The *Escherichia coli* vis*A* Gene Encodes Ferrochelatase, the Final Enzyme of the Heme Biosynthetic Pathway

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Heme is the prosthetic group of cytochromes, hemoglobins, and other respiratory enzymes, and precursors of the heme synthesis pathway are also used for synthesis of chlorophylls, corrins, and bilins in organisms that express them. In *Escherichia coli*, heme synthesis from glutamyl-tRNA requires nine enzymatic steps, and the genes that encode the first five enzymes have been isolated and the nucleotide sequences have been determined (1, 3, 7, 8, 11–14, 16–19, 27, 30, 31). By contrast, much less genetic information is available concerning the genes involved in the late steps of heme synthesis in *E. coli*; mutants were obtained that are reported to be deficient in a late heme synthesis enzyme activity or were predicted to be so on the basis of some other phenotype (6, 24, 26). However, the genes disrupted in those mutants have not been described and those strains are no longer available for experimentation (5). Among the lost strains was a *pop-1* locus mutant; the suggestion that the ferrochelatase gene is located at that locus was put forth (6), but it has never been demonstrated. Recently, *E. coli* mutants were isolated on the basis of sensitivity to visible light, and a considerable body of indirect evidence suggests that the defective gene, designated *vis*A*, is involved in a late step in heme synthesis (20, 21, 23). Despite the volume of data addressing the role of the *vis*A* gene in heme formation, no heme synthesis enzyme activities have been measured in the mutants to determine whether there is indeed a defect in that pathway, nor has an enzyme activity been demonstrated for the *vis*A* gene product. Therefore, a *vis*A* mutant and the gene product were characterized in the present work.

**Characterization of an *E. coli* vis*A* mutant.** *E. coli* ∆Vis contains a deletion in the *vis*A* gene (20); growth of ΔVis in liquid culture was dependent on addition of exogenous hemin (Table 1), and thus that compound can apparently enter *E. coli* even in cells which have not been mutated to a hemin-permeable phenotype. Cells from liquid cultures of the mutant were fluorescent, and the chromophore was purified and identified as protoporphyrin IX by absorption spectroscopy of the methyl ester derivative and by thin-layer chromatography as previously described (10) (data not shown); this conclusion agrees with a recent report (21).

Ferrochelatase catalyzes the chelation of ferrous iron into protoporphyrin IX, and thus a defect in that activity would likely result in accumulation of protoporphyrin IX. However, a protoporphyrinogen oxidase mutant would yield the same phenotype because protoporphyrinogen is unstable and autooxidizes to form protoporphyrin. Therefore, the accumulation of protoporphyrin IX in ∆Vis suggests that either of the last two steps of the heme pathway is defective and it shows that the heme enzymes necessary for protoporphyrinogen synthesis are functional.

Strain ΔVis contained a wild-type level of protoporphyrinogen oxidase activity, but no ferrochelatase activity was detected in that mutant (Table 1). The *vis*A* gene was amplified from strain TBI genomic DNA by the polymerase chain reaction and cloned into pBluescript II KS+ for construction of *pVisA*3. pVisA3 conferred heme prototrophy and loss of the fluorescent-colony phenotype on the mutant, and the transformant expressed a very high level of ferrochelatase activity compared with *vis*A* strain TBI (Table 1). These data show that *vis*A* is essential for ferrochelatase activity and that protoporphyrinogen oxidase activity is affected neither by the *vis*A* deletion nor by the presence of the gene in a high copy number. The high level of ferrochelatase activity expressed in ∆Vis(pVisA3) indicates either that *vis*A* encodes that enzyme or that it encodes a protein involved in its expression.

**Characterization of the *vis*A* gene product.** The *vis*A* gene was modified at the 5' end of the coding region by using the polymerase chain reaction such that it could be cloned into the *NdeI-BamHI* sites of pET3a and express authentic protein (Fig. 1A). This plasmid construct, designated pETVISA, allows the *vis*A* gene to be under the transcriptional control of T7 RNA polymerase when placed into *E.

coli* BL21(ΔDE3)(pLysS) (25). Strain BL21(ΔDE3)(pLysS) (pETVISA) cells induced for expression of the plasmid-borne *vis*A* gene synthesized a peptide of approximately 38 kDa that was easily discerned on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels stained with Coomassie blue (Fig. 1B). This peptide was somewhat larger than the 36-kDa protein predicted by the putative amino acid sequence of the *vis*A* gene product (20), and it was absent in protein from the control strain (Fig. 1B). Because *E. coli* RNA polymerase, but not T7 RNA polymerase, is inhibited by rifampin, only the product of the plasmid-borne *vis*A* gene is synthesized from de novo tran-

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scripts in strain BL21(DE3)(pLysS)(pETVISA) in the presence of that antibiotic. Labeling of cellular protein with [35S]methionine after addition of rifampin showed that the overexpressed 38-kDa protein was radiolabeled (Fig. 1B). This finding demonstrated that the overexpressed protein observed in the Coomassie blue-stained gel is the visA gene product and not a peptide encoded by a chimeric gene which has been induced by expression of visA.

Much of the overexpressed visA product aggregated into presumptive inclusion bodies (Fig. 2A), as is common for proteins overexpressed in E. coli (2). This insoluble fraction was nearly homogeneous for the visA product, as observed on SDS-PAGE gels (Fig. 2A), thus providing a one-step purification scheme. The purified protein possessed ferrochelatase activity (Fig. 2B) with a specific activity of approximately 325 mmol/h/mg of protein in the linear region of time. The comparable fraction from the control cells [BL21(DE3)(pLysS)(pET3c)] contained very little protein, as judged from SDS-PAGE gels and protein assays, and contained no ferrochelatase activity (data not shown). These data show that the visA gene encodes ferrochelatase, and we propose that the visA gene be renamed *hemH*.

**Complementation of strain ΔVis with ferrochelatase-encoding DNA from eukaryotes.** The ferrochelatase gene from *Saccharomyces cerevisiae* (HEM15; reference 15) and the cDNA from a mouse transcript (29) were each cloned into pBluescript downstream of the lacZ promoter and introduced into strain ΔVis. Both the yeast and mouse DNAs conferred hemin prototrophy on strain ΔVis, and the colonies of those transformants were not fluorescent under UV light. Cell extracts of the transformants contained ferrochelatase activities of 13 and 1.2 nmol of mesoheme formed per h per mg of protein for the yeast and mouse enzymes, respectively. Human ferrochelatase has also been expressed in *E. coli* (4). The functional complementation of strain ΔVis with ferrochelatase-encoding mouse and yeast DNAs described herein shows that the eukaryotic proteins not only contained an activity in *E. coli* but must also function in concert with the *E. coli* heme enzymes for heme synthesis. This conclusion is particularly relevant in the context of the proposed substrate channeling between ferrochelatase and protoporphyrinogen oxidase (9), which would require at least dynamic protein-protein interactions, if not stable complex formation. Therefore, it will be interesting to learn the nature of the interactions between the heterologous enzymes in transformed ΔVis cells. A ferrochelatase-protoporphyrinogen oxidase complex has also been proposed to explain the diminution of both enzyme activities in humans with a defect in the structural gene for ferrochelatase (9, 22, 28). In *E. coli*, however, it is clear that protoporphyrinogen

<table>
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<tr>
<th>Strain</th>
<th>Colony fluorescencea</th>
<th>Hemin auxotrophyb</th>
<th>Protoporphyrinogen oxidase activityc</th>
<th>Ferrochelatase activityd</th>
</tr>
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<td>TB1</td>
<td>–</td>
<td>–</td>
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<tr>
<td>ΔVis</td>
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<td>+</td>
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<tr>
<td>ΔVis(pVisA3)</td>
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<td>–</td>
<td>5.3</td>
<td>70.1</td>
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a Fluorescence was observed under UV light.

b Requirement for 15 μM hemin was determined in liquid cultures of Luria-Bertani medium.

c Activity is expressed as nanomoles of protoheme formed per hour per milligram of cell extract protein.

d Activity is expressed as nanomoles of mesoheme formed per hour per milligram of cell extract protein.

**Table 1. Phenotypic characterization of mutant strain E. coli ΔVis with respect to heme biosynthesis**

**FIG. 2.** Overexpression, purification, and ferrochelatase activity of the visA gene product. BL21(DE3)(pLysS)(pETVISA) cells were induced, and fractions were obtained as described in the text. (A) Coomassie blue-stained SDS-PAGE gel of cell fractions. Lanes: 1, broken cells; 2, supernatant fraction of 7,500 × g centrifugation; 3, pellet of 7,500 × g centrifugation which contains the purified visA product in presumptive inclusion bodies. The numbers on the left are molecular sizes in kilodaltons. (B) Ferrochelatase activity of 0.64 mg of the sedimentible visA product (fraction corresponding to lane 3).

**FIG. 1.** Expression of the visA gene in a T7 expression system. (A) Construction of pETVISA. The visA (PCR) sequence shown is the polymerase chain reaction primer homologous to the 5' coding region of the gene. The lowercase bases represent sequences in the primer (thus, the final polymerase chain reaction product) that differ from the authentic gene to create an NdeI site. These changes do not affect the coding sequence. P<sub>T7</sub> represents the T7 gene 10 promoter on pET3c recognized by T7 RNA polymerase. Underlined sequences represent the ribosome-binding site of T7 gene 10. Overlined sequences represent the NdeI recognition site. (B) Coomassie blue staining (lanes 1 and 2) and autoradiogram (lanes 3 and 4) of SDS-PAGE gels of total cell proteins of BL21(DE3)(pLysS)(pETVISA) (lanes 2 and 4) and BL21(DE3)(pLysS)(pET3c) (lanes 1 and 3). The numbers on the left are molecular sizes in kilodaltons.
oxidase activity is not affected in a ferrochelatase-defective strain (Table 1).

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REFERENCES


5. Charles, H. P. Personal communication.


