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Expression of Bioluminescence by *Escherichia coli* Containing Recombinant *Vibrio harveyi* DNA

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When isogenic strains of *Escherichia coli*, RR1 (*rec*$^+$) and HB101 (*recA*), were transformed with mapped recombinant plasmids known to contain *Vibrio harveyi* luciferase genes and large regions of DNA flanking on both sides, a small percentage (0.005%) of the colonies expressed high levels of luminescence (up to 10^{12} quanta s^{-1} ml^{-1}) in the absence of added aldehyde. The altered ability to express light was found to be due to a mutation in the host and not to an alteration in the recombinant DNA. When these bright colonies were cured of plasmid, they could be retransformed with cloned *V. harveyi* gene fragments in *cis* and in *trans* to yield luminescent colonies at 100% frequency. The maximum length of *V. harveyi* DNA required to produce light-emitting *E. coli* was shorter (6.3 kilobase pairs) than that required for expression of the *V. fischeri* system in *E. coli*. Cell extracts from bright clones contained wild-type levels of activity for the heteropolymeric ($\alpha\beta$) luciferase; fatty acid labeling revealed the presence of the three acylated polypeptides of the fatty acid reductase system which is involved in aldehyde biosynthesis for the luminescence reaction. The increased light emission in the mutant bacteria appeared to arise in part from production of higher levels of polycistronic mRNAs coding for luciferase.

The generation of light-emitting *Escherichia coli* transformed with recombinant *Vibrio fischeri* DNA has advanced our understanding of the gene organization and regulation of the bacterial bioluminescence system (8, 9). In transformed *E. coli*, the two genes coding for the nonidentical subunits of luciferase are expressed at levels comparable to that observed in the parental strain. In addition, the three genes coding for the reductase, synthetase, and transferase subunits of the fatty acid reductase complex responsible for long-chain aldehyde biosynthesis are also expressed at high levels (4). Consequently, fatty aldehydes required for the bioluminescence reaction do not need to be supplied exogenously to transformed *E. coli*, and the cells continuously emit light. The five structural genes of *V. fischeri*, as well as the two regulatory genes, are encoded in an 8.5-kilobase-pair (kbp) DNA fragment containing two transcriptional units as defined by transposon mutagenesis (8). Approximately 95% of the coding region is necessary for transcription of the seven polypeptides (9).

Previous attempts to express luminescence systems from other marine bacteria in *E. coli* have met with much less success. High levels of light have been obtained for *E. coli* containing recombinant *V. harveyi* DNA only when the two luciferase genes are placed under control of a suitable external promoter and exogenous aldehyde is added (1, 2, 10). Although large continuous regions of DNA containing the luciferase genes of *V. harveyi* (19) or *Photobacterium phosphoreum* (unpublished data) have been cloned into *E. coli*, the levels of light emission were less than 0.1% of that observed for the native strain even in the presence of exogenous aldehyde. Only the transferase component of the fatty acid reductase system in *V. harveyi* (22) has been detected in transformed *E. coli*, and its expression occurs at a relatively low level (19). Consequently, it was not known whether low luminescence and the need for aldehyde in cloned systems from marine bacteria other than *V. fischeri* reflected inability to express the luminescence genes in *E. coli* or whether the genes were not all present in the recombinant DNA.

The present report describes the discovery of *E. coli* mutants that can be transformed with *V. harveyi* DNA to generate bright-light-emitting *E. coli* in the absence of exogenous aldehyde. Expression of the genes for luciferase and the three polypeptides of the fatty acid reductase was directly demonstrated on transformation of *E. coli* with only 6.3 kbp of *V. harveyi* DNA. As this length of DNA has insufficient coding capacity to synthesize regulatory proteins comparable to those found in the *V. fischeri* system, these results also showed that transcription of regulatory proteins is not required for expression of luminescence in the mutant *E. coli* cells.

MATERIALS AND METHODS

Bacteria and plasmids. *E. coli* RR1 and its isogenic strain HB101 (*recA*), as described by Maniatis et al. (16), were used for propogation of recombinant plasmids. These strains have been tested to be UV resistant in the case of RR1 and UV sensitive in the case of HB101 (20). DNA from *V. harveyi* B392 was subcloned into several plasmids at various sites: pBR322 (3), pACYC184 (6), pOP95-15K (12), P12 (a galactokinase expression vector constructed from pKO-1 [17] in our laboratory; the polylinker of pUC12 [21] was placed in front of the galactokinase gene in pKO-1 to provide more versatility to this vector). For transformation, *E. coli* cells were grown in LB medium and treated with calcium as previously described (16). Selection for transformants was on LB agar plates containing ampicillin at 100 μg/ml, chloramphenicol at 25 μg/ml, or tetracycline at 10 μg/ml (or a combination as in the case of complementation experiments). Once a transformant was obtained, growth was performed in either LB or 1% NaCl complex medium (18) under antibiotic selective pressure. *V. harveyi* was grown in 1% NaCl complex medium.

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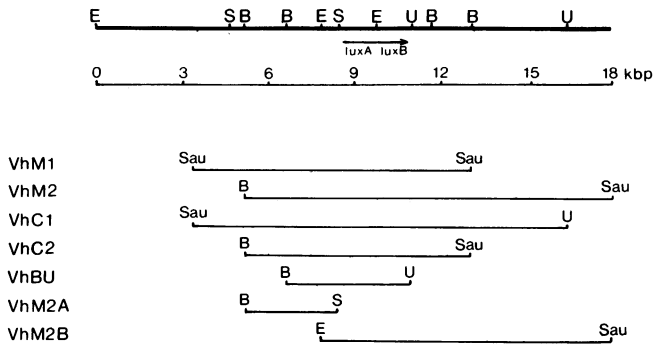

FIG. 1. Restriction map of 18 kbp of *V. harveyi* DNA containing the luciferase genes and the locations of the primary clones studied. The restriction sites shown are those which help to describe the clones indicated; a more complete restriction map has been depicted elsewhere (19). All sites for each restriction enzyme are shown except the numerous sites for *Sau*3AI. Abbreviations: E, *Eco*RI; S, *Sal*I; B, *Bam*HI; U, *Pvu*II; Sau, *Sau*3AI. Plasmid pVhC1 was constructed by *Pvu*II digestion of pVhM1 and pVhM2 DNA and replacement of the DNA downstream from the *Pvu*II site in the VhM1 DNA to the *Pvu*II site of pBR322 with the 5-kbp *Pvu*II fragment of VhM2 DNA. Plasmid pVhC2 was generated similarly by first restricting pVhM2 DNA with *Sal*I to yield pVhM2A (which has lost DNA from the *luxA* gene to the end of VhM2); second, the downstream *Sal*I fragment from pVhM1 was inserted into the *Sal*I site of pVhM2A. Plasmid pVhM2B was a product of partial *Eco*RI digestion of pVhC2 and removal of the upstream DNA from the first *Eco*RI site. The other clones have been described previously (19).

Materials. All of the enzymes used in the recombinant plasmid construction and hybridization studies were purchased from Boehringer Mannheim Biochemicals. Antibiotics were from Sigma Chemical Co., and radiochemicals were from New England Nuclear Corp. Methyl mercury was purchased from Alpha; decanal was from Aldrich Chemical Co., Inc.

Curing *E. coli* of plasmid. When *E. coli* carrying a recombinant plasmid was to be cured of the plasmid, the bacteria were inoculated into LB medium without antibiotic. The culture was incubated at 43°C overnight without being shaken and then spread on LB agar plates at 30°C. Single colonies were picked onto LB plates with or without the antibiotic to which the plasmid is resistant. About three-fourths of the colonies were sensitive to the drug and were also shown to be plasmid free by plasmid preparation analysis. In the case of expressing clones, loss of the plasmid was associated with a concomitant loss of luminescence.

Measurement of luminescence. Luminescence of liquid cultures was measured with a photomultiplier tube calibrated with the light standard of Hastings and Weber (14). One light unit is equal to 5×10^9 quanta/s. To measure aldehyde-dependent luminescence, we added 1 μl of decanal to 1.0 ml of culture just before reading.

Enzyme assays. Assays for luciferase and fatty acid reductase, as well as acylation with [^{3}H]tetradecanoyl-coenzyme A (CoA) in vitro or [^{3}H]tetradecanoic acid in vivo or in vitro were performed as previously described (13, 22).

Other methods. DNA was purified by standard means (16). Most transformations and restrictions were accomplished with "mini preps" of plasmid DNA. RNA was purified as described before (11). Gel electrophoresis of restricted DNA on 1% agarose, that of RNA on methyl mercury-containing agarose (1.2%), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins have also been described before (15, 16, 19). Hybridization of DNA fragments to Northern blots of RNA was performed exactly as described in a previous paper (19), and Southern blot analysis was as described by Maniatis et al. (16).

RESULTS

Transformation of *E. coli* with *V. harveyi* DNA to produce light-emitting colonies. Transformation of *E. coli* with plasmids containing the operons of the *V. fischeri* luminescence system on a 9-kbp segment of DNA results in production of highly luminescent *E. coli* (8). Although large segments of *V. harveyi* DNA containing the luciferase genes have been cloned into *E. coli*, very low levels of light (<0.001% of that for *Vibrio* strains or the cloned *V. fischeri* system in *E. coli*) were observed in the transformed cells (19). VhM1 and VhM2, the larger segments used, are overlapping *Sau*3A fragments which had been constructed by partial *Sau*3A digestion of *V. harveyi* DNA followed by insertion into the *Bam*HI site of pBR322 (19) (Fig. 1). They both contain the *luxA* and *luxB* genes whose positions and orientation on the map are based on the nucleotide sequence (7). Even on addition of exogenous long-chain aldehyde, these large clones emitted very low levels of light either compared with shorter clones that encode only the *lux* genes and in which expression is enhanced by plasmid promoters (1, 2) or compared with the native strain (<0.1% of that of *V. harveyi*). Pairwise complementation of compatible recombinant plasmids encoding the entire 18-kbp region of DNA, which represents 6 to 8 kbp beyond the *lux* genes on either side, also did not result in highly light-emitting *E. coli* (19). However, when pBR322 containing VhM2 DNA (pVhM2) was isolated and retransformed into *E. coli*, approximately 1 in 20,000 colonies emitted a high level of light without aldehyde and could easily be detected within a few seconds in the darkroom. Furthermore, addition of aldehyde did not enhance luminescence.

To determine whether expression of the luminescence system arose from an alteration in the recombinant DNA or in the *E. coli* cells themselves, we isolated the recombinant DNA and cured the transformed luminescent *E. coli* of the plasmid. On restriction analysis, no difference in sites between the recombinant DNA isolated from the luminescent *E. coli* and the original recombinant DNA was detected. Furthermore, on retransformation of *E. coli* with the recombinant DNA isolated from the luminescent cells, all of the 4,000 colonies screened were dark and, on addition of aldehyde, only emitted the low level of light characteristic of the original transformation. In contrast, if the bright *E. coli* cells, after curing of the plasmid, were transformed with the original recombinant DNA, all 2,000 of the transformants were highly luminescent. These manipulations demonstrated that the *E. coli* had mutated to allow expression of the *V. harveyi* system. By transforming different *E. coli* strains, we isolated two distinct *E. coli* mutants: one from transformation of *E. coli* RR1 (43R) and the other from transformation of *E. coli* HB101 (43H). The former grew at the same rate as the parent strain, whereas the latter grew at an extremely slow rate.

Bright, luminous colonies were obtained from transformation of both mutant *E. coli* strains with plasmids containing either VhM1 or VhM2 DNA. Two other plasmids were constructed to contain either the major part of the two regions (VhC1) or the common regions (VhC2) of VhM1 and VhM2 (Fig. 1). VhC1 DNA has some 13 of a maximum of 14.5 kbp of VhM1 and VhM2 clones, whereas VhC2 DNA is 8 kbp long. Both of these plasmids resulted in bright colonies on transformation into the two mutant *E. coli* strains.

In vivo luminescence levels in liquid cultures of mutated cells transformed with pVhM1, pVhM2, pVhC1, or pVhC2 were very similar, with less than a threefold variation in the levels of light emission, depending on the stage of cellular growth. The maximum levels of light emission were between 2,000 (in 43R) and 100,000 (in 43H) times higher than normal *E. coli* RR1 transformed with the same DNA.

As mentioned before, *E. coli* 43R cells grew at normal rates, whereas 43H cells grew extremely slowly. Growth rates were independent of whether or not the mutant cells had been cured or transformed. The growth of 43H recombinants was complicated by a restricted temperature dependence for expression of luminescence. Maximum luminescence at 22°C decreased approximately 100-fold at 24°C and 5-fold at 20°C. Unlike 43H, 43R cells containing recombinant DNA expressed light at their maximum level over a wide range of temperatures (20 to 30°C). The doubling time at 27°C (the temperature used routinely) for 43R cells was about 90 min, whereas that of 43H at 22°C was 8 to 12 h. Vigorous aeration of cells in both systems was helpful in maintaining high levels of luminescence in liquid cultures.

Figure 2 compares the in vivo light emission (as quanta per second per unit of growth [A_{660}]) of *V. harveyi* with those of the two mutant cell lines transformed with pVhM2 DNA. The level of luminescence reached by the transformed 43H cell line was very similar to that of *V. harveyi*, whereas the luminescence of the transformed 43R cell line was about 50

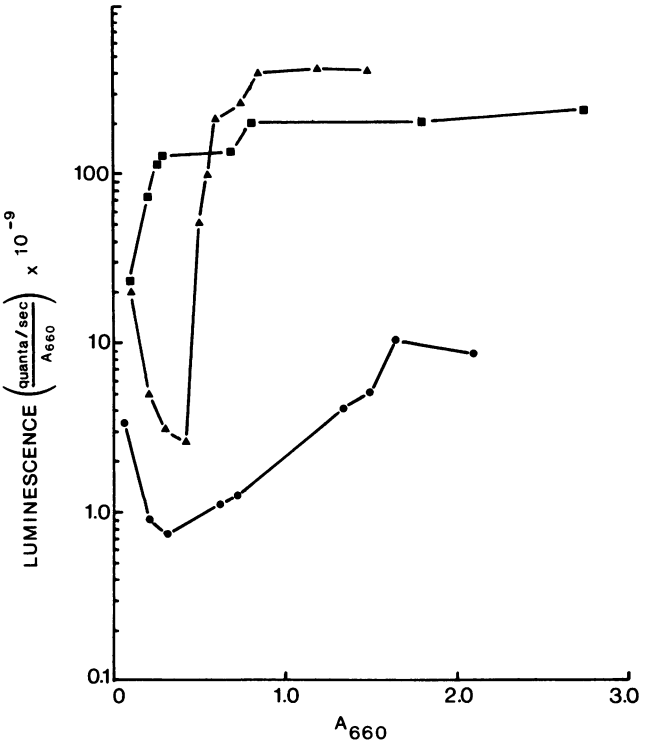

FIG. 2. Expression of luminescence as a function of cell density in *V. harveyi* and mutant *E. coli* strains containing pVhM2. All three bacteria were grown in 1% NaCl complex. *V. harveyi* and *E. coli* 43R harboring pVhM2 were shaken in a 27°C water bath while *E. coli* 43H containing pVhM2 was shaken at 22°C. Samples were taken to measure both light production in a photomultiplier and A_{660} in a spectrophotometer. Luminescence per unit of cell density as a function of cell density was plotted for *V. harveyi* (▲), pVhM2-containing *E. coli* 43R (●), and pVhM2-containing 43H (■).

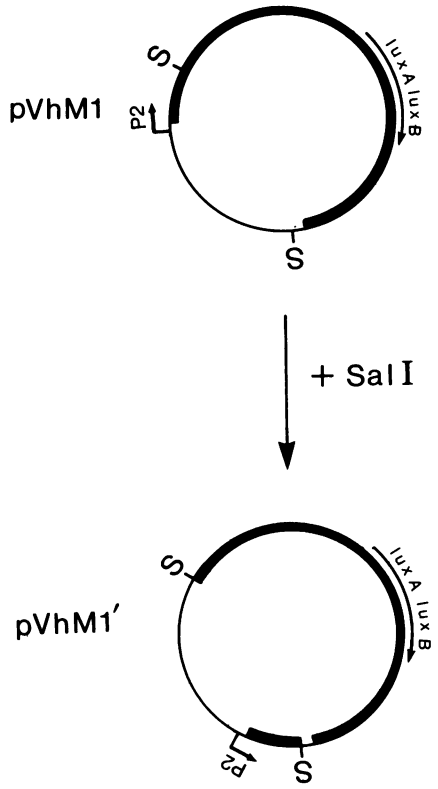

FIG. 3. Construction of the recombinant plasmid pVhM1' from pVhM1. The blackened portion represents the cloned *V. harveyi* DNA in pVhM1 containing the *luxA* and *luxB* genes inserted into the *Bam*HI site of pBR322 in a clockwise fashion after the *tet* promoter (P2). The direction of transcription of the *luxA* and *luxB* genes is indicated. After partial *Sal*I (S) restriction and ligation, the new plasmid (pVhM1') has the *tet* promoter (P2) reversed relative to the direction of transcription of the luciferase genes. All other promoters with the correct orientation in both constructions are at least 2.8 kbp from the cloned insert.

times lower. In addition, it appeared that the relative change in luminescence with growth of the bacteria was less in the mutant *E. coli* than in *V. harveyi*.

As indicated before, the in vivo luminescence of recombinant *E. coli* was very similar whether the DNA used to transform the same strain of mutant cells was pVhM1, pVhM2, pVhC1, or pVhC2. Since the four plasmids contain various lengths of *V. harveyi* DNA upstream (and downstream) from the *luxA* and *luxB* genes, plasmid promoters do not appear to have a major role in facilitating expression of light in transformed *E. coli*. To further establish that plasmid promoters play a limited role in expression of the luminescence system of recombinant *V. harveyi* DNA, we performed the following experiment. The recombinant plasmid pVhM1, which contains an insert in the *Bam*HI site of pBR322 in a clockwise direction downstream from the *tet* promoter, was restricted with *Sal*I, and the fragments were religated. A luminescent transformant (VhM1') was isolated in which the vector portion, along with some 10% of the upstream VhM1 DNA, had been reversed in direction (Fig. 3). *E. coli* 43H cells containing either the parent (VhM1) or the new plasmid (VhM1') produced the same luminescence in vivo (64 and 76 light units/ml, respectively, at A_{660} = 2.5). Thus, the direction and location of the promoters of pBR322

TABLE 1. Luciferase activity in extracts of luminescent *E. coli*[a]

Strain and plasmid	Sp act (LU mg^{-1})
E. coli	
RR1 (pVhM1)	4
RR1 (pVhM2)	5
43R (pVhC1)	100
43R (pVhC2)	100
43R (pVhM2)	300
43H (pVhM1)	3,000
43H (pVhM2)	3,000
V. harveyi	4,000

[a] Cells were grown in either 1% complex or LB medium under conditions described in the text. Cells were harvested at A_{660} readings between 1.0 and 3.0. The luciferase activity in extracts appeared to be partially dependent on the stage of cellular growth and could vary up to two- to threefold from one experiment to another. The values listed are the maximum light levels observed for the different luminescent cells.
[b] LU, Light units.

appeared to have little if any effect on the expression of the luminescence system in *E. coli*.

In vitro luciferase. Since in vivo luminescence reflects both the supply of $FMNH_2$ and O_2 in the bacteria, as well as the expression of the luciferase genes and the genes required for biosynthesis of the aldehyde substrate, luciferase activities were determined in vitro for the recombinant *E. coli*. The specific activity of luciferase in extracts of luminescent 43H cells was very similar to that detected in extracts of *V. harveyi*, whereas the luciferase activity in extracts of luminescent 43R cells was between 3 and 10% of that of the parent strain (Table 1). In contrast, the specific activity of luciferase in extracts of normal *E. coli* RR1 containing the same recombinant DNA (pVhM2) was almost 1,000 times lower than that of *V. harveyi*. The differences in the in vitro luciferase activities of extracts from the various luminescence species correlated reasonably well with the differences in in vivo luminescence except that the relative changes were somewhat smaller, possibly reflecting differences in expression of the genes required for aldehyde biosynthesis.

Expression of the genes required for aldehyde biosynthesis. The enzymes involved in biosynthesis of the aldehyde substrate for the bioluminescence reaction are believed to be part of a fatty acid reductase complex. Three polypeptides involved in the fatty acid reductase system in *V. harveyi* have been specifically identified by acylation in vitro with [^{3}H]tetradecanoic acid (plus ATP) or [^{3}H]tetradecanoyl-CoA, as well as by in vivo acylation with [^{3}H]tetradecanoic acid followed by sodium dodecyl sulfate-gel electrophoresis and fluorography (22). Three acylated polypeptides have also been detected in *E. coli* clones containing the *V. fischeri* operons, indicating that the polypeptides of the fatty acid reductase system are encoded by genes on the *V. fischeri* luminescence operons (4).

Figure 4 shows the in vitro and in vivo acylation of peptide products of the cloned systems in 43H cells. Acylation with [^{3}H]tetradecanoyl-CoA of extracts from the cell lines containing either pVhM1 or pVhM2 DNA showed a specific band (t) of 32 kilodaltons (kDa) (lanes 2 and 3, respectively), analogous to the acylation properties of the transferase subunit of the fatty acid reductase complex from *V. harveyi* (22). As shown in lane 1, however, a labeled band was not seen in the extract from a 43H cell line containing pVhM2A DNA (cf. Fig. 1). Since previous results showed that the 32 kDa was present and acylated with [^{3}H]tetradecanoyl-CoA in extracts of *E. coli* RR1 containing pVhBU (Fig. 1), it appears that the coding region for the transferase polypeptide in VhM2A is incomplete and must extend downstream past the *Sal*I restriction site, in agreement with the nucleotide sequence which shows an open reading frame extending 32 codons past the *Sal*I site (7).

Lanes 4, 5, and 6 show the pattern for in vitro acylation with fatty acid (plus ATP) of pVhM2A-, pVhM1-, and pVhM2-containing mutant cell extracts, respectively. Reaction under these conditions led to the identification of all three polypeptides in the fatty acid reductase system—the reductase (r), the synthetase (s), and the transferase (t)—in extracts containing pVhM1 and pVhM2, whereas none of these polypeptides could be identified in extracts of the upstream clone pVhM2A. Lanes 7 and 8 show that in vivo acylation of pVhM1- and pVhM2-containing *E. coli* 43H was identical to that observed for in vivo acylation of *V. harveyi* (lane 9), whereas no bands were acylated in 43H cells containing the downstream clone pVhM2B (lane 10). (VhM2B, as shown in Fig. 1, includes *luxA* and *luxB* genes and extends some 2 kbp past *luxB*.)

The same polypeptides could be expressed in 43R cells (Fig. 5, lanes 1 and 2) as in 43H cells (lanes 4 and 5) even though luminescence was almost 20 times lower. In these cases, pVhC1 and pVhC2 DNAs were used to transform the cells, which were then labeled with ^{3}H-fatty acid in vivo. Lane 3 was a control for in vivo fatty acid acylation of 43R

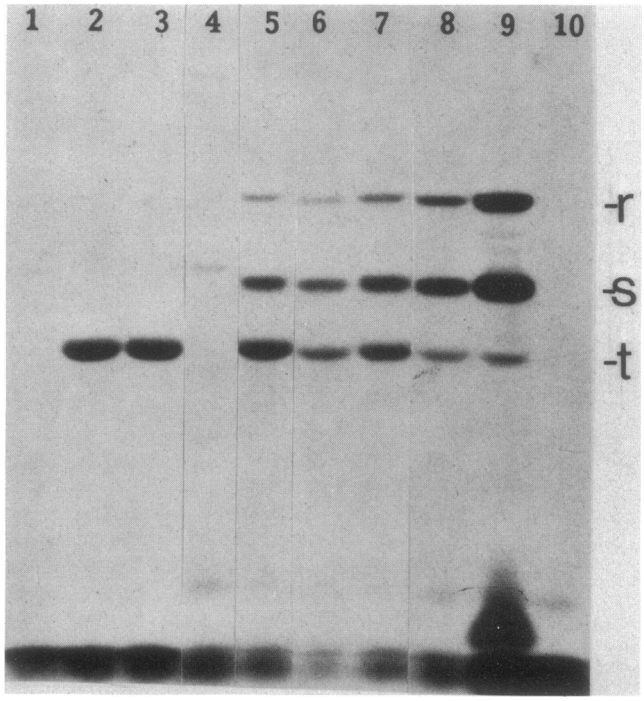

FIG. 4. In vivo and in vitro acylation of proteins from *E. coli* 43H containing recombinant DNA from *V. harveyi*. Proteins were acylated in vitro with [^{3}H]tetradecanoyl-CoA (lanes 1 to 3) or [^{3}H]tetradecanoic acid (plus ATP) (lanes 4 to 6) or labeled in vivo with [^{3}H]tetradecanoic acid (lanes 7 to 10). Cells had been grown to an A_{660} of 2.5 to 2.8. *E. coli* 43H contained pVhM2A (lanes 1 and 4), pVhM1 (lanes 2, 5, and 7), pVhM2 (lanes 3, 6, and 8), or pVhM2B (lane 10). *V. harveyi* was used as a control in lane 9. Proteins of 54, 42, and 32 kDa are designated by r, s, and t, respectively, and refer to the reductase, synthetase, and transferase activities of the fatty acid reductase system.

cells alone. Thus, all three polypeptides involved in aldehyde biosynthesis for the *V. harveyi* luminescence system were detected in clones of both mutant strains when the common region of VhC2 was present.

Complementation of various subclones of *V. harveyi* DNA. To determine whether the polypeptides encoded in the DNA common to VhM1 and VhM2 could be expressed in *trans*, we constructed several pairs of subclones containing *V. harveyi* DNA within this region (8 kbp) in compatible plasmids that conferred differing antibiotic resistances. Each of the individual plasmids was tested and found to be incapable of yielding light-emitting *E. coli* 43R without addition of aldehyde. After transformation of the pairs of plasmids into mutant *E. coli* 43R and selection on LB plates containing the appropriate antibiotics, the plasmids were isolated, checked by restriction analysis, and shown to be present at a molar ratio of about 1:1 in all cases. Clone 1 was complemented with clones 2 to 5, and clone 5 was complemented with clones 6 and 7, as well as clone 1 (Fig. 6). The colonies were then checked for in vivo luminescence (without aldehyde) in liquid culture as described in the legend to Fig. 2.

A number of conclusions can be reached from the complementation results summarized in Fig. 6. In *trans* complementation of two overlapping fragments of *V. harveyi* DNA can result in expression of luminescence (e.g., clones 1 and 2). No light was obtained if the two fragments did not extend far enough or terminated at the same site, disrupting a gene for one of the products in the luminescence system. Thus, in the complementation of clones 1 and 3, the transferase gene was disrupted in both recombinant DNA molecules. The extent of DNA required at the upstream end appears to be

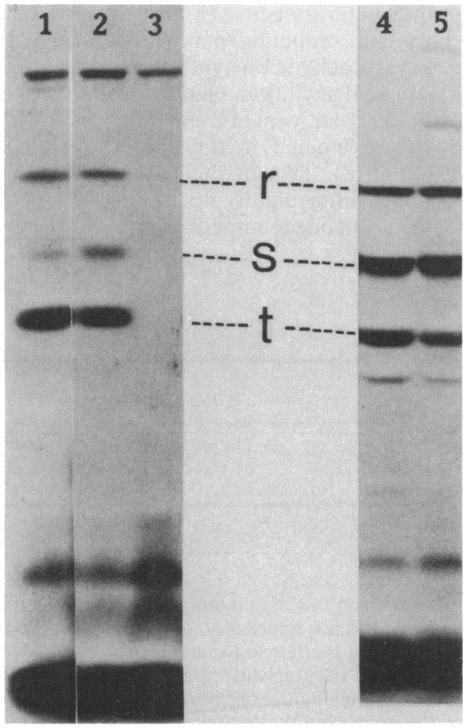

FIG. 5. Comparison of in vivo acylation of proteins from *E. coli* 43R and 43H containing recombinant DNA from *V. harveyi*. Proteins were acylated in vivo with [^{3}H]tetradecanoic acid. The proteins labeled in *E. coli* 43R harboring pVhC1 (lane 1), pVhC2 (lane 2), or no added DNA (lane 3) were compared with those from *E. coli* 43H containing pVhC1 (lane 4) or pVhC2 (lane 5).

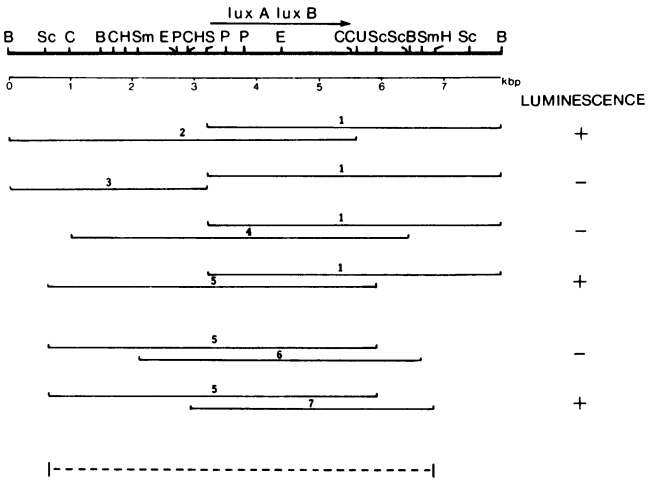

FIG. 6. Complementation and expression of several pairs of recombinant plasmids of *V. harveyi* DNA within the 8-kbp region of DNA encoded in VhC2. At the top, a restriction map of VhC2, including the positions of *luxA* and *luxB*, is diagrammed. Abbreviations: B, *Bam*HI; Sc, *Sac*I; C, *Cla*I; H, *Hin*dIII; S, *Sal*I; Sm, *Sma*I; E, *Eco*RI; P, *Pst*I; U, *Pvu*II. Clone 1 was prepared by inserting an *Sal*I fragment from pVhC2 into the *Sal*I site of pACYC184 (Cm^r). Clones 2, 3, and 4 were inserted into pBR322. Clone 2 was constructed by *Pvu*II restriction of pVhC2, clone 3 is pVhM2A (Fig. 1); and clone 4 was constructed by subcloning the small fragment extending from the first *Cla*I site to the downstream *Bam*HI site (1.5 kbp on the map) into the *Cla*I and *Bam*HI sites of pBR322 and then adding the downstream *Bam*HI fragment (1.5 to 6.4 kbp). Clone 5 was obtained after *Sac*I restriction of pVhC2 and insertion of the DNA (0.6 to 6.0 kbp) into the *Sac*I site of the vector P12, which is compatible with pACYC184. Clones 2 through 5 are Ap^r, and thus selection was on LB plates containing ampicillin and chloramphenicol. Clone 6 was constructed by inserting the *Sma*I fragment of pVhC2 into the *Sma*I site of the plasmid pOP95-15K, which is compatible with clone 5 and is both Ap^r and Tc^r. Selection was on LB plates containing ampicillin and tetracycline. Clone 7 is an *Hin*dIII fragment (3.0 to 6.9 kbp on the map) inserted into the *Hin*dIII site of pACYC184 still containing the chloramphenicol gene. The pairs of compatible recombinant plasmids were complemented in *E. coli* 43R, and their luminescence was tested. The dotted line corresponds to the longest region required for luminescence expression.

located between the *Sac*I site (0.6 kilobase pairs [kbp]) and the *Cla*I site (1.0 kbp), as demonstrated by the absence of luminescence in the complementation of clones 1 and 4 and the positive response in the complementation of clones 1 and 5. The extent of DNA required downstream is between the *Sma*I (6.7 kbp) and *Hin*dIII (6.9 kbp) sites, as shown by the positive response of clones 5 and 7 and the negative response of clones 5 and 6.

Distribution of polycistronic mRNAs for *lux A* in recombinant *E. coli*. A set of four polycistronic mRNAs (2.6, 4, 7, and 8 kb) detected by hybridization of *luxA* DNA to *V. harveyi* RNA has previously been observed in RNA from *E. coli* RR1 containing pVhM2 (19). These larger mRNAs (7 and 8 kb), positioned along the DNA map, extended to the right of the downstream termination of the pVhM1 clone. To test levels of transcription, we extracted RNAs from growing cultures of *E. coli* 43R and 43H cells containing pVhM2. After purification, electrophoresis, and Northern blotting, the RNA was subjected to hybridization with the *luxA* insert. All four *luxA* mRNAs were present in RNAs from pVhM2 in RR1, 43R, and 43H (lanes 1 to 3) with the same relative

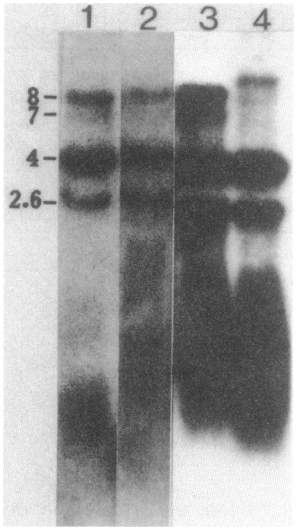

FIG. 7. Hybridization of labeled *luxA* DNA of *V. harveyi* to RNAs from various *E. coli* strains carrying *V. harveyi* recombinant plasmids. *E. coli* RR1, 43R, and 43H, all harboring pVhM2 DNA, were grown in 1% NaCl complex containing ampicillin at 28°C for RR1 and 43R and at 22°C for 43H until A_{660} = 1.0. A fourth culture, *E. coli* 43H containing pVhM1, was also grown at 22°C until A_{660} = 1.0. RNA was isolated from each culture and electrophoresed on 1.2% methyl mercury agarose. A Northern blot was obtained, and hybridization with *luxA* DNA as a probe was performed (19). The hybridization patterns are shown for RNA from RR1 (lane 1), 43R (lane 2), and 43H (lane 3), all carrying pVhM2, and from 43H carrying pVhM1 (lane 4). On the left, the positions and sizes (in kilobases) of *luxA* mRNAs are indicated.

intensities of the different mRNAs (4 ≥ 3 ≥ 8 ≥ 7 kb) (Fig. 7). The total amount of mRNA hybridized to *luxA* was higher for 43H cells and was comparable to that extracted from *V. harveyi* after induction of luminescence (data not shown). However, a substantial level of mRNA was found in all cases. It appears that high expression in mutant cells may be due only in part to higher levels of transcription.

Since the pVhM1 clone also expressed luminescence, it was important to analyze the mRNA from *E. coli* containing this recombinant plasmid. It would be predicted that the larger mRNAs would either be missing or altered in some manner, whereas the major mRNAs (4 and 2.6 kb) would still be present. Only the 4- and 2.6-kb mRNAs could be detected in pVhM1 in *E. coli* 43H; the 7- and 8-kb mRNAs were no longer present, although other mRNA species that can be hybridized with the *luxA* probe could be detected, one at about 8.5 kb and possibly one at about 7.5 kb (Fig. 7, lane 4). These mRNAs could be products of readthrough terminating past VhM1 and the pBR322 *tet* gene.

The role of the polycistronic mRNAs (7 and 8 kb) extending 3 to 4 kb downstream from the luciferase genes (19) is of immediate interest since the terminal 2-kb region is apparently not essential for expression of luminescence in these cells. It will be useful to identify any polypeptides translated from these messages and to check whether any thus far unidentified proteins are involved in some aspect of bioluminescence. One potential candidate is aldehyde dehydrogenase, whose presence is apparently unique to *V. harveyi*, but its role in the bioluminescence system is not yet established (5). In addition, detection of mRNA transcribed off DNA upstream from the polycistronic mRNAs and placement of all of the structural genes along the map will be essential for understanding the gene organization and regulation of bioluminescence in *V. harveyi*.

DISCUSSION

In this study, two mutant *E. coli* strains, 43R and 43H, derived from *E. coli* RR1 and HB101, respectively, produced high levels of luminescence without aldehyde when transformed with plasmid carrying an 8-kbp fragment of *V. harveyi* DNA encompassing the *luxA* and *luxB* genes. This elevated expression appeared to be under the control of *V. harveyi* promoters encoded within the cloned DNA fragment. Furthermore, all five structural genes involved in the luminescence of *V. harveyi* were identified in these recombinant mutant strains.

The high expression of the cloned *V. harveyi* luminescence system was apparently due to an alteration in the host *E. coli*. Southern blot analysis of the mutant *E. coli* DNA with VhM2 DNA as a probe showed no hybridization, which ruled out the remote possibility that some part of the incoming plasmid had been incorporated into the *E. coli* chromosome. We are presently conducting mating experiments to map the alteration of the chromosome. It is interesting that both *recA* and *recA*$^+$ strains gave rise to mutant strains capable of expressing the luminescence system (the mutants were ascertained to have maintained the *rec* properties by UV irradiation). Although retarded growth rate in 43H may be related to the *recA* mutation, the mutation allowing expression of the luminescence system of *V. harveyi* is probably not a result of *recA*.

The maximum length of *V. harveyi* DNA required for expression of the luminescence system in mutant *E. coli* was 6.3 kbp, extending at most 2.8 kbp to the left and 1.4 kbp to the right of the luciferase genes (Fig. 8). This DNA encodes the three fatty acid reductase polypeptides (32, 42, and 54 kDa) required for aldehyde biosynthesis and identified in this system by fatty acid acylation. Genes for three polypeptides with similar molecular weights and required for aldehyde biosynthesis were located in the cloned system from *V. fischeri* MJ1 (Fig. 8). Two of the polypeptides (33 and 53 kDa) are encoded immediately upstream and one polypeptide (42 kDa) is encoded immediately downstream of the luciferase genes (9).

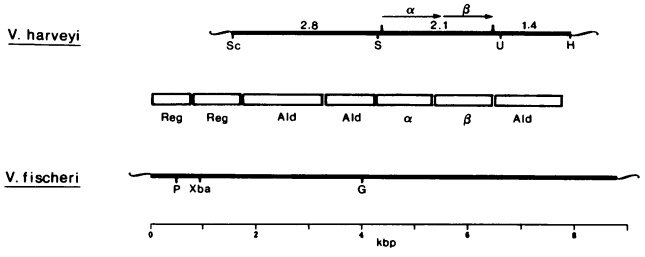

FIG. 8. Comparison of the maximum length of *V. harveyi* DNA required for luminescence expression in *E. coli* with the lengths of the genes required for luciferase (α and β), aldehyde (Ald) biosynthesis, and regulation (Reg) in the *V. fischeri* operon(s). The DNAs were compared by aligning the *luxA* and *luxB* genes (α and β). The locations of the genes for the luciferase subunits (α and β) of *V. harveyi* are based on the nucleotide sequence of Cohn et al. (7), and the lengths and positions of the genes for luciferase, aldehyde biosynthesis, and regulation of the *V. fischeri* operon(s) are from Engebrecht and Silverman (9). The restriction sites are *Sac*I (Sc), *Sal*I (S), *Pvu*II (U), *Hin*dIII (H), *Pst*I (P), *Xba*I (Xba), and *Bgl*II (G). Not all restriction sites are shown.

We have recently identified the start of a gene 950 nucleotides upstream from the luciferase genes in *V. harveyi* cloned DNA which has an open reading frame that could encode for the 32- to 34-kDa polypeptide required for aldehyde biosynthesis (unpublished data). The remaining 1.8 kbp of upstream DNA in the *V. harveyi* system (Fig. 8) would be just sufficient to encode the 53- to 54-kDa polypeptide and would require 90% of this coding capacity. Consequently, there is not sufficient DNA available in the cloned 6.3 kbp of *V. harveyi* DNA to encode regulatory proteins comparable to that found in the *V. fischeri* system. Whether or not the larger fragments of *V. harveyi* DNA encode regulatory genes is unknown since their expression of luminescence in the mutant *E. coli* is the same as that found for the 6.3-kbp fragment of DNA. Neither longer nor shorter fragments of *V. harveyi* DNA resulted in light-emitting cells on transformation of wild-type *E. coli*.

In contrast, expression of the luminescence system of *V. fischeri* in wild-type *E. coli* required that the DNA extend at least 4 kbp upstream of the luciferase genes and code for two regulatory proteins (Fig. 8). Deletion of the DNA to the left of the *V. fischeri* *Pst*I (P) or *Xba*I site eliminated expression of the luminescence system, resulting in dark colonies of *E. coli* RR1 transformed with this DNA. Recently, we transformed mutant 43R cells with the *V. fischeri* DNA to the right of the *Xba*I site (Fig. 8). All colonies were luminescent, even in the absence of aldehyde, and were easily detected on film after only 30 s of exposure. Although the reason for high expression of luminescence in the mutant cells is unknown, these results indicate that alteration in the host allows expression of the *V. fischeri* luminescence system without the necessity for transcription of the regulatory genes. This explanation is also consistent with the high levels of luminescence obtained on transformation of the mutant *E. coli* with the structural genes of the *V. harveyi* system and suggests that regulation of expression of the luminescent system can be bypassed or altered in mutant cells.

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