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Cloning and Nucleotide Sequences of *lux* Genes and Characterization of Luciferase of *Xenorhabdus luminescens* from a Human Wound

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***Xenorhabdus luminescens* HW is the only known luminous bacterium isolated from a human (wound) source. A recombinant plasmid was constructed that contained the *X. luminescens* HW *luxA* and *luxB* genes, encoding the luciferase α and β subunits, respectively, as well as *luxC*, *luxD*, and a portion of *luxE*. The nucleotide sequences of these *lux* genes, organized in the order *luxCDABE*, were determined, and overexpression of the cloned luciferase genes was achieved in *Escherichia coli* host cells. The cloned luciferase was indistinguishable from the wild-type enzyme in its in vitro bioluminescence kinetic properties. Contrary to an earlier report, our findings indicate that neither the specific activity nor the size of the α (362 amino acid residues, M_r 41,389) and β (324 amino acid residues, M_r 37,112) subunits of the *X. luminescens* HW luciferase was unusual among known luminous bacterial systems. Significant sequence homologies of the α and β subunits of the *X. luminescens* HW luciferase with those of other luminous bacteria were observed. However, the *X. luminescens* HW luciferase was unusual in the high stability of the 4a-hydroperoxyflavin intermediate and its sensitivity to aldehyde substrate inhibition.**

Most of the known luminous bacteria are of marine origin and fall within two genera, *Vibrio* and *Photobacterium* (references 13 and 23 and references therein). Luciferases from the marine bacteria *Vibrio harveyi*, *Vibrio fischeri*, *Photobacterium leiognathi*, and *Photobacterium phosphoreum* are all αβ heterodimers (14, 19, 25), with molecular weights of approximately 40,000 for α and 37,000 for β. For the former three species, the *luxA* and *luxB* genes, encoding the luciferase α and β subunits, respectively, have been sequenced (4, 9, 15, 17).

A few nonmarine luminous bacterial species have also been identified (13, 23); among them are *Xenorhabdus luminescens* Hb and Hm, both of which are insect-pathogenic symbionts with nematodes of the family *Heterorhabditidae* (24, 27, 30). The luciferases from *X. luminescens* Hm and Hb have identical α subunits and only a single different amino acid residue in the β subunit. Moreover, they are similar in specific activity and subunit sizes to and have significant sequence homology with luciferases from marine luminous bacteria (16, 27, 29). The *luxA* and *luxB* genes from these two *X. luminescens* strains differ by only one and two bases, respectively (16, 29). In fact, the known sequences of the *lux* operon, starting with the last 142 bases of *luxD* through the first four bases of *luxE* (with *luxA*, *luxB*, and noncoding regions in between) show 99% identity for *X. luminescens* Hm and Hb (16, 29).

A different luminous strain of *X. luminescens* has been isolated from a human wound (8), now designated strain HW. Some unusual properties have been reported for this strain and its luciferase (5) in comparison with all other known luminous bacteria, including *X. luminescens* Hm and Hb. *X. luminescens* HW bioluminescence activities both in vivo and in vitro have been found to be substantially lower in intensity but higher in optimal temperature than those of other luminous bacterial systems. The in vivo bioluminescence of *X. luminescens* HW was optimal at 37°C and reached a peak intensity of about 1.2 quanta/s per cell at 37°C, approximately 1,000 times lower than that of *V. harveyi* at 25°C. The *X. luminescens* HW luciferase in vitro activity, optimal at 40°C, was also about 30-fold lower than that of the *V. harveyi* luciferase. Moreover, the *X. luminescens* HW luciferase has been reported to have molecular

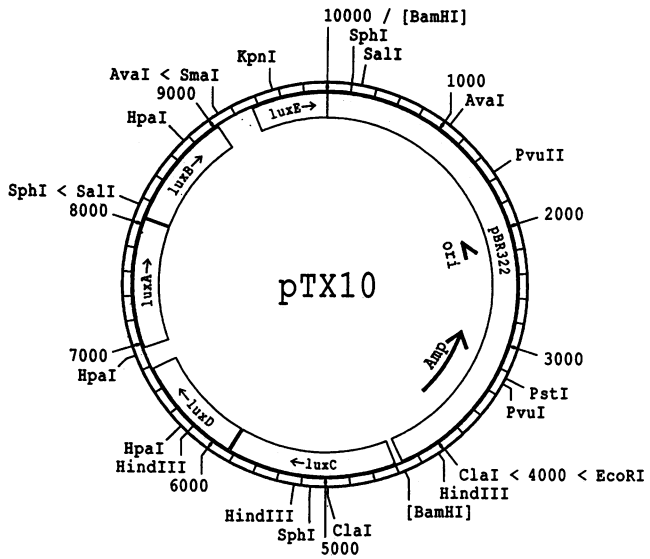

FIG. 1. Physical map of the recombinant plasmid pTX10. A 5.6-kb fragment of *X. luminescens* HW DNA was inserted into the *Bam*HI site of pBR322. This DNA insert contained *X. luminescens* HW *luxCDAB* and part of *luxE*. Sites accessible to cutting by 12 restriction enzymes are shown.

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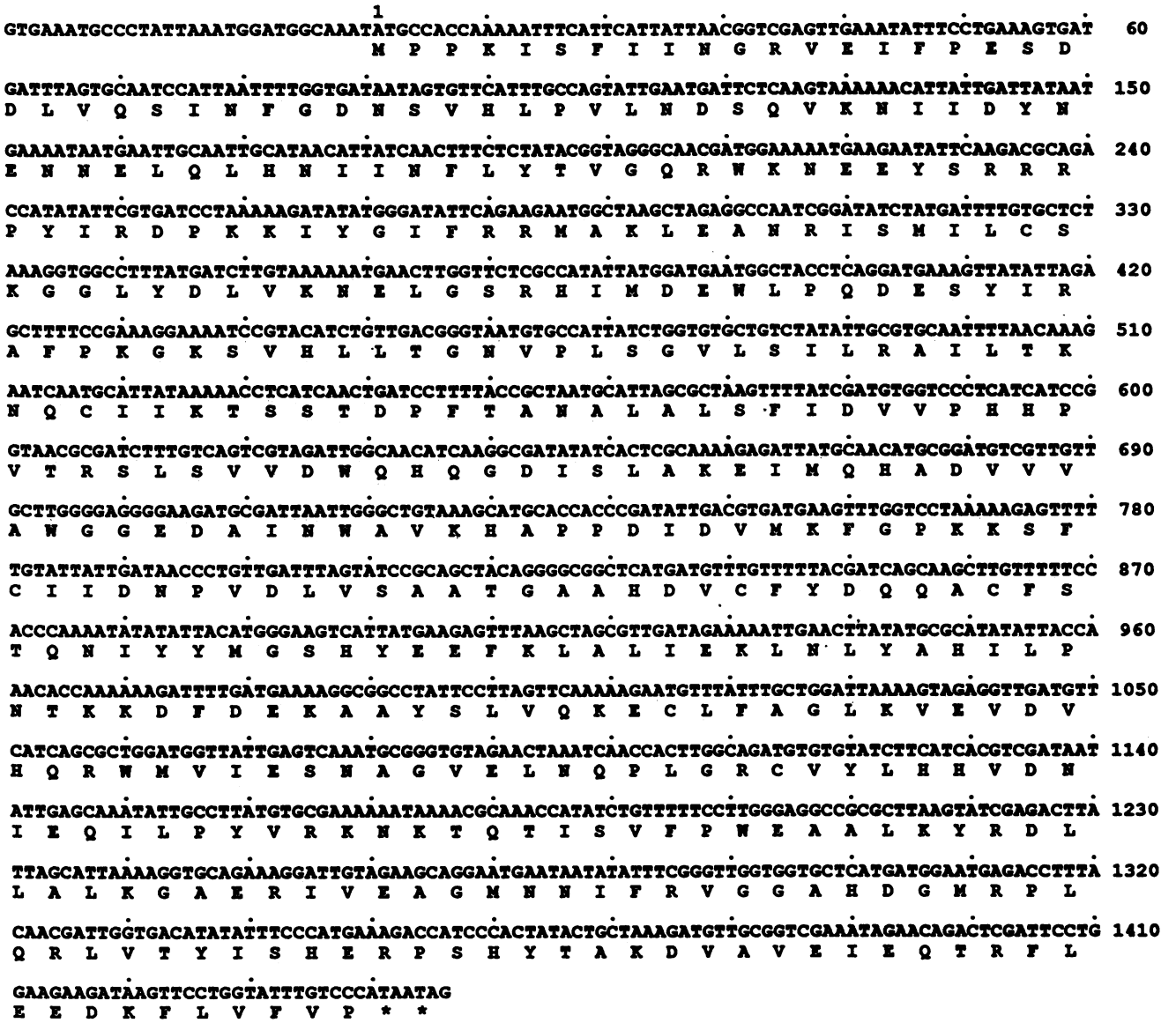

FIG. 2. Nucleotide sequence of the *X. luminescens* HW *luxC* gene and deduced amino acid sequence. The upstream nucleotide sequence is also included. Stop codons are marked by asterisks. Abbreviations for amino acids: G, glycine; A, alanine; V, valine; L, leucine; I, isoleucine; S, serine, T, threonine; C, cysteine; M, methionine; D, aspartic acid; N, asparagine; E, glutamic acid; Q, glutamine; H, histidine; R, arginine; K, lysine; F, phenylalanine; Y, tyrosine; W, tryptophan; P, proline. Amino acid designations are located under the first letter of each codon.

weights of 52,000 and 41,000 for the two subunits, both being significantly larger than the subunits of all other luciferases.

These reported unusual properties of the *X. luminescens* HW system have significant molecular biological and enzymological implications, and this prompted us to carry out a comparative study at the enzyme and gene levels. This study embraced the cloning and sequencing of not only the *X. luminescens* HW *luxA* and *luxB* genes but also *luxC*, *luxD*, and a segment of *luxE*. The latter three genes encode the three enzymes (fatty acid reductase, acyltransferase, and acylprotein synthetase, respectively) that constitute the fatty acid reductase complex responsible for the production of tetradecanal from myristic acid as a luciferase substrate. A system has been developed for overexpressing the cloned *X. luminescens* HW *luxA* and *luxB* genes. The cloned luciferase has been isolated and characterized. The *X. luminescens* HW luciferase was found to be unusual in its sensitivity to aldehyde substrate inhibition and the high stability of its 4a-hydroperoxyflavin intermediate, providing an intriguing system for further enzymological studies.

MATERIALS AND METHODS

Strains and media. *X. luminescens* HW (5) was a generous gift from J. W. Hastings. Cells of this strain and *Escherichia coli* JM107 were grown and plated on LB or YT medium (1) at 37°C.

Molecular cloning, screening, and expression of *lux* genes.

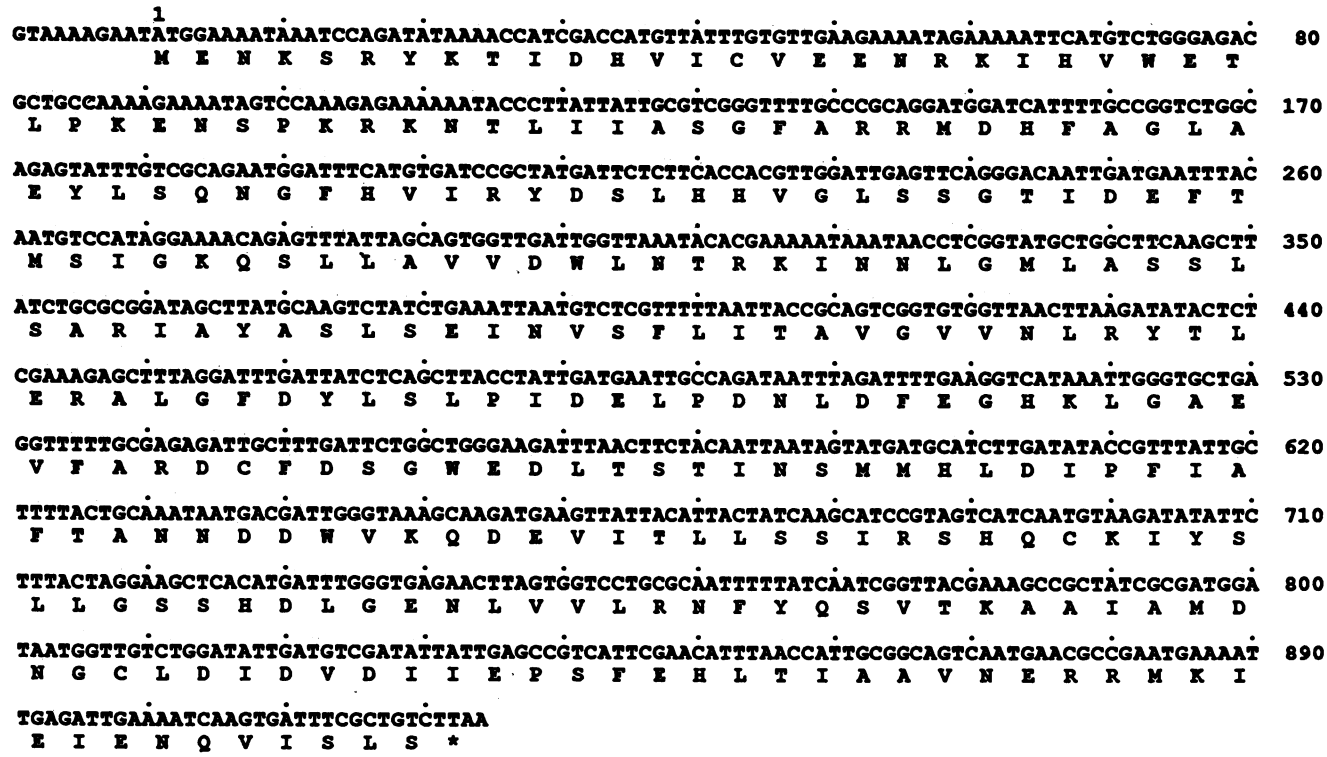

FIG. 3. Nucleotide sequence of the *X. luminescens* HW *luxD* gene and deduced amino acid sequence. The upstream nucleotide sequence that separates *luxC* and *luxD* is included. The stop codon is marked by an asterisk.

Purification of *X. luminescens* HW genomic DNA was carried out by the procedure of Ausubel et al. (1). The DNA was partially fragmented by *Sau*3AI (New England BioLabs) and ligated into *Bam*HI-linearized pBR322 (New England BioLabs). Recombinant DNA was transformed into *E. coli* HB101 (11). Bioluminescence emission from *E. coli* colonies upon exposure to decanal vapor in capped vials was detected by a scintillation counter (Beckman LS7500). An overexpression plasmid (pTX12) was constructed by insertion of *luxAB* into pKK223-3 (Pharmacia).

Nucleotide sequencing. Restriction enzyme fragments were inserted into M13mp18 or M13mp19 (Pharmacia). Nested deletion was carried out with a deletion kit from Pharmacia. Synthetic primers were obtained by using a Biosearch 8600 automated DNA synthesizer. DNA sequencing was carried out with a Sequenase kit from United States Biochemical Corp. and α-^{35}S-dAPT from Du Pont by the dideoxynucleotide method (21, 26). Sequencing data were analyzed with software from the Genetics Computer Group (6).

Luminescence assay and luciferase purification. In vitro bioluminescence activities were assayed by the reduced flavin mononucleotide ($FMNH_2$) injection standard assay (12). The dithionite assay method (20, 32) was used with 50 μM $FMNH_2$ and 0.5 mM decanal to determine the decay constants (k) and peak light intensities (I_0). The *X. luminescens* HW luciferase was purified from *E. coli* JM107 harboring pTX12 (described under Results) by a published method (12) with modifications. The DEAE-cellulose batch adsorption step was replaced by column (5 by 65 cm) chromatography. A three-stage elution was carried out with initially about 1 liter of water, followed by 5 liters of 0.15 M phosphate, pH 7.0, and finally a linear gradient of 0.35 to 0.50 M phosphate, pH 7.0, containing 1 mM EDTA and 0.5 mM dithiothreitol, with luciferase eluted at about 0.4 M phosphate. The luciferase from *V. harveyi* was also purified to $\geq$95% homogeneity as described before (12).

For the determination of the decay rate of the luciferase-bound 4a-hydroperoxy-flavin mononucleotide (FMN) intermediate II, this intermediate was first formed at 23°C by the rapid injection of 0.5 ml of 0.05 M phosphate, pH 7.0, containing 0.1 mM $FMNH_2$ (reduced photochemically in the presence of 1 mM EDTA) and 0 or 0.45 μmole of dodecanol into an equal volume of the same buffer containing *V. harveyi* or *X. luminescens* luciferase. The stabilities of intermediate II and the II-dodecanol complex were then determined by withdrawing 50-μl aliquots after different times of incubation at 23°C and injecting each into 1 ml of a saturating decanal solution in the same buffer for measurement of remaining bioluminescence activities.

Nucleotide sequence accession number. The nucleotide sequences discussed in this article have been assigned GenBank accession number M38525.

RESULTS AND DISCUSSION

From a total of approximately 10,000 transformant clones, 1 colony was found to be bioluminescent when supplied with decanal. This clone was isolated, and the recombinant plasmid, denoted pTX10, was found to contain a 5.6-kb DNA insert. A restriction map was constructed on the basis of digestion patterns with 12 restriction enzymes (Fig. 1) and was later confirmed by DNA sequencing. The nucleotide sequence of the entire 5.6-kb insert in pTX10 was deter-

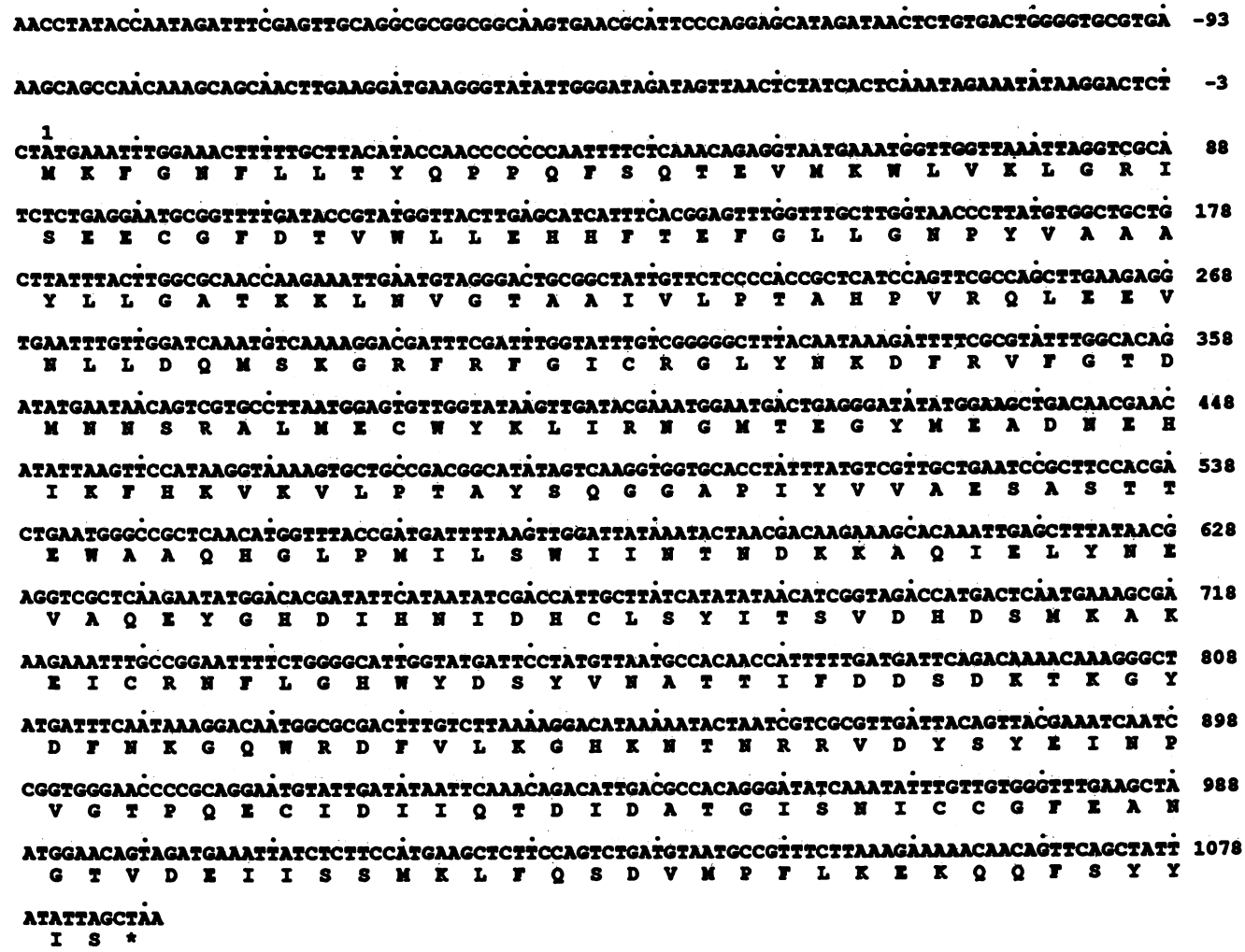

FIG. 4. Nucleotide sequence of the *X. luminescens* HW *luxA* gene and deduced amino acid sequence. The upstream nucleotide sequence that separates *luxD* and *luxA* is included. The stop codon is marked by an asterisk.

mined and found to consist of, sequentially, *luxC* (Fig. 2), *luxD* (Fig. 3), *luxA* (Fig. 4), *luxB*, and a portion of *luxE* (Fig. 5). The DNA sequences are contiguous from Fig. 2 to 5, and the corresponding amino acid sequences for the structural genes are also shown. The *X. luminescens* HW luciferase also contained α and β subunits, encoded by the *luxA* and *luxB* genes, respectively, separated by 10 bases. As in *V. harveyi* (22), *V. fischeri* (3, 7), and *X. luminescens* HM (16, 10), the *X. luminescens* HW *luxC* and *luxD* genes were located immediately upstream of *luxA*, and *luxE* was within the usual range of several hundred bases downstream from *luxB*. However, unlike *P. phosphoreum* (18), *X. luminescens* HW did not have a *luxF* between *luxB* and *luxE*. The complete sequence of *luxC* (Fig. 2) and partial sequence of *luxE* (Fig. 5) of *X. luminescens* HW are the first ones reported for a nonmarine luminous bacterium.

E. coli JM107 cells harboring the recombinant plasmid pTX10 were grown and harvested when they reached optimal in vivo bioluminescence in the presence of exogenously added decanal. Crude lysates prepared from such cells and wild-type *X. luminescens* HW and *V. harveyi* cells were used for measurements of in vitro bioluminescence decay constants (k) and maximal light intensities (I_0). At 23 and 37°C, light decay constants of 0.34 and 0.58 s^{-1}, respectively, were found for the cloned *X. luminescens* HW luciferase; these values were, within experimental errors, essentially the same as that of the wild-type enzyme but were significantly different from the respective decay constants of 0.45 and 0.72 s^{-1} for the *V. harveyi* luciferase. The ratio of the I_0 at 37°C and at 23°C (37°C/23°C) of 1.7 to 1.8 for the wild-type and the cloned *X. luminescens* luciferase was also indistinguishable, whereas the corresponding ratio of 1.5 for the *V. harveyi* luciferase was marginally lower.

High expressions of *X. luminescens* HW luciferase *lux* genes were achieved in *E. coli* harboring pTX12. The maximal in vivo light intensity reached 2,000 quanta/s per cell when cells were supplied with decanal, which was ~1,000 times higher than the maximal in vivo light intensity of the wild-type *X. luminescens* HW without any added aldehyde or ~200 times higher than that with decanal added.

As mentioned earlier, a number of unusual properties have been reported for *X. luminescens* HW and its luciferase (5). We have confirmed the high optimal temperatures for the *X. luminescens* HW in vivo and in vitro bioluminescence activities. However, contrary to the earlier report, the *X. luminescens* HW luciferase is quite similar to other luciferases in

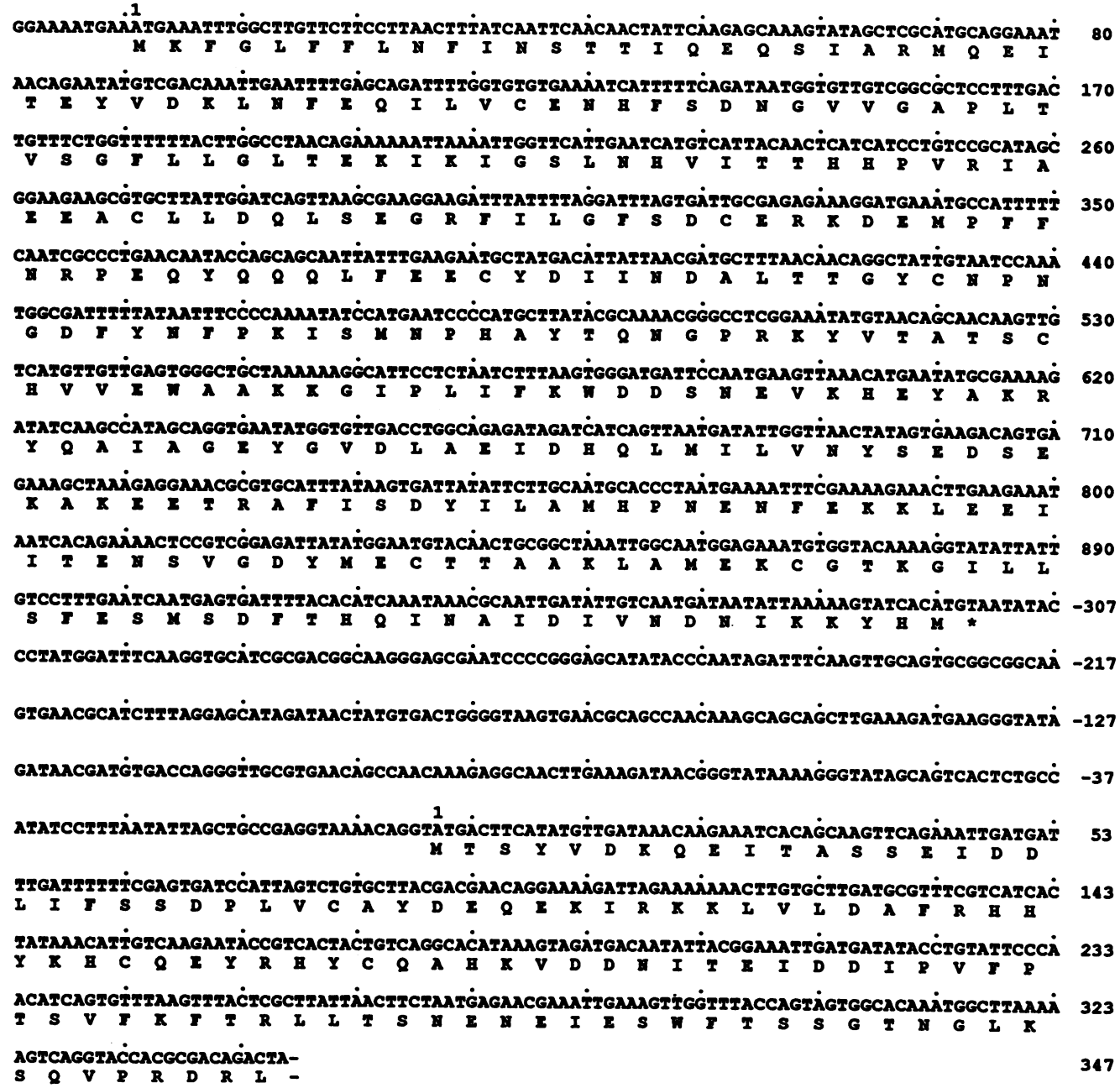

FIG. 5. Nucleotide sequence of the *X. luminescens* HW *luxB* gene and part of the *luxE* gene, and the deduced amino acid sequences. The upstream nucleotide sequence that separates *luxA* and *luxB* as well as the nucleotide sequence between *luxB* and *luxE* are included. The stop codon is marked by an asterisk.

specific activity and in the sizes of the two subunits. The luciferase from *E. coli* harboring pTX12 was prepared to about 80% pure as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The specific activity of this sample was determined to be 2.9×10^{14} quanta/s/A_{280}/ml by the dithionite assay, corresponding to an estimated specific activity of 3.6×10^{14} quanta/s/A_{280}/ml for the pure enzyme. This is comparable to that of other luciferases. The deduced amino acid sequences (Fig. 4 and 5) show that the *X. luminescens* HW luciferase α subunit had 362 amino acid residues (M_r 41,389) and the β subunit had 324 amino acid residues (M_r 37,112), with neither significantly different in size from the corresponding subunits of other luciferases. Moreover, the *X. luminescens* HW luciferase exhibited significant sequence identities with luciferases from the marine bacteria *V. harveyi*, *V. fischeri*, and *P. leiognathi*, ranging from 61 to 83% for the α and 51 to 58% for the β subunit. Much higher identities were observed for the α (93%) and β (84%) subunits of the *X. luminescens* HW luciferase with those of the *X. luminescens* strains Hm (16) and Hb (29).

On the basis of α and β primary sequences, we found that phylogenetically (28), *X. luminescens* was closer to *V. harveyi* whereas *V. fischeri* was closer to *P. leiognathi* in

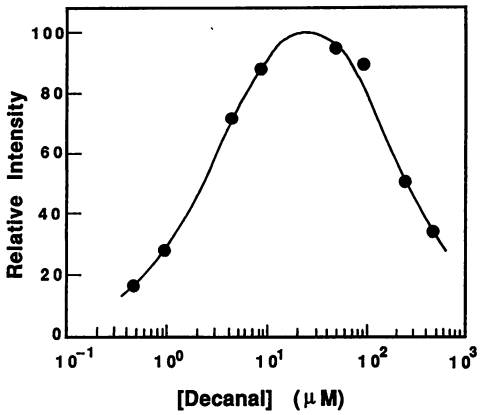

FIG. 6. Aldehyde inhibition of the *X. luminescens* HW luciferase. Luciferase activities were determined by the $FMNH_2$ injection standard assay at 23°C in 50 mM phosphate, pH 7.0, with 50 μM $FMNH_2$ (photochemically reduced in the presence of 1 mM EDTA) and designated amounts of decanal as substrates. The reaction solution did not contain any bovine serum albumin.

evolution. Moreover, the α and β subunits of these luciferases were derived from a single ancestor gene.

In the bacterial bioluminescence reaction, a key 4a-hydroperoxy-FMN intermediate (designated II) is formed by reacting luciferase-bound $FMNH_2$ with O_2. We found that the *X. luminescens* HW luciferase intermediate II was highly stable. In 0.05 M phosphate, pH 7.0, at 25°C, the first-order decay rate constants for the intermediate II formed with the *X. luminescens* HW and *V. harveyi* luciferases were 0.5 and 1.6 min^{-1}, respectively. When complexed with dodecanol for stabilization (31), decay rate constants for the intermediate II of *V. harveyi*, *V. fischeri*, and *P. phosphoreum* luciferases were, respectively, 0.02, 0.05, and 0.21 min^{-1}, all faster than that of the *X. luminescens* HW luciferase (0.01 min^{-1}). The high stability of the *X. luminescens* HW luciferase intermediate II makes it most suitable for detailed biochemical and biophysical characterizations.

Among all known bacterial luciferases, aldehyde substrate inhibition has only been reported for the *V. harveyi* luciferase (14). We now have found that the *X. luminescens* HW luciferase was also sensitive to aldehyde inhibition, with an apparent optimal activity at 25 μM decanal in 50 mM phosphate, pH 7, when assayed by the $FMNH_2$-initiated standard assay in the absence of bovine serum albumin (Fig. 6). This optimal decanal concentration was about three- to fivefold higher than that of the *V. harveyi* luciferase.

When the *V. harveyi* luciferase α subunit Cys-106 is mutated to serine (2), alanine, or valine (2, 17a), these luciferase variants are also subject to aldehyde inhibition but at much higher aldehyde concentrations. It has been speculated that the aldehyde inhibition of the *V. harveyi* luciferase may involve thiohemiacetal formation between the aldehyde and the Cys-106 residue (33). It is interesting that the two luciferases that are most sensitive to aldehyde inhibition (the *V. harveyi* and *X. luminescens* HW luciferases) are the two luciferases known to have a cysteine residue at the 106 position in the alpha subunit. Our results certainly indicate that the *X. luminescens* HW luciferase should be an invaluable system for further elucidation of the nature of aldehyde inhibition.

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