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Identification of the Genes Encoding NAD(P)H-Flavin Oxidoreductases That Are Similar in Sequence to *Escherichia coli* Fre in Four Species of Luminous Bacteria: *Photorhabdus luminescens*, *Vibrio fischeri*, *Vibrio harveyi*, and *Vibrio orientalis*

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Genes encoding NAD(P)H-flavin oxidoreductases (flavin reductases) similar in both size and sequence to Fre, the most abundant flavin reductase in *Escherichia coli*, were identified in four species of luminous bacteria, *Photorhabdus luminescens* (ATCC 29999), *Vibrio fischeri* (ATCC 7744), *Vibrio harveyi* (ATCC 33843), and *Vibrio orientalis* (ATCC 33934). Nucleotide sequence analysis showed Fre-like flavin reductases in *P. luminescens* and *V. fischeri* to consist of 233 and 236 amino acids, respectively. As in *E. coli* Fre, Fre-like enzymes in luminous bacteria preferably used riboflavin as an electron acceptor when NADPH was used as an electron donor. These enzymes also were good suppliers of reduced flavin mononucleotide ($FMNH_2$) to the bioluminescence reaction. In *V. fischeri*, the Fre-like enzyme is a minor flavin reductase representing <10% of the total FMN reductase. That the *V. fischeri* Fre-like enzyme has no appreciable homology in amino acid sequence to the major flavin reductase in *V. fischeri*, FRase I, indicates that at least two different types of flavin reductases supply $FMNH_2$ to the luminescence system in *V. fischeri*. Although Fre-like flavin reductases are highly similar in sequence to *luxG* gene products (LuxGs), Fre-like flavin reductases and LuxGs appear to constitute two separate groups of flavin-associated proteins.

In the bacterial luminescence reaction, reduced flavin mononucleotide ($FMNH_2$) and long-chain aliphatic aldehyde (R-CHO) are simultaneously oxidized by molecular oxygen, with blue-green light being emitted (5, 12, 25). The $FMNH_2$-supplying system involves NAD(P)H-flavin oxidoreductase (flavin reductase), which catalyzes the reduction of flavins, including FMN, at the expense of reduced pyridine nucleotides [NAD(P)H] (5, 12, 25). The overall reaction is as follows:

$$FMNH_2 + \text{R-CHO} + O_2 \xrightarrow[\text{(luciferase)}]{} FMN + \text{R-COOH} + H_2O + \text{light (490 nm)}$$

$$FMN + \text{NAD(P)H} + H^+ \xrightarrow[\text{(flavin reductase)}]{} FMNH_2 + \text{NAD(P)}^+$$

Most luminous bacteria appear to contain multiple flavin reductases different in enzymatic nature and molecular size (12). For example, *Vibrio harveyi* contains at least three types of flavin reductases (15, 47). The 30-kDa enzyme is specific to NADH, whereas the 40-kDa enzyme is NADPH specific, and the third enzyme possibly uses both NADH and NADPH as electron donors. *Vibrio fischeri* may contain two to three species of flavin reductase. Duane and Hastings (9) identified the major flavin reductase whose molecular weight was estimated at 43,000 by gel filtration. Recently, we cloned the gene encoding the major FMN reductase in *V. fischeri*, FRase I, and purified it virtually to homogeneity from *Escherichia coli* cells expressing the cloned FRase I (51). FRase I was capable of using both NADH and NADPH as electron donors (9, 51). As electron acceptors, FMN was the most effective, flavin adenine dinucleotide (FAD) was considerably effective, and riboflavin was the least effective. When FMN and NADH were used as an electron acceptor and donor, respectively, FRase I represented >90% of the total flavin reductase activity in crude extracts of *V. fischeri* cells (51). Gel filtration analysis of the crude extract of *V. fischeri* also showed that a minor flavin reductase or reductases were included in *V. fischeri* cell extracts, which preferentially use riboflavin as an electron acceptor (51). The minor flavin reductase or reductases in *V. fischeri* may correspond to the 24-kDa enzyme which was found by Puget and Michelson (30) and suggested to be widely distributed in both luminous and nonluminescent bacteria. Most, if not all, flavin reductases so far identified in luminous bacteria have been capable of serving as good suppliers of $FMNH_2$ to the in vitro bioluminescence reaction (9, 15, 30, 38, 51).

We do not know whether these flavin reductases of luminous bacteria are related in sequence or evolution, since *V. fischeri* FRase I is the only flavin reductase in luminous bacteria whose amino acid sequence has been determined (51). The amino acid sequence of FRase I showed no significant homology with the reported sequences of two bacterial flavin reductases, *E. coli* Fre (42), which is a member of a ribonucleotide reductase multiprotein system (10) and is the flavin reductase representing most activity of flavin reductase in *E. coli* (37), and BaiH from *Thermoanaetobium brockii* (11), which has considerable homology with disulfide reductases. As with the minor flavin reductase or reductases in *V. fischeri*, *E. coli* Fre preferentially uses riboflavin as an electron acceptor. Previous experiments showed that there is considerable sequence homology between the *E. coli* Fre and LuxG proteins in luminous bacteria (1, 49).

Here, we searched for *fre*-like genes in luminous bacteria to examine whether luminous bacteria contain Fre-like enzymes. Genes encoding flavin reductase highly similar in sequence to

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E. coli Fre were found to be widely distributed in luminous bacteria. These Fre-like enzymes were capable not only of using riboflavin as the most preferred electron acceptor but also of serving as a supplier of $FMNH_2$ to the in vitro bioluminescence reaction. Thus, at least in *V. fischeri*, two flavin reductases unrelated in sequence and evolution are suggested to be simultaneously used as catalysts for supplying $FMNH_2$ to the bioluminescence system. Although our results indicated that *luxG* gene products (LuxGs) of luminous bacteria are highly similar in amino acid sequence to Fre-like enzymes, LuxGs constitute a group of proteins distinctly different from that for Fre flavin reductases.

MATERIALS AND METHODS

Enzymes and chemicals. *V. fischeri* luciferase was purchased from Boehringer Mannheim (Indianapolis, Ind.), while restriction enzymes and various DNA-modifying enzymes (polynucleotide kinase, T4 DNA ligase, and so on) were obtained from Nippon Gene (Toyama, Japan), Toyobo (Osaka, Japan), and Takara Shuzo (Kyoto, Japan). [α-^{32}P]dCTP (3,000 Ci/mmol) was purchased from ICN Biomedicals (Costa Mesa, Calif.). Oligonucleotides were synthesized with a Biosearch Cyclone DNA synthesizer. FMN sodium salt, FAD disodium salt, riboflavin, and rabbit anti-goat immunoglobulin G polyclonal antibody labeled with horseradish peroxidase were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). NADH sodium salt was from Oriental Yeast (Osaka, Japan), while NADPH tetrasodium salt was from Merck (Darmstadt, Germany). Glutathione, goat anti-glutathione *S*-transferase (GST) polyclonal antiserum, thrombin, isopropyl-β-D-thiogalactopyranoside (IPTG), glutathione Sepharose 4B, and Superose 12 were obtained from Pharmacia (Uppsala, Sweden). 4-Chloro-1-naphthol was purchased from Bio-Rad (Richmond, Calif.). All of these and other chemicals were of the highest grade commercially available.

Bacterial strains and plasmids. Both marine luminous bacteria (*V. fischeri* ATCC 7744, *Alteromonas hanedai* ATCC 33224, *V. harveyi* ATCC 33843, *Photobacterium phosphoreum* ATCC 11040, and *Vibrio orientalis* ATCC 33934) and terrestrial luminous bacteria (*Photorhabdus luminescens* ATCC 29999) (6) were obtained from American Type Culture Collection. Marine bacteria were cultured in *Photobacterium* medium (Difco Laboratories, Detroit, Mich.) at 26°C, while terrestrial bacteria were cultured in Luria-Bertani medium (35) at 37°C. The following *E. coli* strains were also used for cloning and expression of cloned fragments: JM83 [*ara* Δ(*lac-proAB*] F^- *rpsL*(*φ80lacZΔM15*)] (27) and C600 (r_K^- m_K^+ F^- *thi-1 thr-1 leuB6 lacY1 tonA21 supE44*) (2, 32). As cloning vehicles, pCR1000 (Invitrogen), pUC13 (26), pTV119N (Takara Shuzo) (22, 46), and pGEX-2T (Pharmacia) were used.

Molecular cloning. (i) PCR, Southern blotting, and genomic DNA library construction. PCR was carried out with the AmpliTaq PCR kit (Perkin-Elmer, Norwalk, Conn.) essentially as described by Saiki et al. (34): 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 60 s, and extension at 72°C for 90 s with a Perkin-Elmer thermal cycler. Southern hybridization was performed under low-stringency conditions (51). Nucleotide sequences were determined by the dideoxy sequencing method (13) with the *Bca*Best dideoxy sequencing kit (Takara Shuzo) (45). Sequence information was analyzed with DNASIS software (Hitachi Software Engineering, Yokohama, Japan) implemented on an NEC-compatible personal computer. Genomic DNA libraries were constructed with *P. luminescens* and *V. fischeri* DNAs partially digested with *Sau*3AI or completely digested with *Hin*dIII. Other techniques for molecular cloning have been described by Sambrook et al. (35).

(ii) Cloning of *E. coli* and *P. luminescens fre* genes. After PCR amplification of the genomic DNA of *E. coli* C600 with the oligonucleotides FRE1 and FRE2 (see Fig. 3), amplified DNA was cloned into pCR1000. Nucleotide sequence analysis showed that all clones examined had the entire *fre*-coding sequence (42). The insert of pFre2, a clone arbitrarily chosen, was used as a probe for Southern blotting and isolation of pPH3. Moreover, the insert of pPH3 was used as a probe to isolate pPS40 from a *Sau*3AI partial genomic DNA library of *P. luminescens*. A 1.1-kb *Sal*I-*Hpa*I fragment of pPS40 was inserted into the *Sal*I-*Sma*I site of pUC13 to construct pPFR1, in which a putative *fre* gene of *P. luminescens* is under control of the *lac* promoter-operator so that JM83 cells harboring pPFR1 can overproduce *P. luminescens* Fre protein.

(iii) Cloning of *fre* genes of *V. fischeri*, *V. harveyi*, and *V. orientalis*. With FRE3 and FRE4 oligonucleotides (see Fig. 3) as PCR primers, *fre* gene fragments were amplified from the genomic DNA of *V. fischeri*, *V. harveyi*, and *V. orientalis*. Amplified *fre* gene fragments 550 bp long were cloned into pUC13 and subjected to sequence analysis. In the case of *V. fischeri*, pVS2, a clone containing the entire *fre* sequence, was further isolated from a *Sau*3AI partial genomic DNA library of *V. fischeri* with PCR-amplified cloned DNA as a probe. For expression in *E. coli*, a 1.2-kb *Xho*I-*Bgl*II fragment of pVS2 was inserted into the *Sal*I-*Bam*HI site of pUC13 to construct pfFR1.

(iv) Cloning of *lux* genes. By using as probes the *luxC* and *luxG* DNA fragments prepared in a separate experiment, pLux7, a clone containing the entire *lux* operon of *V. fischeri* (3, 43), was isolated. For construction of pG, an expression plasmid of *luxG*, a 1.7-kb *Eco*RV-*Stu*I fragment was inserted into the *Hin*cII site of pTV119N. For expression of the GST-*luxG* fusion gene, a 47-bp-long double-stranded DNA consisting of two synthetic deoxyoligonucleotide chains (5′-CGGATCCATGATTGTTGATGGCAGAGTTTCAAA GATAGTTTTAGCAT-3′ and 5′-CGATGCTAAAACTAT CTTTGAAACTCTGCCATCAACAATCATGGATC-3′) was inserted into the *Cla*I site of pG to make pG′. The 1.5-kb *Bam*HI fragment of pG′ was inserted into the *Bam*HI site of pGEX-2T to generate pGST-G. In *E. coli* cells with pGST-G, the GST-*luxG* fusion gene is expressed.

Protein analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was carried out essentially as described by Laemmli (19) under reducing conditions with a 15% polyacrylamide gel (1-mm thickness). Protein bands were visualized by staining with Coomassie brilliant blue R-250 (Bio-Rad). Western blot (immunoblot) analysis was performed as described previously (48). The protein concentration was determined by the dye-binding method of Bradford (7) with bovine gamma globulin as a standard (Bio-Rad).

Enzyme assay. The preparation of cell extracts, column chromatography, the flavin reductase assay, and the bioluminescence reaction were carried out essentially as described previously (51).

Nucleotide sequence accession number. DDBJ, EMBL, and GenBank accession numbers for the complete DNA sequences of the *fre* genes of *P. luminescens* and *V. fischeri* are D17745 and D17744, respectively, while those for the partial DNA sequences of the *V. harveyi* and *V. orientalis fre* genes are D17746 and D17747, respectively.

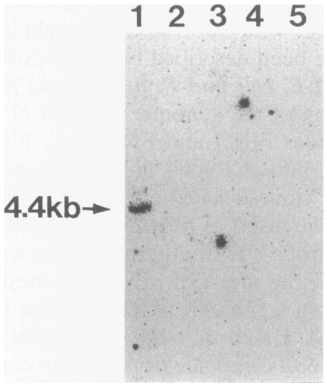

FIG. 1. Identification of a DNA fragment of the putative flavin reductase gene of *P. luminescens* similar in sequence to *E. coli fre*. Genomic DNA from five species of luminous bacteria was digested with *Hin*dIII, size fractionated on an agarose gel by electrophoresis, and subjected to low-stringency blot hybridization with an *E. coli fre* fragment as a probe. The arrow indicates a hybridized band of 4.4 kb. Lanes: 1, *P. luminescens*; 2, *V. orientalis*; 3, *V. harveyi*; 4, *V. fischeri*; 5, *A. hanedai*.

RESULTS AND DISCUSSION

Identification of the *fre* gene of *P. luminescens*. As a first step to examine whether luminous bacteria contain the gene encoding Fre-like flavin reductase, chromosomal DNA from five species of luminous bacteria was digested with *Hin*dIII, size fractionated on an agarose gel by electrophoresis, and subjected to Southern hybridization with the insert of pFre2 as a probe (Fig. 1). Under the less-stringent condition used, only a single hybridization band of 4.4 kb was detected in the lane for *P. luminescens* (lane 1). Since the long open reading frame found in the cloned 4.4-kb DNA fragment was truncated, a more complete clone (pPS40) was isolated from a different library of *P. luminescens* (Fig. 2A). Nucleotide sequence analysis showed the open reading frame in pPS40 to code for a polypeptide consisting of 233 amino acids (estimated molecular weight, 26,439 [Fig. 2B]) highly similar in sequence to *E. coli* Fre (42) (233 amino acids long). Identity and similarity in amino acid sequence were estimated as 73 and 84%, respectively; nucleotide sequence homology was 67%. Similarity in nucleotide sequence can be extended into both 5′ and 3′ flanking regions (Fig. 2B). A 12-nucleotide-long consecutive sequence was found to be conserved in the region situated

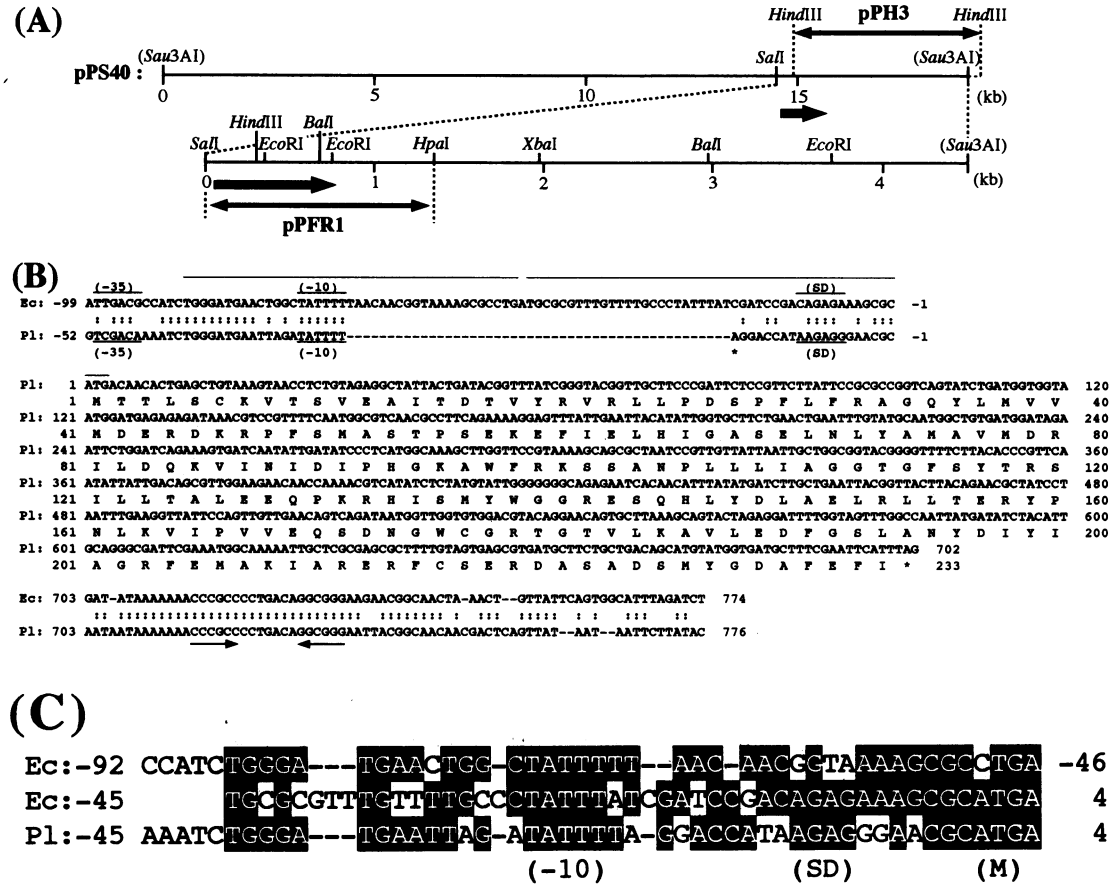

FIG. 2. Physical map and nucleotide sequence of the *P. luminescens fre*-like gene. (A) Physical map of the DNA insert in pPS40. Horizontal lines with double arrowheads show the cloned regions. Thick arrows show the size, location, and orientation of transcription of the putative *fre*-coding sequence. (B) Nucleotide sequence of the *P. luminescens fre*-like gene and the deduced amino acid sequence of its putative gene product. Nucleotide sequences of the 5′ and 3′ flanking regions of *E. coli fre* (42) are also shown for comparison. Ec, *E. coli fre*; Pl, *P. luminescens fre* homolog. Thin lines labeled −10 and −35, respectively, show both sizes and locations of −10 and −35 boxes (33). SD, Shine-Dalgarno sequence (40). Thin horizontal lines show a possible duplication found in the 5′ regulatory region of the *E. coli fre* gene (see panel C). A pair of arrows in the 3′ untranslated region indicates the location of a stem-loop structure, a possible transcription terminator (33). (C) Duplicated sequences found in the 5′ untranslated region of the *E. coli fre* gene. Identical bases are indicated by white letters in black boxes. M, putative initiation methionine.

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      1 Met-Thr-Thr-Leu-Ser-Cys-Lys-Val 8
 FRE1:5' ATG-ACA-ACC-TTA-AGC-TGT-AAA-GT 3'
(23-mer)

    226 Gly-Asp-Ala-Phe-Ala-Phe-Ile-*** 233
 FRE2:  3' G-CTA-CGC-AAA-CGT-AAA-TAG-ACT 5'
(22-mer)

     35 Gln-Tyr-Leu-Met-Val-Val-Met 41
 FRE3:5' CAA-TAT-TTT-ATG-GTT-GTT-ATG 3'
(21-mer)   G   C C C       C   C
                A       A   A
                G       G   G

    201 Ala-Gly-Arg-Phe-Glu-Met-Ala 207
 FRE4:3' CGA-CCA-GCA-AAA-CTT-TAC-CG 5'
(20-mer)   G   G T G   G   C
           T   T   T
           C   C   C
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FIG. 3. Design of PCR primers. FRE1 and FRE2 correspond to the 5′- and 3′-terminal sequences of the *E. coli fre*-coding region (42). Mixed primers FRE3 and FRE4 were made on the basis of the amino acid sequences conserved between *E. coli* Fre and *P. luminescens* Fre-like flavin reductase.

between the −10 and −35 boxes (33). An apparent 47-bp-long deletion in *P. luminescens* appears to be caused by a duplication in *E. coli*, as shown in Fig. 2C. The 3′ untranslated region contains a 29-nucleotide-long conserved sequence including a stem-loop structure surrounded by AT-rich sequences, a presumptive transcription terminator (Fig. 2B) (33). Taken together, these results suggest that the open reading frame identified here in *P. luminescens* is the counterpart of the *E. coli fre* gene (42).

Identification of *fre* homologs in luminous bacteria other than *P. luminescens*. To further search for *fre* homologs in luminous bacteria, redundant PCR primers, FRE3 and FRE4 (Fig. 3), were made on the basis of the amino acid sequence homology between *E. coli* Fre and the *P. luminescens* Fre homolog (Fig. 4). DNA extracted from *V. fischeri*, *V. harveyi*, *V. orientalis*, *P. phosphoreum*, and *A. hanedai* was subjected to PCR amplification. A single band of 550 bp was detected in all samples except for those for *P. phosphoreum* and *A. hanedai*. Nucleotide sequence analysis of cloned, PCR-amplified fragments showed that *V. fischeri*, *V. harveyi*, and *V. orientalis* contain a gene capable of encoding a polypeptide similar in sequence to both *E. coli* Fre (42) and the *P. luminescens* Fre homolog (Fig. 4). This finding was further confirmed by molecular cloning and subsequent sequencing of a *V. fischeri* DNA fragment with a complete sequence encoding the Fre-like polypeptide (Fig. 5). The *V. fischeri* gene encodes a polypeptide with a length of 236 amino acids (estimated molecular weight, 26,067). Except for a 3-amino-acid-long deletion or insertion, the identity and similarity in amino acid sequence to *E. coli* Fre (42) were 52 and 72%, respectively, whereas those to *P. luminescens* were 51 and 67%, respectively (Fig. 4). The 3′ untranslated region of the *V. fischeri fre*-like gene contains a putative transcription terminator with a stem-loop structure (33), as was found with *P. luminescens fre* homolog and *E. coli fre* (Fig. 5B).

By X-ray crystallography, the atomic structure of spinach ferredoxin-NADP$^+$ reductase, having similarity in sequence to Fre, has been examined and four amino acid sequences (RLYS, TGTG, CG, and EVY) are suggested to be situated at or near the flavin-nicotinamide interaction site (16). As shown in Fig. 4, all of these sequences are replaced by chemically similar sequences, but their functional conservation in *E. coli* Fre and luminous bacterial Fre homologs is feasible. Furthermore, *P. luminescens* and *V. fischeri* Fres were associated with

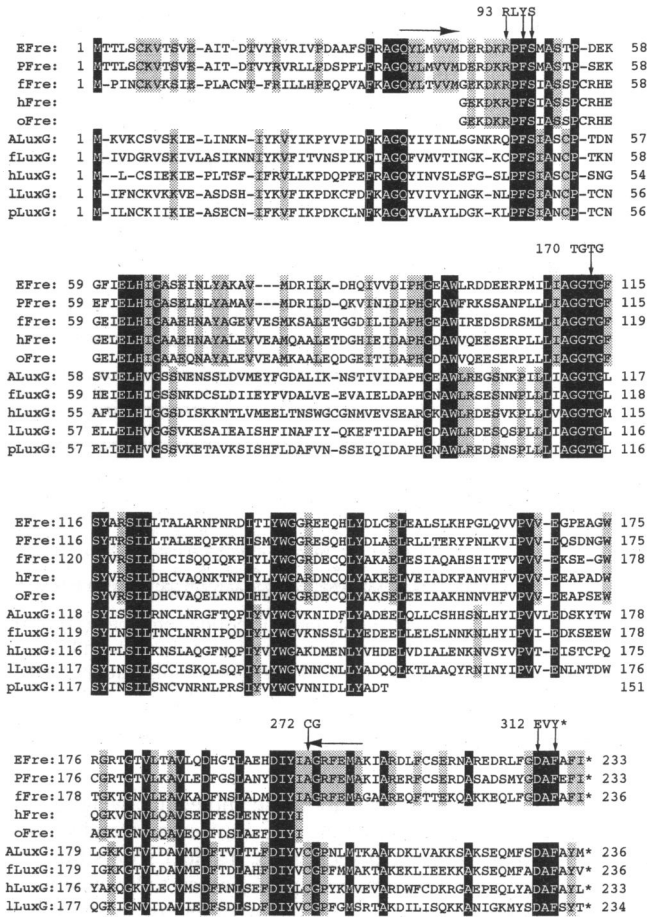

FIG. 4. Amino acid sequence comparison of Fre and LuxG proteins. EFre, *E. coli* Fre (42); PFre, *P. luminescens* Fre; fFre, *V. fischeri* Fre; hFre, *V. harveyi* Fre; oFre, *V. orientalis* Fre; ALuxG, *A. hanedai* LuxG (52); fLuxG, *V. fischeri* LuxG (43); hLuxG, *V. harveyi* LuxG (44); lLuxG, *Photobacterium leiognathi* LuxG (20); pLuxG, *P. phosphoreum* LuxG (44). Identical amino acids are indicated by white letters in black boxes. Amino acids conserved only in Fres or LuxGs are indicated by shaded boxes. Four key sequences situated at or near the flavin-nicotinamide interaction site of spinach ferredoxin-NADP$^+$ reductase (16) are indicated by vertical arrows marked with their sequences and positions. Horizontal arrows show the amino acid sequences corresponding to the PCR primers FRE3 and FRE4. Amino acids belonging to the same group are as follows: A, S, P, T, and G; N, D, E, and Q; H, R, and K; M, L, I, and V; and F, Y, and W (39). Gaps (−) were inserted to increase the sequence homology.

the activity of flavin reductase having substrate preference similar to that of *E. coli* Fre (described below) (10). Thus, we conclude that not only *P. luminescens* but also *V. fischeri*, *V. harveyi*, *V. orientalis*, and *P. luminescens* have a gene encoding flavin reductase very similar to Fre in *E. coli*. We hereafter refer to these genes as *fre* genes of luminescent bacteria.

Fre proteins of *P. luminescens* and *V. fischeri* are flavin reductase capable of enhancing the in vitro bioluminescence reaction. To examine whether *P. luminescens* and *V. fischeri fre* gene products are flavin reductase, their expression was stimulated in *E. coli* with the *lacZ* operator-promoter system. In *V. fischeri*, a new protein band of 32 kDa was clearly seen (Fig. 6), whereas a very faint 29-kDa band was detected in *P. luminescens* (data not shown), possibly suggesting that the *V. fischeri fre* gene product is more stable in *E. coli* than is the counterpart

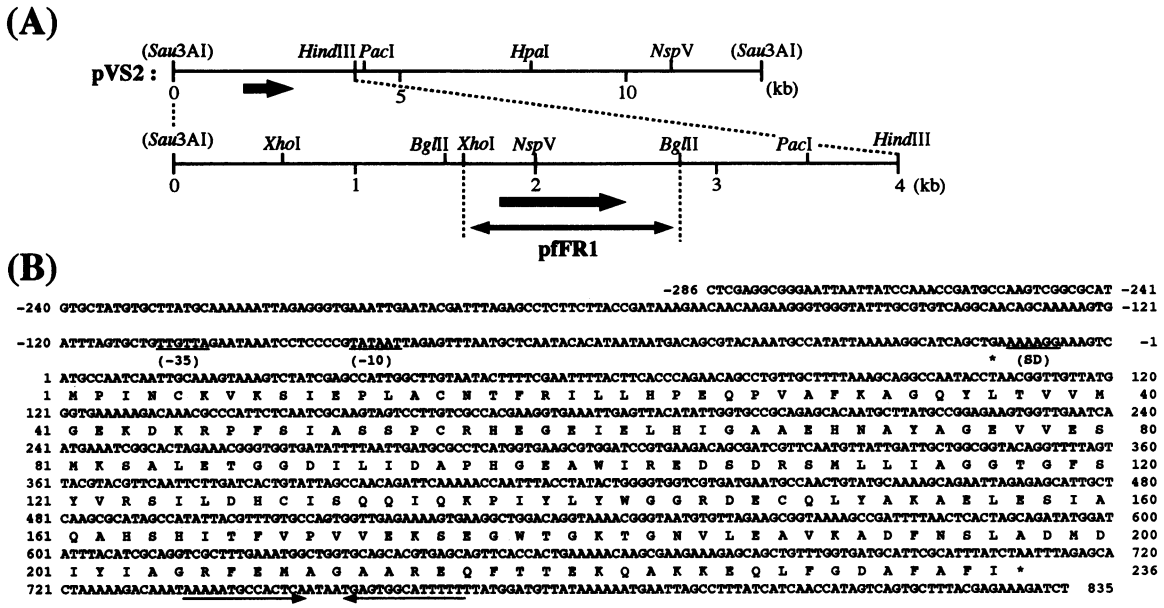

FIG. 5. Genomic structure (A) and nucleotide sequence (B) of the *V. fischeri fre* gene. (A) Restriction map of the DNA insert of pVS2. The horizontal line with double arrowheads indicates the size and location of the DNA insert in pfFR1. Thick arrows show the size, location, and orientation of transcription of the *V. fischeri fre*-coding sequence. (B) The deduced amino acid sequence of the *V. fischeri* Fre-like protein is shown below the nucleotide sequence. −10, −10 box (33); −35, −35 box (33); SD, Shine-Dalgarno sequence (40). Two pairs of palindromic sequences are indicated by arrows.

of *P. luminescens*. In both cases, the molecular weights obtained were slightly higher than the molecular weights estimated by using amino acid sequences. Table 1 shows both *fre* gene products of *P. luminescens* and *V. fischeri* to be capable of exhibiting a significant level of flavin reductase activity if a suitable electron donor and acceptor are chosen. When NADH was used as an electron donor, both FMN and riboflavin could serve as effective electron acceptors, while NADPH could transfer electrons almost exclusively to riboflavin. Only a low level of electron transfer, if any, occurred when FAD was used as an electron acceptor. Thus, the substrate preference of Fre flavin reductases of *P. luminescens* and *V. fischeri* is suggested to resemble that of *E. coli* Fre (10) (Table 1).

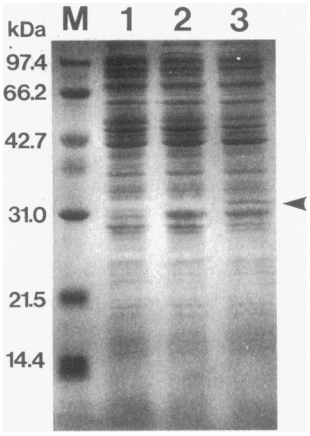

FIG. 6. Expression of *V. fischeri* Fre in *E. coli*. A 30-μl aliquot of extracts was applied to each lane. Molecular mass is shown in the left margin in kilodaltons. Lanes: M, protein size markers (Bio-Rad) phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa); 1, extracts of JM83 cells; 2, extracts of JM83 cells harboring pUC13; 3, extracts of JM83 cells harboring pfFR1. The arrowhead shows the location of the expressed *V. fischeri* Fre protein (32 kDa).

Figure 7A shows a gel filtration pattern of crude *V. fischeri* extracts. It is evident that, in addition to FRase I, *V. fischeri* contains a minor flavin reductase or reductases (fraction II) representing <10% of the total FMN reductase activity and capable of reducing riboflavin effectively. The molecular weight of the flavin reductase or reductases in fraction II is 26,000 and is identical to that of the Fre flavin reductase of *V. fischeri* (compare Fig. 7A with B). Furthermore, as described above, *V. fischeri* Fre preferably uses riboflavin as an electron acceptor. Thus, *V. fischeri* Fre is suggested to represent most, if not all, of the flavin reductase activity found in fraction II.

TABLE 1. Flavin reductase activity in *E. coli* cells expressing *P. luminescens* or *V. fischeri fre* genes

Pyridine nucleotide	Extract	Flavin reductase activity (nmol/min/mg of protein) on:		
		FMN	FAD	Riboflavin
NADH	JM83	26	27	29
	JM83(pUC13)	25	31	25
	JM83(pXFR1)	136	51	154
	JM83(pfFR1)	603	125	732
	Fre[a]	350	350	1,000
NADPH	JM83	6	4	21
	JM83(pUC13)	2	2	16
	JM83(pXFR1)	107	2	510
	JM83(pfFR1)	88	3	689
	Fre[a]	120	0	1,500

[a] Fontecave et al. (10).

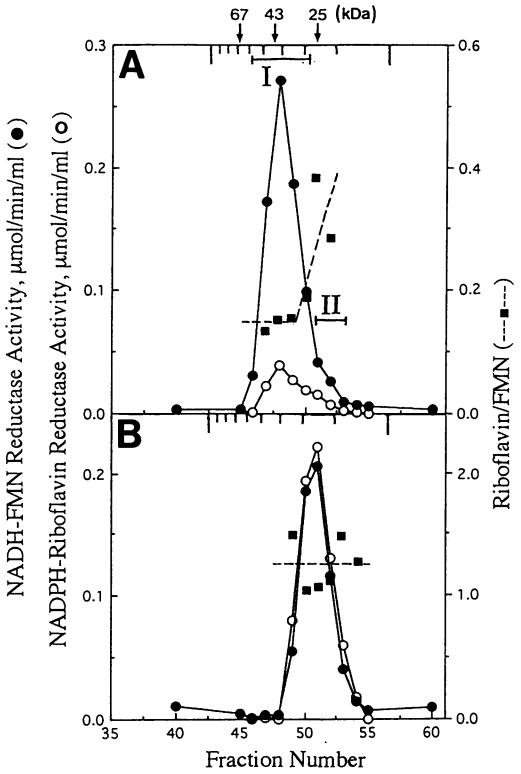

FIG. 7. Superose 12 elution profiles of crude extracts of *V. fischeri* (A) and *E. coli* (B) cells expressing the *V. fischeri* Fre-like enzyme. ●, NADH-FMN reductase activity; ○, NADPH-riboflavin reductase activity; ■, ratio between riboflavin reductase and FMN reductase activities. The locations of molecular size markers (albumin [67,000], ovalbumin [43,000], chymotrypsinogen A [25,000]) are shown by vertical arrows at the top. I and II, respectively, show fractions rich in FRase I and a minor flavin reductase or reductases. The molecular weight of the Fre-like enzyme is identical to that of the minor flavin reductase in fraction II and is estimated as 26,000. Fraction II represents <10% of the total flavin reductase activity in *V. fischeri* extracts.

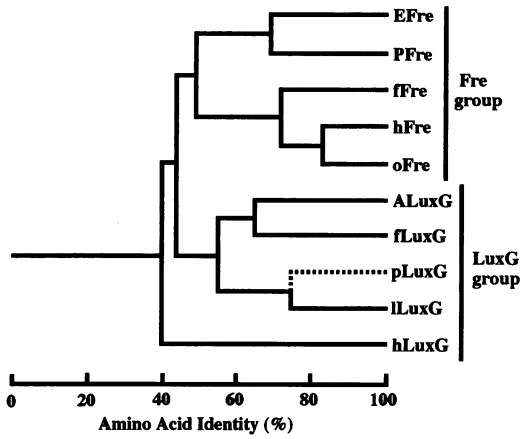

FIG. 8. Homology tree of Fre and LuxG proteins of luminous bacteria. Amino acid sequences corresponding to the 42 to 200 region of *E. coli Fre* (42) were used for comparison. In the case of *P. phosphoreum* LuxG, only a partial sequence was used for calculation. Abbreviations are the same as those defined in the legend to Fig. 4. The homology tree was constructed by using amino acid sequence homologies. A deletion was presumed to be equivalent to a 1-amino-acid substitution.

When *E. coli* extracts expressing *V. fischeri* Fre were used as a source of flavin reductase, about 34-fold stimulation of the bioluminescence reaction was detected (data not shown). Incubation with *E. coli* extracts expressing *P. luminescens* Fre resulted in a 10-fold increase bioluminescence. These findings and the fact that FRase I of *V. fischeri* is capable of enhancing the in vitro bioluminescence reaction (51) may suggest that, at least in *V. fischeri*, two flavin reductases, evolutionarily unrelated, and hence having little sequence homology with each other, are involved in supplying $FMNH_2$ to the bioluminescence reaction, although additional in vivo experiments will be needed.

LuxG and Fre proteins form two separate groups of flavin-associated proteins in luminous bacteria. In previous experiments (1, 49), LuxG proteins of luminous bacteria were shown to be similar in sequence to *E. coli* Fre (Fig. 4). Furthermore, as summarized in Fig. 4, three of four key sequences forming the flavin-nicotinamide interaction site (16) are conserved or replaced by chemically similar amino acid sequences in LuxG proteins (20, 43, 44, 52). Unlike Fre-like enzyme, the arginine residue in the remaining key sequence, RLYS, which is presumed to be involved in the interaction with pyrophosphate in FAD, was deleted. The identity and similarity in amino acid sequence between LuxGs and Fres are estimated as 35 to 46 and 56 to 68%, respectively. Thus, it is quite feasible that LuxG proteins of luminous bacteria belong to a class of flavin-associated proteins (1, 16, 49) highly related in evolution and/or function to Fres. A homology tree shown in Fig. 8 demonstrates that Fres and all LuxGs other than *V. harveyi* LuxG constitute two different subgroups of flavin-associated proteins. *V. harveyi* LuxG may form a third group, possibly indicating that there are two cycles of gene duplication involved in the evolution of *fre* and/or *luxG* genes. However, we do not know whether the LuxG protein is associated with flavin reductase activity. So far, neither additional flavin reductase activity nor an appreciable amount of LuxG protein has been detected in cell extracts of *E. coli* with the cloned *luxG* gene or the entire *lux* enzyme genes (*luxCDABEG*) of *V. fischeri*. When a GST-*luxG* fusion gene was expressed in *E. coli*, almost all GST-LuxG proteins were recovered as inclusion bodies.

Additional discussion. In the present work, we showed evidence that Fre-like flavin reductases with a molecular weight of 30,000 are widely distributed in luminous bacteria. In *V. fischeri*, the Fre-like enzyme represents only a minor portion of the total flavin reductase activity, while in *E. coli*, Fre is the major flavin reductase (37). In contrast, FRase I represents >90% of the total flavin reductase activity in *V. fischeri* (51) (Fig. 7A), while its counterpart in *E. coli* is the *nfnB* nitroreductase (18, 36), which is associated with a very low level of flavin reductase activity (50). These results may suggest that it is the total amount of the reduced form of FMN not the level of expression of individual flavin reductases that is important to bacteria. In other words, flavin reductases are functionally redundant.

The phylogenetic tree shown in Fig. 8 suggests that the *luxG* and *fre* genes were generated in evolution by duplication. Although we do not know whether the *luxG* gene product is flavin reductase or not, its intimate relationship in sequence and evolution to the *fre* gene product strongly suggests that the *luxG* gene product is a protein capable of binding to various flavins, as in the case of proteins encoded by the *lux* operon, which include α and β subunits of luciferase (LuxA and LuxB

[5, 12, 25]), nonfluorescent flavoprotein (LuxF [14, 17, 23, 24, 28, 29, 31, 41]), and yellow fluorescent protein (LuxY [4, 5, 8, 21]). For bioluminescence in vivo, luciferase ($\alpha\beta$ heterodimer) must be supplied with reduced FMN and tetradecanal (5), and nonfluorescent flavoprotein is shown to be associated with myristylated FMN (17). Yellow fluorescent protein is also capable of binding to FMN (5, 8, 21). However, Fre-like enzymes showed no appreciable homology in sequence to any of them. So far, no report showing the functional requirement of LuxG in the bioluminescence reaction has been published.

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