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Freshwater Bioluminescence in *Vibrio albensis* (*Vibrio cholerae* biovar *albensis*) NCIMB 41 Is Caused by a Two-Nucleotide Deletion in *luxO*

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We previously proposed that the function of the *lux* operon is to produce a halotolerant flavodoxin, FP_{390} or P-flavin binding protein, and not to produce light. A crucial basis of this hypothesis is that almost all species of luminous bacteria emit light in culture media containing over 2% NaCl. However, *Vibrio albensis* (*Vibrio cholerae* biovar *albensis*) NCIMB 41 emits light in freshwater and this appears to be in direct conflict with our hypothesis. To determine why this exceptional freshwater bioluminescence is emitted, we studied the *lux* operon and the regulatory system of the operon in this strain, and found that expression of the operon is regulated by a system involving a derivative of 4,5-dihydroxy 2,3-pentanedione, DPD, as an inducer, and the repressor gene for the *lux* operon, *luxO*, is damaged by deletion of two nucleotides. Furthermore, to study the effect of damage to the *luxO* gene, pUC18 derivatives containing the damaged and repaired *luxO* sequences were prepared. Cells transfected with the damaged *luxO* sequence emitted light like the parental strain, whereas ones transfected with the repaired one did so only sparingly. Here we show that the light emission in freshwater by this strain is not in conflict with our hypothesis.

Key words: electrotransformation, freshwater bioluminescence, *luxO*, SUGDAT, *Vibrio cholerae*.

One of the most important findings supporting the hypothesis that the function of the *lux* operon is to produce a halotolerant flavodoxin, FP_{390} or P-flavin binding protein, and not to produce light, is that most strains of luminous bacteria show enhanced light emission when the culture medium contains more than 2% NaCl (*1–3*). The crucial importance of NaCl for bioluminescence-emitting activity has been recognized since the 1950s (*4*). Farghaly reported over 50 years ago that for *Vibrio fischeri*, maximum growth occurs with 1.5–2% NaCl in the medium while maximum luminescence occurs with between 2.7–3.5% NaCl (*5*). Although today this pivotal evidence seems to be mostly overlooked, certain reports do still deal with this topic; we recently reconfirmed the finding for *Photobacterium phosphoreum* (*2*), and Stabb et al. reported a correlation between osmolarity and the luminescence of symbiotic *V. fischeri* (*6*).

Vibrio albensis was first isolated as a luminous bacterial strain similar to *V. cholerae* by Kutscher from water of the river Elbe in Hamburg, Germany, in 1893 and was named *Elbvibrio* (*7*). Later, it was renamed *V. albensis* by Lehmann and Neumann (*8*). As the strain emits light even in freshwater it attracted special attention (*4*). This was because the researchers at that time thought it to be an exceptional luminous strain as the common species only emit light in media containing NaCl, such as seawater. We have recognized for a long time now that the bioluminescence of *V. albensis* in freshwater is strong evidence against our hypothesis, and Dunlap and Kita-Tsukamoto explicitly pointed out this inconsistency of our hypothesis in their review of the subject (*3*). We had previously thought that it would be impossible to obtain this strain because it was isolated over 110 years ago. However, we noticed that the strain had been used in recent studies under the name "*V. cholerae* biovar *albensis*" (*9*, *10*), and on follow-up, we discovered that in fact it is available from several depositories including the NCIMB, from where we subsequently obtained it. For this reason, we use the name *V. cholerae* NCIMB 41 for this strain throughout this report. The precise route by which this strain arrived at the NCIMB is not clear, but according to the NCIMB catalogue, the strain was initially held by Sonnenschein, who isolated it independently of Kutscher from fish caught in the river Elbe in August 1930 (*11*). Because Reichelt et al. found greater than 80% DNA complementarity between pathogenic *V. cholerae* ATCC 14035 and *V. cholerae* NCIMB 41 (*12*), and the genomic sequence of *V. cholerae* El Tor N 16961 was published in 2000 (*13*), a great deal of useful data including genetic information on this unusual emission of light by *V. cholerae* NCIMB 41 is now available, and such a favorable investigative situation induced us to undertake this study.

MATERIALS AND METHODS

Bacteria and Preparation of Genomic DNA—V. cholerae NCIMB 41 was purchased from the NCIMB and cultured at 25°C overnight with shaking in Luria-Bertani (LB)

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medium. Cells were harvested by centrifugation. Genomic-tip 500/G (QIAGEN) was used for the isolation of DNA according to the manual supplied.

PCR and DNA Sequencing—The conditions for PCR and DNA sequencing were reported previously (*2*, *14*). The primers used are listed in Table 1 and the respective 20-mer nondegenerate primers were designed with reference to the genomic sequence of *V. cholerae* N 16961 (*13*). These sequences were not included among those submitted to the database, because nucleotide replacement(s) might be present even though the sequences were amplified using these primers by PCR. A new protocol for SUGDAT (Sequencing Using Genomic DNA As a Template) was substituted for the previous one (*14*): 5 μl of genomic DNA (800 ng μl^{-1}), 1 μl of 16 μM sequencing primer, 2 μl of Terminator Ready Reaction Mix (Applied Biosystems), 3 μl of 5× Sequencing Buffer (Applied Biosystems), and 9 μl of water were placed in a 0.2-ml PCR tube, and then the cycle sequencing reaction was conducted using a program of 10 s at 95°C, 5 s at 50°C, and 3 min at 60°C for 80 cycles. Previously (*14*), a mixture of the genomic DNA and a sequencing primer was preheated at 95°C for 5 min before the main cycle sequencing reaction, but we omitted this process and subsequently could read longer sequences: for *V. fischeri* and *P. phosphoreum*, the nucleotide sequences which could be read in one run were 700–800 bp long, whereas, for *V. cholerae* NCIMB 41, they were 400–500 bp long, for unknown reasons. The nucleotide sequences reported in this paper have been submitted to the DDBJ/EMBL/GenBank nucleotide sequence database.

Construction of pUC18 Derivatives Containing the Damaged and Repaired luxO *Sequences*—A scheme for construction of the respective plasmids is shown in Fig. 1. The front and rear of the open reading frame of the damaged *luxO* sequence of *V. cholerae* NCIMB 41 are designated as *luxO*Front and *luxO*Rear, respectively. *luxO*Front with the 131-bp upstream sequence along with *luxO*Rear with the 12-bp downstream one (1,551 bp) was amplified by PCR using as primers, 5′-ATCGTGCCAACTCAAATCGG-3′ (*luxO*For1) and 5′-TTCAAGCTTCGCTTACCGTTCCTTCTCTT-3′ (*luxO*Rev), with genomic DNA of *V. cholerae* NCIMB 41 as a template and a high fidelity DNA polymerase, *GeneAmp* (Applied Biosystems), according to the instructions provided. The PCR product was digested with two restriction enzymes, *Xba*I and *Hin*dIII, purified by gel electrophoresis, and then ligated into the vector pUC18, which was digested before use with the same enzymes and purified. The completed plasmid, designated as pUC18VcluxOD (AB235904), was transferred into competent cells of *Escherichia coli* JM109 (Toyobo) and amplified. To repair the *luxO* gene, the rear segment of the *luxO* sequence of *V. cholerae* NCIMB 41 was amplified using as primers, 5′-TGAGCGCGC**GT**GGTGATGATGTGATCGAG-3′ (*luxO*For2) and *luxO*Rev. In the former, the two nucleotides in bold, GT, were inserted to repair the mutation found in *luxO* of *V. cholerae* NCIMB 41. The PCR product was digested with two restriction enzymes, *Bss*HII and *Hin*dIII, and then purified by electrophoresis. The digest was ligated into pUC18VcluxOD, which was digested with the same enzymes and purified before use, and the completed plasmid was transferred into JM109 cells. Because in pUC18VcluxOD, CG resides four times in tandem upstream of the deletion point, two restriction sites of *Bss*HII are present side by side. Therefore, we obtained two kinds of plasmid, one having the designed sequence and one in which two nucleotides, GC, were deleted from the designed sequence. We isolated eight transformants, extracted plasmids from the respective cells and then sequenced the junction between the two segments. Seven transformants contained the GC-deleted plasmid but only one contained the designed one, which was designated as pUC18VcluxOR (AB235903). All PCR products cloned into the plasmids were sequenced on both strands to confirm that no mutations had been introduced during PCR amplification.

Electrotransformation of V. cholerae *NCIMB 41*—*V. cholerae* NCIMB 41 was cultured in 100 ml of LB medium at 25°C with shaking overnight. Forty milliliters of the culture, in which bacteria had grown to a turbidity of 0.5–0.7, were added to 100 ml of fresh LB medium. On a further 2.5 h incubation, the cells grew to a turbidity of approximately 0.5 and were then harvested by centrifugation at 11,000 × *g* for 10 min at 4°C. Cells were washed with a cold 10% sucrose solution eight times by repeated suspension and centrifugation. Finally, the cells were suspended in 100 μl of a 10% sucrose solution and 40 μl aliquots were each mixed on ice with 1 μl of each plasmid, which was dissolved in 10 mM Tris EDTA buffer (pH 8.0) at a concentration of approximately 1 μg μl^{-1}. Each mixture of cells and DNA was transferred to a 1 mm electroporation cuvette and then pulsed on a MicroPulser (Bio-Rad) using the program Ec1. The cell suspension was diluted with 300 μl of LB medium and then the mixture was maintained at 25°C for 1 h. A 200 μl aliquot was plated on LB medium containing 1.5% agar and ampicillin (75 μg ml^{-1}). The plates were incubated at 25°C and after 48 h, 2–50 colonies of transformants appeared. The transformation efficiency was not high but we were able to effectively transfer pUC18 and its derivatives into the cells of *V. cholerae* NCIMB 41, according to the protocol, although we were unsuccessful in transferring pBR322 and its derivatives into the cells of this strain for unknown reasons. The four transformants used in the following experiments have been deposited at the Biological Resource Center (NBRC), National Institute of Technology and Evaluation, Japan.

Growth and Bioluminescence Curves—The parental strain and a dark mutant were cultured in LB medium, and four transformants were grown in LB medium supplemented with ampicillin at 75 μg ml^{-1}, so that the cells would retain the plasmids. The strains were precultured in the respective medium until a turbidity of approximately 1 was attained. Then, 8 ml of each respective seed culture was transferred to 100 ml of fresh medium, followed by incubation at 25°C with shaking. Growth was monitored by measuring turbidity at 600 nm. Bioluminescence was measured in arbitrary units (AU) from the bottom of the flask in a dark box using a Photosensor Module (Hamamatsu Photonics, H6780MOD) connected to an Amplifier Unit (Hamamatsu Photonics, C2719) and a Power Supply for a PMT Module (Hamamatsu Photonics, C7169). This light detection system has an excellent linear dynamic range, *i.e.*, 1 to 7,000,000 AU.

To obtain mutants, which had lost the plasmids, the four transformants were cultured in ampicillin-free LB medium

Table 1. **Identification of genes inferred to be involved in bioluminescence in *V. cholerae* NCIMB 41.**

Gene Acc. No.	Protein	Sequencing method	Primers used 5′—3′	Present/damaged		Length (nt/aa)[a]		Identity[b] (nt/aa)
				NCIMB 41	N16961	NCIMB 41	N16961 (3083)	
lux operon AB115761	Lux A, B, C, D, E & G	PCR (Degenerate primers & Nondegenerate primers) + SUGDAT	GARGCNAAYTGGATHGCNATG GCRTGNGCNGTRAANGGRTC CAYCAYTTYACNGARTTYGG CGNCCYTTNGACATRTGRTC ATHATHACNGGNGGNGGNTGG GCRTANACCCANG GNGGNAC	Present	Not present	–	–	–
fld1 AB115759	Flavodoxin1	PCR (Deg. primers) + SUGDAT	ATHCCNACNTGGTAYTAYGG ACNGTNCCCATNGCRTCRCA	Present	Present	528/175	528/175	99.6%/100%
fld2 AB115760	Flavodoxin2	PCR (Nondeg. primers)	CGGTATTTTCTGCCAAACCG CCGGTTTGGTGTTTGACTGG	Present	Present	597/198	597/198	98.8%/100%
lux R & I –	LuxR & I	PCR (Nondeg. primers)	CAGCGGTTAGTTGTATTGAG AGCAAAACGRCTTAATTC	Not present	Not present	–	–	–
luxR2 AB113245	LuxR2	PCR (Deg. primers) + SUGDAT	GGNGGNCAYGCNGAYATHGC TCRAACCANACYTTNARCCA	Present	Damaged	612/203	(612/203)	(99.3%/99.0%)
luxM –	LuxM	PCR (Deg. primers)	SANAARTGGTAYGARATGYT GGYTGYTCDATDATNGTRAA	Not present	Not present	–	–	–
luxN –	LuxN	PCR (Deg. primers)	GCNCAYGARATGMGNAAYCC TCDATNCCNGGNCCNGTRTC	Not present	Not present	–	–	–
luxS AB114425	LuxS	PCR (Nondeg. primers)	TATTTTGCCTACCTACAGGC CTCGAGCTGGAAGAGAATCG	Present	Present	519/172	519/172	99.0%/100%
Pfs AB114424	SAH nucleosidase	PCR (Nondeg. primers)	GAAAAGCTCACGCTGTCTGG ACGAGGAGCGCACCATTGGC	Present	Present	696/231	696/231	99.6%/100%
luxP AB113244	LuxP	PCR (Nondeg. primers)	GTCGATTTAACCGCGTTTGG TATAAAGTGGCGATCCCGGC	Present	Present	1095/364	1095/364	97.6%/98.6%
luxQ AB113244	LuxQ			Present	Present	2574/857	2574/857	94.8%/97.2%
luxO AB114423	LuxO	PCR (Nondeg. primers)	ATCGTGCCAACTCAAATCGG CAGAGTTAGAACAGCGTTGG	Damaged	Present	–	1368/455	–

[a]nt, nucleotides and aa, amino acids, respectively. [b]Identity between sequences of NCIMB 41 and those of N 16961 or 3083 is shown.

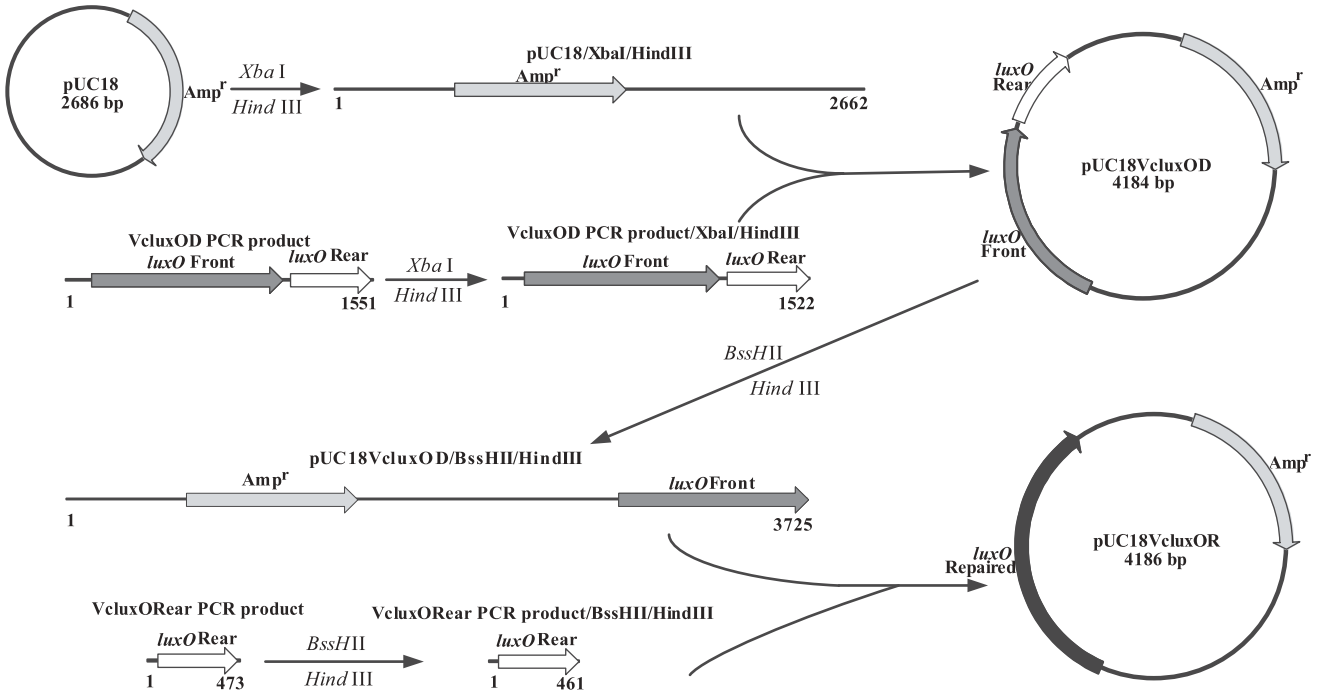

Fig. 1. **A scheme for construction of modified pUC18s, containing damaged and repaired *luxO* sequences.** A PCR product of the damaged *luxO* sequence with both flanking regions was inserted into pUC18 and the newly constructed plasmid was designated as pUC18VcluxOD. The rear segment of the *luxO* sequence, containing the repaired sequence, was amplified by PCR and exchanged with the damaged one, and the altered plasmid was designated as pUC18VcluxOR. For details, see "MATERIALS AND METHODS."

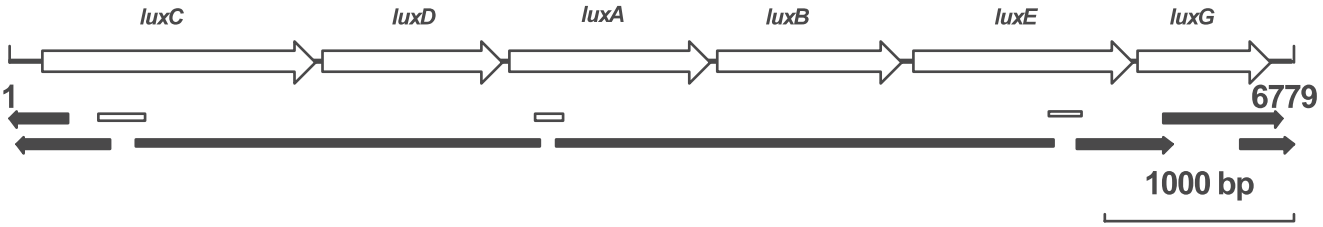

Fig. 2. **An outline of the sequencing of the *lux* operon in *V. cholerae* NCIMB 41.** Three partial sequences of the operon, as indicated by the open bars, were amplified by PCR using three pairs of degenerate primers (Table 1), and the respective amplified DNA sequences were sequenced without cloning. Two intervening sequences, indicated by the black solid bars, were also amplified by PCR using nondegenerate primers, and were sequenced. Both flanking regions were sequenced in each direction by SUGDAT as indicated by the five black arrows.

overnight and then plated on ampicillin-free plates. Because the respective mutants could grow neither in the LB medium containing ampicillin nor on the ampicillin plates, we concluded that these four mutants had lost the respective plasmids. To prepare growth and bioluminescence curves, the parent strain and the four mutants were cultured in LB medium. Other conditions were the same as above.

Southern Hybridization—The plasmid pUC18VcluxOR, and genomic and plasmid DNAs from two transformants, TrR1 and TrR2 (see below), were digested with *Eco*RI, *Hin*dIII and *Sma*I. The digests were analyzed by gel electrophoresis using 0.8% agarose. The gel was stained with ethidium bromide. DNA fragments were transferred to a nylon membrane and then hybridized with a probe for the gene encoding β-lactamase, which is present on pUC18. The probe was prepared using a DIG High Prime DNA Labeling and Detection Kit II (Roche) using a template prepared by PCR with a pair of primers, 5′-CTCATGAGACAATAACCCTG-3′ and 5′-CAAAAAGGATCTTCACCTAG-3′. The hybridized bands were detected using the same kit according to the instructions provided.

RESULTS

An ordinary and Complete lux *Operon Is Present in* V. cholerae *NCIMB 41*—The *lux* operon is not present in the genomic sequence of *V. cholerae* N 16961 (*13*). To confirm the presence of a complete *lux* operon in *V. cholerae* NCIMB 41, we sequenced the operon with both flanking regions (6,779 bp) using three pairs of degenerate primers (Table 1), in combination with SUGDAT, as shown in Fig. 2. Six *lux* genes were found and we performed homology searches on six Lux proteins: LuxA, LuxB, LuxC,

Table 2. **Homology of six Lux proteins, LuxA, LuxB, LuxC, LuxD, LuxE and LuxG, of *V. cholerae* NCIMB 41 with those of seven species.**

Lux proteins	LuxA		LuxB		LuxC		LuxD		LuxE		LuxG	
	Identity (%)	No. aa	Identity (%)	No. aa	Identity (%)	No. aa	Identity (%)	No. aa	Identity (%)	No. aa	Identity (%)	No. aa
Vibrio cholerae NCIMB 41	–	355	–	324	–	481	–	315	–	383	–	235
Vibrio harveyi	86	355	64	324	62	477	70	305	60	378	38	233
Photorhabdus luminescens	84	360	64	324	64	480	71	307	63	370	–	–
Shewanella hanedai	67	354	55	326			66	308	63	376	45	236
Vibrio fischeri	65	354	52	326	61	479	68	307	63	378	46	236
Vibrio salmonicida	65	354	54	327	62	476	68	258	62	373	44	235
Photobacterium leiognathi	63	354	52	325	61	478	62	315	61	373	46	234
Photobacterium phosphoreum	61	357	51	328	59	488	61	306	61	373	40	234

LuxD, LuxE and LuxG of *V. cholerae* NCIMB 41, using the FASTA program at DDBJ. The results are summarized in Table 2. Except for the outstandingly high similarity scores for LuxA of *V. cholerae* NCIMB 41 and LuxA of *Vibrio harveyi* or *Photorhabdus luminescens*, the homology of LuxA, LuxB, LuxC, LuxD and LuxE between *V. cholerae* NCIMB 41 and seven species was in the range of 51–71% similarity. On the other hand, the similarity between LuxG of *V. cholerae* NCIMB 41 and that of six species was 38–46%. The numbers of amino acid residues comprising the respective proteins are also listed in Table 2. The respective homologous proteins have nearly the same numbers. In the *lux* operon of *Photobacterium*, the *luxF* gene is present in addition to six genes (*15*, *16*), whereas in that of *Photorhabdus*, *luxG* is absent (*17*). On the basis of that in *V. cholerae* NCIMB 41, there are six *lux* genes homologous to genes in other species such as the luminous *Vibrio* species described above, and the strain emits light, we conclude that an ordinary and complete *lux* operon is present in this species.

Both Flavodoxin Genes Are Present Intact in V. cholerae *NCIMB 41*—Since we had proposed that the final product of the *lux* operon, FP_{390} or P-flavin binding protein, acts as a flavodoxin substitute in a saline environment (*1*, *16*, *18*), we assumed that the bioluminescence in freshwater exhibited by *V. cholerae* NCIMB 41 might be caused by damage to the flavodoxin genes. Because it has been reported that *V. cholerae* NCIMB 41 is not closely related to *V. cholerae* El Tor (*19*), we first tried to amplify a partial sequence of the flavodoxin 1 gene, *fld1*, using degenerate primers (Table 1). We were able to amplify and sequence this PCR product, and determined both of the flanking sequences by SUGDAT. On comparison of the newly determined 1,023-bp sequence with the corresponding region of *V. cholerae* N 16961 (*13*) (DDBJ/EMBL/GenBank accession numbers; AE004283 and AE004284), the identity at the nucleotide level between the two sequences was found to be 99.6% and the amino acid sequence of flavodoxin 1, deduced from the nucleotide sequence of *V. cholerae* NCIMB 41, was identical with that of *V. cholerae* N 16961. Thus, with orthologues of the target genes present in the genomic sequence of *V. cholerae* N 16961, the sequencing strategy consisted of PCR amplification of the target sequence using nondegenerate primers, designed according to the genomic sequence, and sequencing of the PCR product. Because in the genomic sequence of *V. cholerae* N 16961 a gene for flavodoxin 2, *fld2*, is present, we also sequenced this gene of *V. cholerae* NCIMB 41 with both flanking regions, 1,032 bp. The deduced amino acid sequence of the protein was identical with that of *V. cholerae* N 16961. As a result, we conclude that both flavodoxin genes are present intact in *V. cholerae* NCIMB 41.

Expression of the lux *Operon Is Not Constitutive but Regulated by a System Involving a DPD Derivative as an Inducer in* V. cholerae *NCIMB 41*—We next assumed that the freshwater bioluminescence in this strain is caused by damage to one or more genes involved in the regulation of the *lux* operon. The *luxR* and *luxI* regulatory system for the *lux* operon is the most classical such system reported to date. These genes were proposed to regulate the *lux* operon in *V. fischeri* when genetic studies on luminous bacteria first started (*20*, *21*). Today, orthologues of these genes have been found in luminous species as well as in many non-luminous ones (*22*). In *V. fischeri*, these genes are present just upstream of the operon and *luxI* terminates only 53 bp upstream of *luxC*. We determined a 175-bp upstream sequence of the *luxC* gene in an effort to find *luxI*, as described above, but did not detect the gene in this region. Nishiguchi and Nair succeeded in amplifying a partial sequence of *luxRI* (AY292964) by PCR with the pair of primers listed in Table 1, using the genomic DNA of *V. cholerae* (an Environmental isolate) as a template (*22*). We tried to amplify the corresponding sequence using the same primers and the genomic DNA of *V. cholerae* NCIMB 41 as a template, but failed. On the basis of these results, it is highly probable that the *luxR* and *luxI* genes are not present in *V. cholerae* NCIMB 41.

The regulatory genes for the *lux* operon have been extensively and systematically studied in *V. harveyi* by the group of Silverman and Bassler (*23–27*). Although we doubt that bacteria emit light to sense population density according to the proposal of Bassler *et al.* (*28*), as discussed below, we do acknowledge that the proposed regulatory genes regulate the *lux* operon. Among these genes, one generally referred to as *V. harveyi luxR*, which we have

named *luxR2* in this report for simplicity's sake, is reported to be absolutely required for the expression of the *lux* operon (*23*), although the mechanistic effect on the *lux* operon remains obscure. We were not able to find an orthologue of *luxR2* in the genomic sequence of *V. cholerae* N 16961, but did find one in *V. cholerae* strain 3083 (AF000716) (*29*). Therefore, we tried to sequence this gene in *V. cholerae* NCIMB 41 using degenerate primers (Table 1) in combination with SUGDAT. We successfully sequenced the *luxR2* gene with both flanking regions, 1,530 bp, in *V. cholerae* NCIMB 41 and the amino acid sequence deduced from the nucleotide sequence exhibited 99% identity with that of strain 3083. The valine and alanine residues (97th and 191st) in the amino acid sequence of LuxR2 of strain 3083 were exchanged with leucine and valine ones, respectively. Such mutations are not likely to abolish the function of the protein and we concluded that *luxR2* is present in *V. cholerae* NCIMB 41. After finishing the sequencing, we performed a FASTA search, DNA query versus DNA database, and found that a damaged *luxR2*, in which a single nucleotide is deleted and by which a stop codon is inserted at about one third point of the full length, is present in the genomic sequence of *V. cholerae* N 16961 (AE004143). The finding that the gene is intact in *V. cholerae* NCIMB 41 but is damaged in strain N 16961 strongly supports the gene is involved in regulation of the *lux* operon, because the operon is not present in the latter strain.

In *V. harveyi*, induction of the expression of the *lux* operon is regulated by two low molecular weight inducers, acylhomoserine lactone and DPD. It has been reported that the former is synthesized by the LuxM protein (*24*, *30*) and we tried to identify two genes, *luxM* and *luxN*, the latter of which is always located downstream of *luxM* and seems to participate in the regulation of the operon (*24*). Because these genes are found in neither the genomic sequence of *V. cholerae* N 16961 nor other reported sequences of *V. cholerae*, we tried to amplify a partial *luxN* sequence using a pair of degenerate primers (Table 1). These primers are thought to be quite competent because we used them successfully to sequence these genes of *P. phosphoreum* IFO 13896 and *V. fischeri* ATCC 7744 (data not shown). However, because we could not amplify the partial gene, we further designed a pair of degenerate primers (Table 1) to amplify a partial *luxM* in *V. cholerae* NCIMB 41. This effort was also fruitless. It is highly probable that in *V. cholerae* NCIMB 41, these genes are not present.

On the other hand, at least four genes are involved in the expression of the *lux* operon involving a DPD derivative as an inducer. DPD is synthesized in a process for salvaging homocysteine from *S*-adenosylhomocysteine, in which two genes, *pfs* and *luxS*, are involved: Pfs is S-adenosylhomocysteine nucleosidase (*31*) and LuxS is an *S*-ribosylhomocysteine cleavage enzyme (*27*). The other two genes are *luxP* and *luxQ*, which are involved in the signal transport system (*26*, *32*). Because orthologues of these four genes are present in the genomic sequence of *V. cholerae* N 16961, we sequenced these four orthologues of *V. cholerae* NCIMB 41, using nondegenerate primers (Table 1) in a similar way to as described above. The sequencing was successful, and the amino acid sequences of LuxS, Pfs, LuxP and LuxQ, deduced from the respective nucleotide sequences of *V. cholerae* NCIMB 41, exhibited, respectively, 100%, 100%, 99% and 97% identity with that of *V. cholerae* N 16961. On the basis of these results, we concluded that these four genes are intact and that expression of the *lux* operon is not constitutive but regulated like in *V. harveyi*, by a system involving a DPD derivative as an inducer, in *V. cholerae* NCIMB 41.

Identification of a Two-Nucleotide Deletion in luxO *of* V. cholerae *NCIMB 41*—The *lux* operon is positively regulated as described above, and while it is known to be negatively regulated by *luxO* (*25*, *33*), LuxO is reported to control *luxR2* expression (*34*). Because *luxR2* is present in *V. cholerae* NCIMB 41, as stated above, and an orthologue of *luxO* was found in the genomic sequence of *V. cholerae* N 16961 as VC1021 (AE004184), we tried to identify this gene in the genome of *V. cholerae* NCIMB 41. By designing a pair of nondegenerate primers (Table 1) referenced to the genomic sequence of *V. cholerae* N 16961, we were able to amplify a 1,988-bp sequence by PCR. The sequence comprises the 121-bp upstream and 894-bp downstream sequences of the *luxO* gene. The identity of the entire sequenced segment (including the upstream and downstream elements) with that of AE004184 was 98%. However, in the sequence of *luxO* of *V. cholerae* NCIMB 41, two nucleotides succeeding the 957th nucleotide from the initiation site are deleted, and due to the resulting frameshift, a stop codon appears just after this point, as shown in Fig. 3. The *luxO* orthologues are widely distributed in Proteobacteria, regardless of bioluminescence, and in *V. cholerae* N 16961, *Vibrio parahaemolyticus*, *V. harveyi*, *Vibrio vulnificus*, and *V. fischeri*, the LuxO proteins consist of 455, 453, 453, 466, and 468 amino acid residues, respectively. In *V. cholerae* NCIMB 41, however, owing to this deletion, the number of amino acid residues is reduced to 320, and it is highly probable that the protein thereby loses its function as the repressor for the *lux* operon.

The Repaired luxO *Gene Acts as Proposed in Cells of* V. cholerae *NCIMB 41*—On the basis of the considerable results obtained above, the conclusion may be reasonably made that *V. cholerae* NCIMB 41 emits light even in freshwater because the strain has undergone serious damage to *luxO* and the expression of the *lux* operon is not repressed even under non-saline conditions, under which it would be normally repressed with an intact LuxO. However, it is natural to pose the question of whether the intact *luxO* gene really acts in cell as expected. To answer this question, an effort was made to restore the damaged *luxO* gene in this bacterium by transferring a plasmid containing the repaired *luxO* sequence into the cells.

To achieve this, we selected plasmid pUC18 as the vector, because *V. cholerae* NCIMB 41 is sensitive to ampicillin and cells which have received this plasmid or its derivatives may be selected on plates containing antibiotics, and furthermore, the plasmid has a simple structure and small size. pUC18 derivatives containing the damaged and repaired *luxO* sequences, pUC18VcluxOD and pUC18VcluxOR, respectively, were constructed as described under MATERIALS AND METHODS (Fig. 1). On comparison of the *luxO* sequence of *V. cholerae* NCIMB 41 with that of N 16961, it was found that two nucleotides, GT, are deleted in NCIMB 41, as shown in

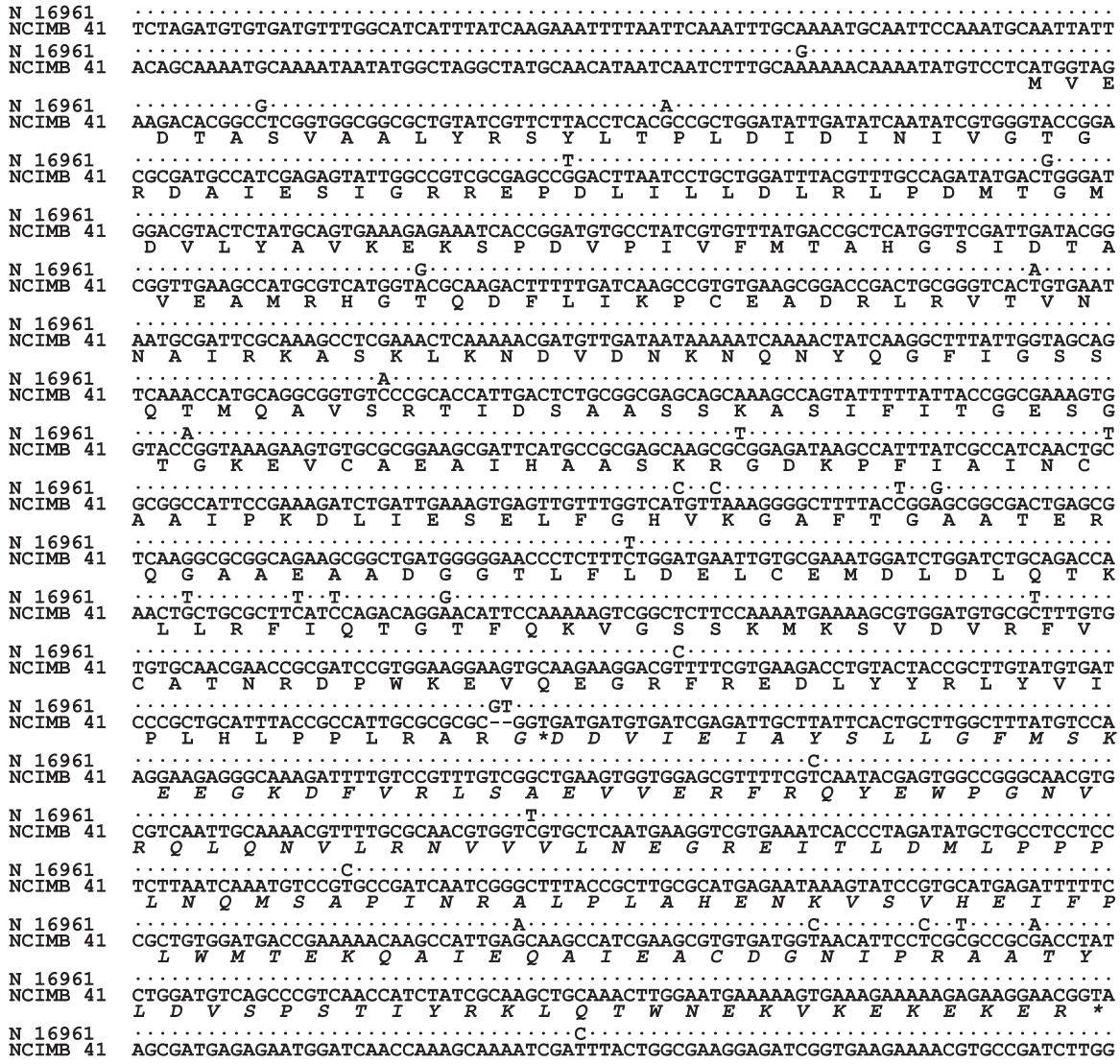

Fig. 3. **Nucleotide sequence of *luxO* with both flanking regions of *V. cholerae* NCIMB 41, along with that of *V. cholerae* N 16961.** Only nucleotides of *V. cholerae* N 16961 not identical with those of *V. cholerae* NCIMB 41 are shown by letters. The initial 1,598-bp sequence is shown in this figure, although the total length of the sequence of accession number AB114423 is 1,948 bp. Note that two nucleotides are deleted in the sequence of *luxO* of *V. cholerae* NCIMB 41. The deleted positions are indicated by dashes. Due to this deletion, a stop codon, which is indicated by an asterisk, appears just after the deletion point. The deduced amino acid sequence of *V. cholerae* NCIMB 41 is shown under the nucleotide sequence in normal letters but after the deletion point, the sequence of *V. cholerae* N 16961 is shown in italics.

Fig. 3. It is reasonable that the two nucleotides originally residing here should also reside in NCIMB 41. Therefore, to restore the sequence to its original state, two nucleotides, GT, were inserted at the deletion point in pUC18VcluxOD. Either pUC18VcluxOD or pUC18VcluxOR was transferred into cells of *V. cholerae* NCIMB 41 by electroporation, as described under MATERIALS AND METHODS. Two typical transformants, which received pUC18VcluxOD, were isolated and designated as TrD1 and TrD2, respectively, whereas two others, which received pUC18VcluxOR, were designated as TrR1 and TrR2, respectively.

The growth and bioluminescence curves for the parental *V. cholerae* NCIMB 41 strain (A-1), a dark mutant of this strain (B-1) which appeared spontaneously and then was isolated in our laboratory, as well as TrD1 (C1-1) and TrD2 (C2-1), and TrR1 (D1-1) and TrR2 (D2-1), are shown in Fig. 4. On comparison of the bioluminescence curve of the parental strain (A-1) with those of two transformants, TrD1 (C1-1) and TrD2 (C2-1), the parental strain appeared to emit light more strongly; however, as seen on comparison of the growth curves of these three cultures, the parental strain grew more rapidly and densely, because the culture medium of the parental strain did not contain ampicillin, whereas those of the transformants did. For this reason, we concluded that the damaged *luxO* sequence received by the transformants did not affect the light emission from the cells. On the other hand, we were unable to detect by the naked eye, light from either of transformants TrR1 and TrR2 which had received a repaired *luxO* sequence, indicating that repair of the *luxO* gene had been successful in these cells (D1-1 and D2-1). Because

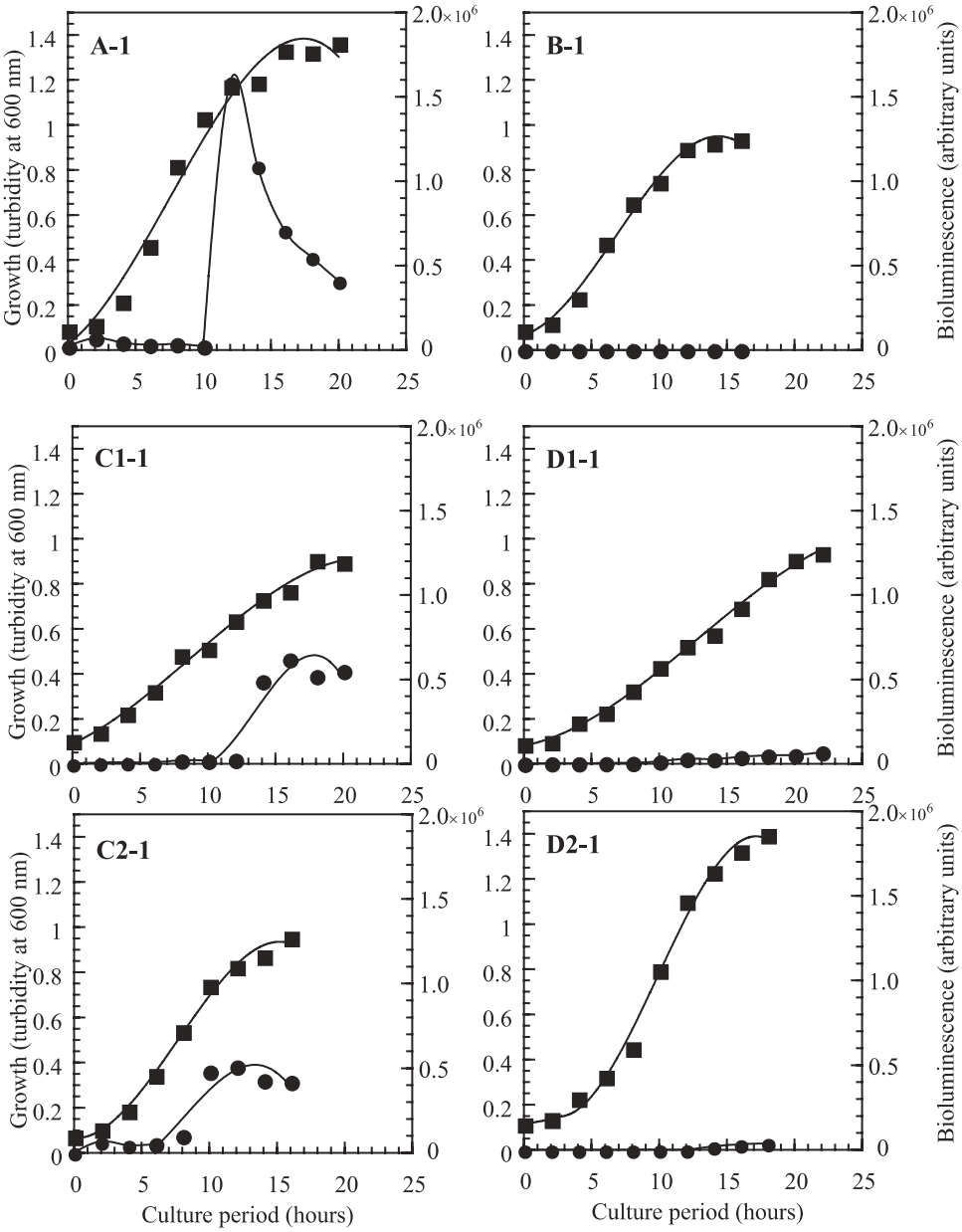

Fig. 4. **Growth and bioluminescence curves of the parental *V. cholerae* NCIMB 41 strain and a dark mutant, in LB medium, and four transformants TrD1, TrD2, TrR1 and TrR2, in medium containing ampicillin.** In panels (A-1) and (B-1), the growth curves, depicted as solid squares, and the bioluminescence curves, depicted as solid circles, of the parental strain and a dark mutant are shown, respectively, whereas in (C1-1), (C2-1), (D1-1) and (D2-1), those of the transformants TrD1, TrD2, TrR1 and TrR2, respectively, are shown.

the TrR2 cells grew like those of the parental strain even in the presence of ampicillin but emitted light more weakly than the TrR1 cells, we assumed that the plasmid, pUC18VcluxOR, must be integrated into the genome of TrR2. To examine this, DNAs extracted from TrR1 and TrR2 were digested with *Eco*RI, *Hin*dIII and *Sma*I, and then subjected to electrophoresis. DNA fragments were transferred to a nylon membrane and hybridized with a probe to detect DNA sequences for β-lactamase. If the plasmid sequence is indeed integrated into the genome, two or more bands should be detected, because each transformant retained the plasmid. However, a single band was found in all lanes at the same position as for pUC18VcluxOR, as shown in Fig. 5. Because the DNA sequence for β-lactamase was not integrated, we further amplified the *luxO* sequences by PCR with both flanking regions using a pair of primers listed in Table 1, and with the genomic DNAs of TrR1 and TrR2 as templates, and subsequently sequenced the PCR products. In TrR2, two nucleotides, GT, were inserted at the deletion point found in the parental strain, although in TrR1 such a repair was not found.

Because *luxO* in the genome of TrR2 was repaired, a mutant of TrR2, which had lost the plasmid, seemed to be useful for examining the effects of the repair of the genomic *luxO* alone on the light emission and growth.

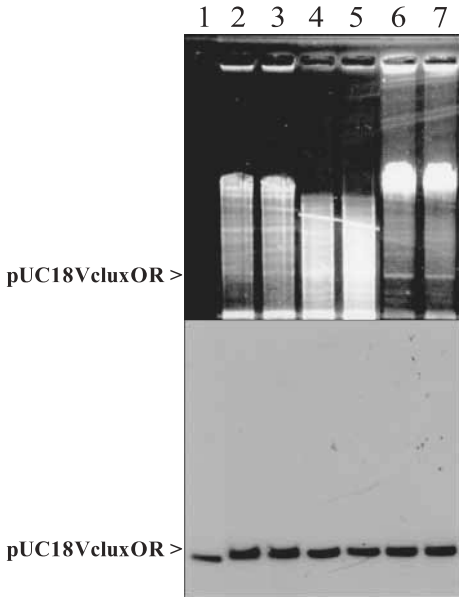

Fig. 5. **Southern hybridization with a probe for the gene encoding β-lactamase.** Lane 1, pUC18VcluxOR; lanes 2, 4 and 6, genomic and plasmid DNAs from TrR1; and lanes 3, 5 and 7, those from TrR2. Lanes 1, 2 and 3, DNAs, digested with *Eco*RI; lanes 4 and 5, with *Hin*dIII; and lanes 6 and 7 with *Sma*I. After electrophoresis, the gel was stained with ethidium bromide and is shown in the upper panel. Southern hybridization with a probe for the gene encoding β-lactamase is shown in the lower panel.

We successfully obtained four mutants, which had lost the plasmids, from the four transformants, TrD1, TrD2, TrR1 and TrR2, by culturing them in ampicillin-free LB medium, and designated them as TrD1m, TrD2m, TrR1m and TrR2m. We cultured the respective mutants in LB medium. The growth and bioluminescence curves of the parental *V. cholerae* NCIMB 41 strain (A-2), as well as TrD1m (C1-2) and TrD2m (C2-2), and TrR1m (D1-2) and TrR2m (D2-2), are shown in Fig. 6. Three mutants, TrD1m, TrD2m and TrR1m, emitted light similarly to the parent strain because the damaged genomic *luxO* gene of each mutant remains unrepaired. However, TrR2m did not change to a luminous state like TrR1m on lose of the plasmid, remaining dim. TrR2m grew more rapidly and densely than the other strains also in this experiment, indicating that the repair of the *luxO* gene had favorable effects on the cells, in addition to repression of the *lux* operon. Because orthologues of *luxO* are distributed widely among Proteobacteria, the function of this gene seems not to be limited to repression of the *lux* operon.

These findings unequivocally show that a two-nucleotide deletion in *luxO* causes the light emission, even with low concentrations of salt, observed for *V. cholerae* NCIMB 41.

DISCUSSION

We have proposed that most luminous bacterial strains are dim or dark, and that strongly luminous strains are rare (*2*), and to determine whether this proposal is correct or not, have conducted the present study and confirmed it. However, it was then realized that this project had been accomplished only in part. As described above, *V. cholerae* NCIMB 41 was initially held by Sonnenschein, who isolated it in 1930 (*11*), and has been maintained for over 70 years, and initially is likely to have repeatedly undergone subculture for maintenance. Typically when such strains are maintained by repeated subculturing, almost all luminous strains become dim or dark, so we were very surprised to find that the culture obtained from the NCIMB still emits light, not powerfully, but nevertheless definitely. It was concluded that the bioluminescent property of this strain is remarkably stable. However, we subsequently found dark mutants during subculturing of this strain and isolated one typical example. Shewan and Veron referred to the loss of luminescence by *V. cholerae* NCIMB 41 after repeated subculturing in their review (*10*). The growth and bioluminescence curves for the dark mutant we isolated are presented in Fig. 4 (B-1). In this figure, the bioluminescence appears to be zero but in fact a barely detectable amount of light, about two hundred AU according to our detector at the maximum luminescent stage, was emitted from the cells. Because we sequenced the *luxO* gene of this mutant and confirmed that the gene was indeed still damaged, the mutation of the parental strain to the dark mutant was not caused by spontaneous repair of the gene. These results indicate there are more than two factors that cause the bioluminescence observed for *V. cholerae* NCIMB 41 in freshwater: one is damage to the *luxO* gene, as shown above, and the other is the rather high level of expression of the *lux* operon. It is difficult to determine how much the expression of the operon is stimulated in common strains. Because we hold that most strains of luminous bacteria are dim or dark, we expect that the normal expression of the operon is essentially maintained at a very low level. Our proposal is supported by the following evidence. Loss of luminescence has been observed by a number of researchers studying luminous bacteria (*2*, *35*), but the opposite, i.e., dark or dim strains being luminous mutants, is quite rare; we have never encountered such mutations, for example, and we speculate that this change, from a luminous to dark or dim strain, is caused by reverse mutations (*2*). Furthermore, Palmer and Colwell reported that in about 50% of strains of *V. cholerae* isolated from the environment, luciferase gene sequences were detected, but most were nonluminescent (*9*), and Ramaiah et al. reported that luciferase gene sequences were detectable in many strains of nonluminous bacteria isolated from the environment (*36*). In this study, we could not identify points of mutation other than in the *luxO* gene that brought about a change from a luminous to dark state. However, we believe that we will soon be able to confirm that dim or dark strains are in fact normal by means of the specific identification of such mutation points.

Although it is obvious that two inducers, acylhomoserine lactone and a DPD derivative, are able to induce the expression of the *lux* operon, we hypothesize that the critical function of both compounds is not to transmit information on cell density to the surrounding cells but rather to detect the damage to flavodoxin (*18*), which is crucial for reactivation of methionine synthase damaged on autoxidation, or alternatively the rate of metabolism of *S*-adenosylmethionine, because if methionine is not satisfactorily regenerated from homocysteine, homoserine, and as a consequence, acylhomoserine lactone, accumulates in the

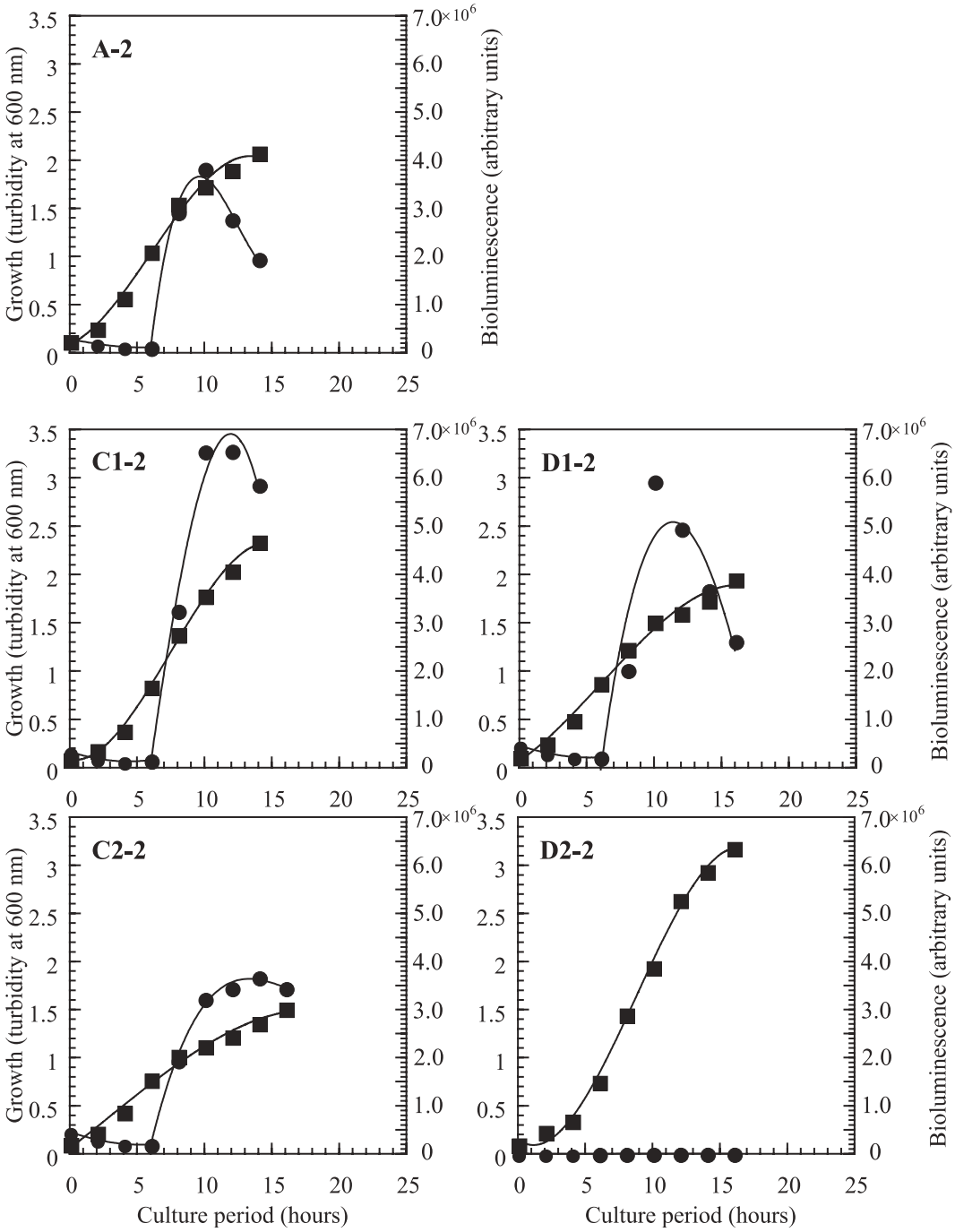

Fig. 6. **The growth and bioluminescence curves of the parental *V. cholerae* NCIMB 41 strain, and four mutants TrD1m, TrD2m, TrR1m and TrR2m, in LB medium.** In panel (A-2), the growth curves, depicted as solid squares, and the bioluminescence curves, depicted as solid circles, of the parental strain are shown, respectively, whereas in (C1-2), (C2-2), (D1-2) and (D2-2), those of the mutants TrD1m, TrD2m, TrR1m and TrR2m, respectively, are shown.

cell and DPD is produced in the metabolic pathway from S-adenosylhomocysteine to homocysteine. We speculate that these two compounds appear to be sensors for population density, as they apparently do to most researchers studying luminous bacteria, because they diffuse easily across membranes and cell densities in artificial cultures are extraordinarily high compared with those in the environment. For this reason, we cannot agree with the proposal that the function of the *lux* operon in luminous bacteria is to produce light and the expression of the operon is induced as a function of sensing the bacterial population density.

The habitats of the *V. cholerae* strains are terrestrial environments and optimum growth is achieved in media containing less than 1% NaCl (*3*, *37*). These characteristics are distinct from those of other *Vibrio* species. We have proposed that the function of the *lux* operon is to produce halotolerant flavodoxin and, as a consequence, expression

of the operon is induced when the concentration of NaCl in the medium increases above 2%. However, we were not able to confirm this induction of expression because the growth of *V. cholerae* NCIMB 41 is extremely slow in a medium containing over 2% NaCl. The *lux* operon is considered to be present in about 50% of *V. cholerae* strains isolated from the environment (*9*), but we suspect that all ancestral strains of this species had the operon without exception because most of the genes for regulation of the operon are still present and one, *luxR2*, is damaged in the genome of *V. cholerae* N 16961, although the *lux* operon itself is not present. The ancestral strains may have grown even in seawater like other *Vibrio* species, but the main habitats of *V. cholerae* strains may have shifted to estuarine or terrestrial environments, and strains living in such locations, having no need for the operon, could have lost it.

The nucleotide sequences reported in this paper have been submitted to the DDBJ/EMBL/GenBank nucleotide sequence database and are available under accession numbers AB115759, AB115760, AB115761, AB113244, AB113245, AB114423, AB114424, AB114425, AB235903 and AB235904. Four transformants, TrR1, TrR2, TrD1 and TrD2, reported in this paper have been deposited at the Biological Resource Center (NBRC), National Institute of Technology and Evaluation, Japan, and are available under accession numbers NBRC 101273, NBRC 101274, NBRC 101275 and NBRC 101276, respectively. We thank Dr. A. Bacher, for carefully reading the manuscript, Dr. T. Honda, for generously providing the *V. cholerae* NCIMB 41, Drs. M. Kitamura and M. Miyata for the discussion and technical assistance, and Dr. T. Nakamura for the assistance in operating the DNA sequencer.

REFERENCES

1. Kasai, S. (1999) Occurrence of P-flavin binding protein in *Vibrio fischeri* and properties of the protein. *J. Biochem.* **126**, 307–312
2. Kasai, S. and Sumimoto, T. (2002) Stimulated biosynthesis of flavins in *Photobacterium phosphoreum* IFO 13896 and the presence of complete *rib* operons in two species of luminous bacteria. *Eur. J. Biochem.* **269**, 5851–5860
3. Dunlap, P.V. and Kita-Tsukamoto, K. (2001) Luminous bacteria, Ch. 329 in *The Prokaryotes: An Evolving Electronic Resource for the Microbial Community* (Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H. and Stackebrandt, E., eds.) Springer-Verlag, New York, NY (Online)
4. Harvey, E.N. (1952) *Bioluminescence*, Academic Press, New York, NY
5. Farghaly, A.H. (1950) Factors influencing the growth and light production of luminous bacteria. *J. Cell. Comp. Physiol.* **36**, 165–184
6. Stabb, E.V., Butler, M.S., and Adin, D.M. (2004) Correlation between osmolarity and luminescence of symbiotic *Vibrio fischeri* strain ES114. *J. Bacteriol.* **186**, 2906–2908
7. Kutscher, F. (1893) Ein Beitrag zur Kenntniss der den Choleravibrionen ähnlichen Wasserbacterien. *Dtsch. med. Wschr.* **15**, 44–46
8. Lehmann, K.B. and Neumann, R. (1896) *Atlas und Grundriss der Bakteriologie und Lehrbuch der speziellen bakteriologischen Diagnositk*, 1st ed., J. F. Lehmann, München, Germany
9. Palmer, L.M. and Colwell, R.R. (1991) Detection of luciferase gene sequence in nonluminescent *Vibrio cholerae* by colony hybridization and polymerase chain reaction. *Appl. Environ. Microbiol.* **57**, 1286–1293
10. Shewan, J.M. and Veron, M. (1974) Genus I Vibrio *Pacini* 1854, in *Bergey's manual of determinative bacteriology*, 8th ed. (Buchanan, R.E. and Gibbons, N.E. eds.) pp. 340–345, Williams and Wilkins, Baltimore, MD
11. Sonnenschein, C. (1931) Fortzüchtung von Leuchtvibrionen in Rindergalle. *Zbl. Bakt.* (Abt. 1) **123**, 92–93
12. Reichelt, J.L., Baumann, P., and Baumann, L. (1976) Study of genetic relationships among marine species of the genera *Beneckea* and *Photobacterium* by means of *in vitro* DNA/DNA hybridization. *Arch. Microbiol.* **110**, 101–120
13. Heidelberg, J.F., Eisen, J.A., Nelson, W.C., Clayton, R.A., Gwinn, M.L., Dodson, R.J., Haft, D.H., Hickey, E.K., Peterson, J.D., Umayam, L., Gill, S.R., Nelson, K.E., Read, T.D., Tettelin, H., Richardson, D., Ermolaeva, M.D., Vamathevan, J., Bass, S., Qin, H., Dragoi, I., Sellers, P., McDonald, L., Utterback, T., Fleishmann, R.D., Nierman, W.C., White, O., Salzberg, S.L., Smith, H.O., Colwell, R.R., Mekalanos, J.J., Venter, J.C., and Fraser, C.M. (2000) DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature* **406**, 477–483
14. Kasai, S. and Yamazaki, T. (2001) Identification of the cobalamin-dependent methionine synthase gene, *metH*, in *Vibrio fischeri* ATCC 7744 by sequencing using genomic DNA as a template. *Gene* **264**, 281–288
15. Soly, R.R., Mancini, J.A., Ferri, S.R., Boylan, M., and Meighen, E.A. (1988) A new *lux* gene in bioluminescent bacteria codes for a protein homologous to the bacterial luciferase subunits. *Biochem. Biophys. Res. Commun.* **155**, 351–358
16. Kita, A., Kasai, S., Miyata, M., and Miki, K. (1996) Structure of flavoprotein FP_{390} from a luminescent bacterium *Photobacterium phosphoreum* refined at 2.7 Å resolution. *Acta Crystallogr.* **D52**, 77–86
17. Duchaud, E., Rusniok, C., Frangeul, L., Buchrieser, C., Givaudan, A., Taourit, S., Bocs, S., Boursaux-Eude, C., Chandler, M., Charles, J.F., Dassa, E., Derose, R., Derzelle, S., Freyssinet, G., Gaudriault, S., Medigue, C., Lanois, A., Powell, K., Siguier, P., Vincent, R., Wingate, V., Zouine, M., Glaser, P., Boemare, N., Danchin, A., and Kunst, F. (2003) The genome sequence of the entomopathogenic bacterium *Photorhabdus luminescens*. *Nat. Biotechnol.* **21**, 1307–1313
18. Kasai, S. (1997) Evidence that FP_{390}, the final product of the *lux* operon in luminous bacteria, has flavodoxin function and takes part in biosynthesis of methionine in *Flavins and Flavoproteins 1996* (Stevenson, K.J., Massey, V., and Williams, C.H. Jr., eds.) pp. 367–372, University of Calgary Press, Calgary, Canada
19. Thompson, F.L., Thompson, C.C., Vicente, A.C., Theophilo, G.N., Hofer, E., and Swings, J. (2003) Genomic diversity of clinical and environmental *Vibrio cholerae* strains isolated in Brazil between 1991 and 2001 as revealed by fluorescent amplified fragment length polymorphism analysis. *J. Clin. Microbiol.* **41**, 1946–1950
20. Engebrecht, J., Nealson, K., and Silverman, M. (1983) Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*. *Cell* **32**, 773–781
21. Devine, J.H., Countryman, C., and Baldwin, T.O. (1988) Nucleotide sequence of the *luxR* and *luxI* genes and structure of the primary regulatory region of the *lux* regulon of *Vibrio fischeri* ATCC 7744. *Biochemistry* **27**, 837–842
22. Nishiguchi, M.K. and Nair, V.S. (2003) Evolution of symbiosis in the *Vibrionaceae*: a combined approach using molecules and physiology. *Int. J. Syst. Evol. Microbiol.* **53**, 2019–2026
23. Showalter, R.E., Martin, M.O., and Silverman, M.R. (1990) Cloning and nucleotide sequence of *luxR*, a regulatory gene

controlling bioluminescence in *Vibrio harveyi*. *J. Bacteriol.* **172**, 2946–2954
24. Bassler, B.L., Wright, M., Showalter, R.E., and Silverman, M.R. (1993) Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. *Mol. Microbiol.* **9**, 773–786
25. Bassler, B.L., Wright, M., and Silverman, M.R. (1994) Sequence and function of LuxO, a negative regulator of luminescence in *Vibrio harveyi*. *Mol. Microbiol.* **12**, 403–412
26. Bassler, B.L., Wright, M., and Silverman, M.R. (1994) Multiple signalling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway. *Mol. Microbiol.* **13**, 273–286
27. Schauder, S., Shokat, K., Surette, M.G., and Bassler, B.L. (2001) The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. *Mol. Microbiol.* **41**, 463–476
28. Miller, M.B. and Bassler, B.L. (2001) Quorum sensing in bacteria. *Annu. Rev. Microbiol.* **55**, 165–199
29. Jobling, M.G. and Holmes, R.K. (1997) Characterization of *hapR*, a positive regulator of the *Vibrio cholerae* HA/protease gene *hap*, and its identification as a functional homologue of the *Vibrio harveyi luxR* gene. *Mol. Microbiol.* **26**, 1023–1034
30. Hanzelka, B.L., Parsek, M.R., Val, D.L., Dunlap, P.V., Cronan, J.E. Jr., and Greenberg, E.P. (1999) Acylhomoserine lactone synthase activity of the *Vibrio fischeri* AinS protein. *J. Bacteriol.* **181**, 5766–5770
31. Cornell, K.A. and Riscoe, M.K. (1998) Cloning and expression of *Escherichia coli* 5′-methylthioadenosine/S-adenosylhomocysteine nucleosidase: identification of the *pfs* gene product. *Biochim. Biophys. Acta* **1396**, 8–14
32. Chen, X., Schauder, S., Potier, N., Van Dorsselaer, A., Pelczer, I., Bassler, B.L., and Hughson, F.M. (2002) Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* **415**, 545–549
33. Miyamoto, C.M., Lin, Y.H., and Meighen, E.A. (2000) Control of bioluminescence in *Vibrio fischeri* by the LuxO signal response regulator. *Mol. Microbiol.* **36**, 594–607
34. Miyamoto, C.M., Dunlap, P.V., Ruby, E.G., and Meighen, E.A. (2003) LuxO controls *luxR* expression in *Vibrio harveyi*: evidence for a common regulatory mechanism in *Vibrio*. *Mol. Microbiol.* **48**, 537–548
35. Silverman, M., Martin, M., and Engebrecht, J. (1989) Regulation of luminescence in marine bacteria in *Genetics of Bacterial Diversity* (Hopwood, D.A. and Chater, K.F., eds.) pp. 71–85, Academic Press, London
36. Ramaiah, N., Chun, J., Ravel, J., Straube, W.L., Hill, R.T., and Colwell, R.R. (2000) Detection of luciferase gene sequences in nonluminescent bacteria from the Chesapeake Bay. *FEMS Microbiol. Ecol.* **33**, 27–34
37. Baumann, P., Furniss, A.L., and Lee, J.V. (1984) Genus I Vibrio *Pacini* 1854 in *Bergey's Manual of Determinative Bacteriology*, Vol. 1 (Krieg, N.R. and Holt, J.G., eds.) pp. 518–538, Williams and Wilkins, Baltimore, MD