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Accumulation of protoporphyrin IX in light-sensitive mutants of *Escherichia coli*

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The accumulation of protoporphyrin IX (Proto IX) in light-sensitive mutants of *Escherichia coli* was detected by spectrofluorimetry. Fluorescence emission and excitation spectra were recorded from extracts of bacterial cells. Proto IX clearly accumulated in cells with mutations in the *visA* (*hemH*) gene but not in the wild-type strain CA274 or in *visA* mutants that had been rendered light-resistant by introduction of the wild-type *visA*$^+$ gene. Accumulation of Proto IX was also not observed in cells with a mutation in the *visB* gene. These results confirm the hypothesis that the sensitivity of the *visA* mutants to light is caused by the abnormal accumulation of Proto IX, a substrate of ferrochelatase, as the result of a genetic defect in the gene for ferrochelatase.

Light-sensitive mutant; *visA* (*hemH*); Protoporphyrin IX; Ferrochelatase

1. INTRODUCTION

We previously isolated and characterized several light-sensitive mutant of *E. coli* [1]. These mutants can be classified into two groups: *visA* mutants [1,2] and *visB* mutants [3]. Cells with mutations in the *visA* gene are killed by illumination with visible light at about 460 nm. It has been shown that the gene, designated *visA*, in which almost all the mutations have been mapped, is the same gene as *hemH*. The *visA* (*hemH*) gene encodes ferrochelatase, the enzyme that catalyzes the final step in the biosynthesis of heme. The photosensitivity of *visA* mutants is probably caused by the accumulation of Proto IX, a substrate for ferrochelatase. Proto IX is a photosensitizer and generates an active species of oxygen via photochemical reactions [4,5]. The mutant bacterial cells might be killed by the harmful effects of such active oxygen.

In this study, we confirmed that Proto IX accumulates in the light-sensitive mutant bacteria by recording fluorescence spectra from the cell extracts. Utilizing various mutant strains, we also investigated the relationship between the sensitivity to light and the accumulation of Proto IX.

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Abbreviations: Proto IX, protoporphyrin IX; ΔALA, δ-aminolevulinic acid; LB medium, Luria–Bertani medium.

2. MATERIALS AND METHODS

All the bacterial strains used in this study have been reported previously [1–3]. The relevant phenotypes are listed in Table I.

λ*visA*$^+$ and pVRL1 are λgt-γC [6] phage clone and a pUC118 [7] plasmid that carry the 3.5-kb *Eco*RI fragment that includes the *visA*$^+$ gene, respectively [1].

The basic medium used was LB medium. For growth of the VS200 strain, LB medium were supplemented with 0.3% glucose.

For illumination, plates were exposed to light from two fluorescent lamps (Sunlight FL40SSD/37-G, 40 W, Hitachi, Tokyo, Japan) at a distance of 15 cm (approximately 7500 lx). Dark controls were wrapped in aluminium foil and placed at the same location. For cultures of light-sensitive mutants, test tubes were wrapped in aluminium foil and shaken. All cultures were incubated at 37°C.

For spectrofluorometry, the cultures of bacterial strains were centrifuged and cells were collected. The cells were suspended in extraction buffer, a mixture of acetone and 0.1 N NH_4OH (9:1, v/v), and vortexed thoroughly. These cell extracts were recentrifuged and each supernatant was collected. The supernatants were used for spectrofluorometry. Fluorescence emission (633 nm) and excitation (405 nm) spectra were recorded on a model 650-10S spectrofluorometer (Hitachi, Tokyo, Japan). The slit width was 3 nm for recording of the emission spectra and 6 nm for excitation spectra. The peaks of fluorescence were clearly due to Proto IX [8], as confirmed by measurements of fluorescence of pure Proto IX in solution. We used the pure Proto IX to generate a calibration curve and used the curve for the determination of approximately amounts of Proto IX in cell extracts.

Proto IX and hemin were obtained from the Sigma Chemical Co. (St. Louis, MO, USA).

3. RESULTS AND DISCUSSION

3.1. *Evidence that a light-sensitive mutant of* E. coli *accumulated Proto IX*

Fluorescence spectra were used to demonstrate the accumulation of Proto IX directly. Five ml of an overnight culture of the mutant strain VS101 and of the wild-type strain CA274 were centrifuged and cells were

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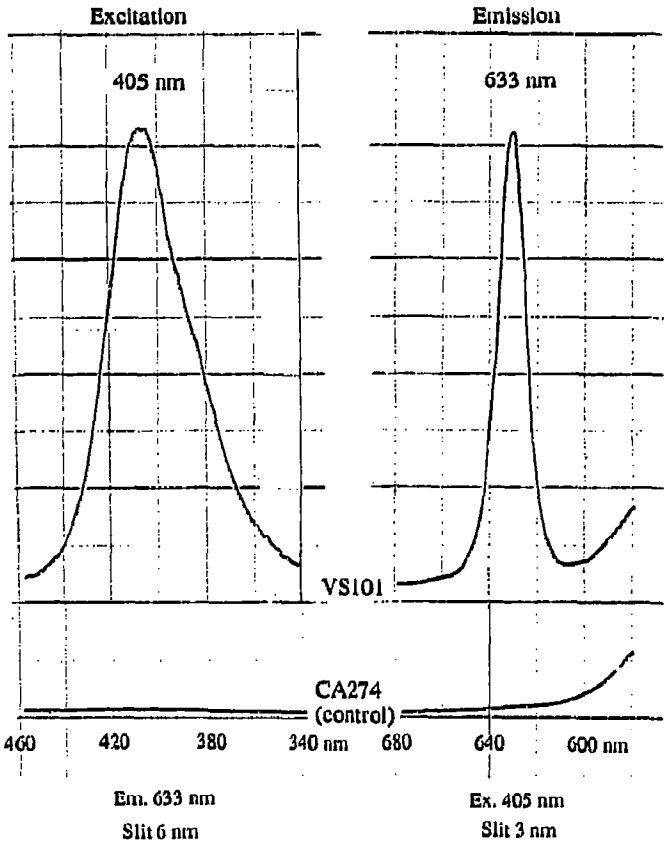

Fig.1. Fluorescence emission and excitation spectra were recorded by spectrofluorometry from cell extracts of the light-sensitive mutant VS101 and the wild-type CA274. An emission peak was observed at 633 nm and an excitation peak at 405 nm in the case of the extract of VS101. These peaks were not observed in the case of the extract of CA274.

resuspended in extraction buffer and vortexed. These suspensions of cells were recentrifuged and the supernatants were used for recording of fluorescence spectra. As shown in Fig. 1, an emission peak at 633 nm and an excitation peak at 405 nm were found in the case of the VS101 extract but not in that of the CA274 extract. These peaks corresponded to those peaks generated by Proto IX [8].

Table I

Bacterial strains

Strain	Relevant genotype*	Reference
CA274		9
VS101	*visA1*$^-$	1
VS102	*visA2*$^-$	1
VS103	*visA3*$^-$	1
VS104	*visA4*$^-$	1
VS200	*ΔvisA*	2
VS217	*ΔvisA, hemA*$^-$	2
VS550	*visB*$^-$	3

*Other genotypes are *HfrC*, lac_{am}, trp_{am}

3.2. *Relationship between light sensitivity and the level of Proto IX in cell extracts*

VS101 and cells with other mutations in the *visA* gene (VS102 through VS104) accumulated Proto IX at approximately 10^{-9}–10^{-10} M, as determined from the standard curve. We examined the relationship between the sensitivity to light and the level of Proto IX. VS200 (*ΔvisA*) is more sensitive to visible light than is VS101 [2]. As shown in Table II, the concentration of Proto IX in the extract of VS2000 (*ΔvisA*) cells was about twice that in the extract of VS101 cells. This result indicates that the level of Proto IX is directly related to the degree of sensitivity to light. However, we could not detect significant differences in the levels of Proto IX that corresponded to the different degree of sensitivity to light of the mutants VS101 through VS104. Differences in the recovery of Proto IX from the various cultures might explain this result.

3.3. *Evidence that no accumulation of Proto IX occurs in the visA mutant after complementation with the wild-type* visA$^+$ *gene or in other mutants*

We examined the accumulation of Proto IX in mutant cells when the wild-type *visA*$^+$ gene had been introduced by either *λvisA*$^+$ or the plasmic pVRL1. VS101 cells carrying either *λvisA*$^+$ or pVRL1, which reverse the photosensitivity, did not accumulate Proto IX (data not shown). This result shows directly that the substrate for the product of the *visA* gene is Proto IX and that the *visA* gene encodes ferrochelatase.

A double mutant with mutations in *visA* (*hemH*) and the *hemA* gene has been isolated in our laboratory and designated VS217 [2]. The double mutant VS217 is not sensitive to light. We hypothesized that the mutation in the *hemA* gene blocks the biosynthesis of ΔALA (δ-aminolevulinic acid) and that no Proto IX is synthesized. We examined this hypothesis by measuring the accumulation of Proto IX in the VS217 mutant. As expected, no Proto IX accumulated in the VS217 mutant (data not shown).

We also examined the accumulation of Proto IX in the *visB* mutant (VS550) and found no accumulation of Proto IX in these cells (data not shown). Thus, Proto IX does not participate in the sensitivity of the *visB* mutant to light.

Table II

Accumulation of Proto IX in VS101 and VS200 (*ΔvisA*) mutants, as measured in cell extracts

Strain	OD_{600} of original culture	Proto IX (M)	Proto IX/OD_{600} ($\times 10^{-10}$)
CA274	1.47	$>10^{-12}$	>0.01
VS101	1.51	1.08×10^{-9}	7.15
VS200	0.25	3.55×10^{-10}	14.2

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