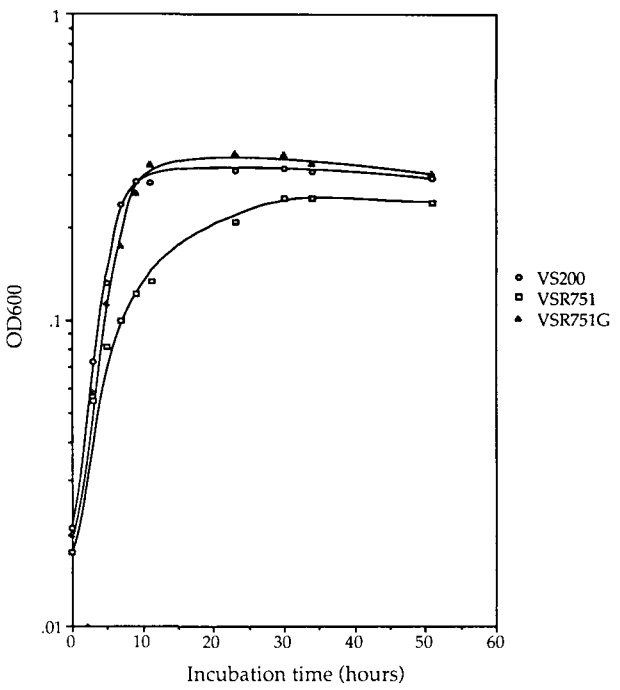

Figure 5. Growth curves of mutants. Overnight cultures from single colonies were diluted to OD_{600}=0.02 and the diluted cultures were incubated at 37°C. Mutants grew very poorly because they were all Δ*hemH*. It took 12–14 hrs for VS200 to reach the stationary phase (OD_{600}=0.35), while VSR751 required 35 hrs to do so (OD_{600}=0.25). VSR751G (λ*hemG* lysogen of VSR751) was similar to that of VSR200, the parental strain.

be modified and lowered. The reduced level of expression of the *hemG* gene might have made it possible to clone the *hemG* gene on a phage vector, since it was impossible to clone the *hemG* gene on a plasmid vector even when we used a low-copy number vector or tightly regulated expression vector. When the latter expression vector was used, although we prepared the DNA fragment by PCR using a set of primers with a particular restriction site to fuse in frame to the start codon, we only obtained clones in which the DNA had been deleted at its end and had been ligated divergently to the promoter of the vector.

The second possibility is as follows. ORF181 might encode one of the subunits of PPO, and VSR751 might have another mutation in the gene encoding the subunit. For example, the purified PPO of anaerobic bacterium *Desulfovibrio gigas* is composed of three dissimilar subunits.[41] Furthermore, in our previous study,[12,13] we obtained only three *hemG* mutants out of total 129 isolated mutants in heme biosynthesis, and in another study aimed at the isolation of mutants in heme biosynthesis of *S. typhimurium*, *hemG* mutants were shown to be rare.[23] The second putative mutation might have only been partial and, therefore, ORF181 could complement the mutation sufficiently to restore growth and viability even though the enzymatic activity of PPO was not restored completely. A similar situation can be proposed for VS100, in which the *hemH* gene was partially defective and large amounts of proto IX accumulated, but growth was just the same as that of the wild-type strain.

A newly identified gene, *hemK*,[25] located just downstream of *hemA* and *prfA* (these three genes probably form an operon) might be involved in the oxidation of protogen IX. We hypothesized that another mutation in VSR751 might be located in this gene, however we could not detect any change in the accumulation of proto IX when we introduced $hemK^+$ into VSR751 in addition to $hemG^+$ (data not shown).

During preparation of this report, we found the nucleotide sequence for the *hemG* gene of *E. coli* in the DNA database. Sasarman et al. reported the isolation and sequencing of the *hemG* gene of *E. coli*.[42] Our results indicating that the ORF181 is the *hemG* gene are consistent with theirs.

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