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Mutant Analysis and Enzyme Subunit Complementation in Bacterial Bioluminescence in *Photobacterium fischeri*

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Chemical mutagens were used to obtain mutants deficient in bioluminescence in the marine bacterium *Photobacterium fischeri* strain MAV. Acridine dyes were effective in the production of dark mutants but not in the production of auxotrophs. These dark mutants were all of one type and appeared to contain lesions blocking the synthesis of luciferase. ICR-191 was especially effective in the production of aldehyde mutants, i.e., dark strains that luminesce when a long-chain aldehyde such as *n*-decanal is added to them. However, other mutant types were isolated after treatment with ICR-191. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine induced many bioluminescence-deficient types with respect to both the site of the lesion and the quantitative effect on the luminescent system. We characterized the dark and dim mutants with respect to their response to exogenous decanal, levels of in vivo and in vitro luminescence, and their rates of reversion to wild type. In addition, the luciferases of the mutant strains were examined by subunit complementation. On the basis of these analyses, we identified mutants which synthesize altered luciferase, strains which are deficient in synthesis of luciferase, and aldehyde mutants. The results of analysis of luciferase from the aldehyde mutants and the complementation studies indicate that the lesions in these strains are in the luciferase itself. Results obtained with wild-type cells grown in minimal medium, and aldehyde mutant cells grown either in complete or minimal medium, indicate that a "natural aldehyde factor" is involved in in vivo light emission. These same studies showed that the long-chain aldehyde(s) could only partially substitute for the natural "aldehyde factor." The possibility that the in vivo aldehyde factor is not a long-chain aldehyde is discussed.

Bacterial bioluminescence is a flavine-mediated luminescence which was studied by many investigators with respect to both its physiology and enzymology. The cell-free system was first demonstrated by Strehler (16); the in vitro light-emitting reaction was shown to involve the oxidation of reduced flavine mononucleotide (FMN) by molecular oxygen with a long-chain aliphatic aldehyde function as a cofactor or cosubstrate (2, 6, 17). The reaction sequence is shown below.

$$NADH + H^+ + FMN \xrightarrow{\text{reductase}} NAD + FMNH_2 \quad (1)$$

$$FMNH_2 + O_2 + \text{aldehyde} \xrightarrow{\text{luciferase}} \text{luciferase-flavine(excited state)} \quad (2)$$

$$\text{Luciferase-flavine (excited state)} \longrightarrow \text{light} \quad (3)$$

Reaction 2 is catalyzed by a specific luciferase, a protein of molecular weight 80,000, comprised of two nonidentical subunits (9). Neither the chemistry of the aldehyde function nor the identity of the molecular species involved in light emission has been established; the latter is thus designated as an excited state of a luciferase-flavine complex (12). Bioluminescence in crude extracts is also stimulated by reduced nicotinamide adenine dinucleotide (NADH), which serves to reduce

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FMN, via reaction 1, catalyzed by a specific flavine reductase (7).

In this study, mutants defective in the bioluminescent system were isolated and characterized. Mutants were obtained in which the luciferase molecule is defective; in others there were lesions that result in the synthesis of low levels of luciferase. These two classes were distinguished by complementation studies.

In addition, a large number of aldehyde mutants were isolated. These were characterized as dark mutants in which the intact cells emit light when exposed to vapors of *n*-decanal, similar to mutants first reported by Rogers and McElroy (13). A variety of different aldehydes are active, and in spite of the fact that aldehyde is also required for maximal activity with the isolated luciferase in the in vitro reaction, there is no information concerning the molecular identity of the presumed "natural aldehyde." Furthermore, aside from the fact that the aldehyde mutants respond to externally added aldehyde, there is no substantial evidence from this study to indicate that any of the aldehyde mutants are actually defective in aldehyde synthesis. In fact, some of the properties of luciferases from several of the aldehyde mutants were studied and found to differ significantly from wild-type luciferase. Thus, some of the aldehyde mutants might presumably involve lesions in the luciferase that alter the binding of, or reaction with, the natural aldehyde.

MATERIALS AND METHODS

The buffers and solutions for experiments in vivo contained 3% NaCl. Phosphate buffers were made by mixing the dibasic potassium salt with the monobasic sodium salt to give the desired *p*H. Synthetic sea water was made with 200 g of Neptune salts (General Biological Supply House, Inc., Chicago, Ill.) dissolved to a total volume of 17 liters in distilled water. Urea (8 M) was purified by treatment (batchwise) with 15 g of a mixed-bed ion-exchange resin (Bio-Rad 11A8) per liter and filtered. Guanidine hydrochloride (5 M) was purified by mixing with activated charcoal (2 g/liter) and filtered.

FMN was a gift of Sigma Chemical Co., and was chemically reduced by bubbling hydrogen in the presence of platinized asbestos (E. H. Sargent and Co.). Long-chain aldehydes were purchased from K & K Laboratories, Plainview, N.Y., and emulsions of 100-fold dilution of the aldehyde in distilled water were prepared by sonic disruption with an ultrasonic power unit (Measuring and Scientific Equipment, Ltd., London, England). *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was purchased from Aldrich Chemical Co. Acridine dyes (acriflavine, proflavine, and acridine orange) were kindly supplied by R. W. Tuveson. ICR-191, the acridine half mustard, was a gift of H. J. Creech.

Photobacterium fischeri strain MAV was obtained from J. W. Hastings and designated as MAV (9) to indicate that it is different from *Photobacterium* (*Achromobacter*) *fischeri* (ATCC 7744), the species on which much of the previously published work is based.

Minimal medium was identical with that of Farghaly (3), containing 30 g of NaCl, 5.3 g of NaH_2PO_4, 2.1 g of K_2HPO_4, 0.5 g of $(NH_4)_2PO_4$, 0.1 g of $MgSO_4$, and 3 ml of glycerol dissolved to a total volume of 1 liter with distilled water. Complex medium was made by adding 0.5 g of yeast extract (Difco), 5.0 g of peptone (Difco), and 3 ml of glycerol to one liter of synthetic sea water. For a solid medium, 11 g per liter of agar (Difco) was added. When a medium with a lower salt concentration was required, the broth was prepared by adding the yeast extract and peptone to 1 liter of minimal medium made with the desired concentration of NaCl.

Growth experiments were done in liquid cultures aerated by shaking at 100 rev/min on a New Brunswick reciprocal shaker at 25 to 27 C. Flasks with a side arm (16 mm outer diameter) were used to grow cultures (50-ml volumes). Optical density (OD) at 660 nm was measured (in the side arms) with a Coleman Jr. II spectrophotometer. The number of bacteria per milliliter in complex medium (Fig. 1 and 4) was directly proportional to OD at 660 nm up to a value of 0.3 which equaled 1.2×10^8 bacteria per ml. OD values of 0.5, 0.7, and 1.0 corresponded to 2.5×10^8, 5.5×10^8, and 1.0×10^9 bacteria per ml, respectively. In complex medium, the generation time was approximately 20 min, whereas in a minimal medium it was approximately 250 min. Viable bacteria per ml in minimal medium, reported in the figures, were determined directly from counts on dilution plates.

Cells for NTG mutagenesis were grown aerobically in complete medium, washed, and resuspended in minimal medium at an OD of 10.0. NTG was added as solid crystals to this suspension in a screw-cap tube, and the tube was shaken at 30 C. After treatment, the cells were dilution-plated onto complex medium for observation of mutants. The precise time of treatment with the mutagen varied between experiments to achieve the same level of killing. The maximal efficiency of mutagenesis was observed at a level of 1% survivors.

Mutagenesis with either the acridine dyes or ICR-191 required medium with a reduced NaCl concentration (1%). The mutagen, dissolved in distilled water, was sterilized by filtration (membrane filters, Millipore Corp.) and added to wild-type cells diluted to an OD of 0.02 in the low salt medium. After growth to stationary phase, the mutagenized cells were dilution-plated.

Light measuring equipment was kindly lent to us by J. W. Hastings and consisted of a high-voltage power supply, a photomultiplier tube (RCA 1P21), and the appropriate amplifier. The light output was recorded on an Esterline Angus Speed Servo Recorder. The sample to be assayed (in a standard liquid scintillation counter vial) was placed in a light-tight chamber, and exposed to the phototube, by means of a shutter

mechanism. Addition of compounds to the reaction vial was accomplished by injection via a syringe. Light intensity is expressed in light units, calibrated by using the standard of Hastings and Weber (8). One light unit equals 2×10^{10} quanta/sec.

In vitro enzyme assays were initiated by injecting reduced flavine mononucleotide ($FMNH_2$), 1 ml of 5×10^{-5} M, into the vial containing the other components. The assay mixture for in vitro luciferase determination consisted of 0.01 to 0.1 ml of the enzyme in 0.8 ml of 0.25 M phosphate buffer (*p*H 7.6) and 0.1 ml of a 10^{-2} dilution of emulsified aldehyde. The maximal initial intensity of light emission (I_0) was proportional to the amount of luciferase present.

The measurement of in vivo light was accomplished by simply placing a 1-ml sample of cells in a standard scintillation vial and recording the intensity of light emission. With dense cultures, oxygen quickly became a limiting factor; thus, the sample was aerated by shaking, and light emission was recorded promptly.

For enzyme assays of small samples, a sample of the culture was harvested either on a membrane filter (Millipore Corp.) or by centrifugation at $15,000 \times g$ for 10 min. Cell lysis was accomplished by suspending the cells in 1 ml of distilled water containing 0.01 M ethylenediaminetetracetic acid (EDTA) and 0.001 M dithiothreitol (DTT) with the *p*H adjusted to 7.0 (lysis buffer).

Enzyme complementation was done in crude extracts. Luciferase was denatured by dilution into guanidine hydrochloride (one volume of crude extract plus four volumes of 6.5 M guanidine hydrochloride). Samples (1 ml) of these denatured extracts were then placed in dialysis bags and renatured by dialysis versus 20 volumes of 0.25 M phosphate buffer (*p*H 7.0) containing 10^{-3} M DTT at 4 C with three changes of buffer. By using crude extracts of luciferase from wild-type cells, approximately 50% of the original activity was recovered after 48 hr. Each dialysis bag contained either 1 ml of a mutant extract (control) or a mixture of 0.5 ml of each of two different mutant extracts. Controls and mixtures were renatured for 48 hr or longer, the dialysis sack was opened, and the luciferase was assayed. From the activity in the controls, one can predict the mean activity for the mixture of the two. Results are then recorded as fold stimulation above that predicted from measurements of the controls.

Luciferase was purified by a modification of the method of Hastings et al. (7) devised by R. L. Heinrikson (*personal communication*). Cells were grown to maximal luminescence with aeration by a mixture of equal volumes of O_2 and air, harvested by centrifugation in a Sharples continuous-flow centrifuge, and quick-frozen in an acetone-dry ice bath. Cells were thawed, and 6 ml of lysis buffer was added per gram of wet-packed cells. This mixture was allowed to sit for about 1 hr at 4 C with occasional dispersal with a glass stirring rod and was then centrifuged at $20,000 \times g$ for 1 hr to remove debris; the supernatant was saved. Three grams of dry diethylaminoethyl (DEAE) cellulose (Whatman DE-32) was added per gram of total protein, as determined by a biuret assay (18). The DEAE cellulose was added slowly with stirring, while 0.5 N acetic acid was used to keep the *p*H from exceeding 8.5 at any time and to adjust the final *p*H to 7.0. The suspension was allowed to equilibrate with slow stirring for 15 to 30 min. A sample was filtered to verify that all of the luciferase activity was absorbed onto the gel. The gel was washed twice with 0.15 M phosphate buffer (*p*H 7.0), and luciferase was then eluted with 0.35 M phosphate buffer (*p*H 7.0). The resulting eluate was precipitated by the addition of solid ammonium sulfate; the fraction which precipitated between 40 and 75% saturation was resuspended in 0.25 M phosphate buffer (*p*H 7.0) containing 10^{-3} M EDTA and 10^{-3} M DTT and dialyzed overnight versus the same buffer.

RESULTS

Dark and dim mutants. All mutagens tested (acridines, ICR-191, and NTG) were effective both in killing and in the production of dim and dark mutants. When growth and light emission were measured in these mutants, two important facts emerged. First, the light emission per cell was not the same at all times during growth in a shake flask. The synthesis and expression of the luminescent system occurred only after a few hours of delay and then proceeded at a rate faster than growth (Fig. 1). All mutants, irrespective of their origin or the specific nature of the lesion, exhibited this same pattern (Fig. 1). Measurements of the luminescence in the wild type and in different mutants must therefore be made at

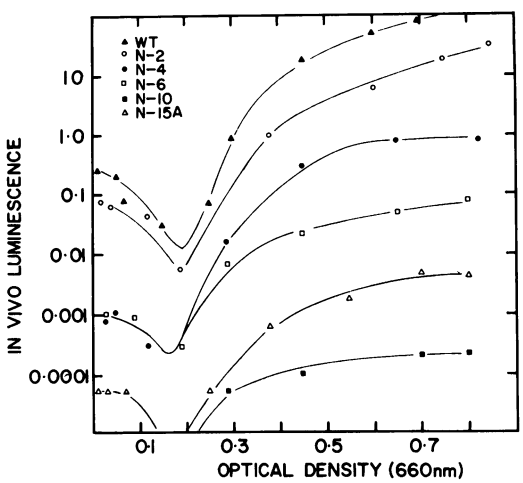

FIG. 1. *In vivo luminescence as a function of cell density. Wild-type and mutant strains were grown in shake flasks at 25 C in complex medium. Optical density was measured in side arms of the flasks in a Coleman Jr. spectrophotometer. See Materials and Methods for the correspondence between OD at 660 nm and viable count. Luminescence is expressed as light units per ml of culture (one light unit equals 2×10^{10} quanta/sec) measured on a 1-ml sample.*

comparable times in the growth cycle. Second, lesions in the luminescent system resulted in a wide array of mutants from the quantitative point of view (Fig. 1 and Table 1). However, none were found in which luminescence could not be detected at all. The sensitivity of the detection method is, admittedly, unusually good; activity levels between 10^{-6} and 10^{-7} of that of the wild type could be easily measured. Mutants were arbitrarily designated as dark when in vivo light emission was less than 1% of that of the wild type and dim when in vivo light emission was more than 1% of wild type.

Acridine. Although the cells were quite resistant to killing by acridines when grown in the normal medium, they became sensitive to killing at a lower salt concentration (1%). Sensitivity to ICR-191, actinomycin D, and puromycin was also achieved by using 1% NaCl; presumably 1% NaCl allows the compounds to penetrate more readily.

Upon inoculation of the cells into the low salt medium containing the dye, a long lag (12 to 16 hr) occurred before the OD began to increase. During this lag, a great deal of cell death occurred, the viable count falling by a factor of about 10^3. The lag was followed by a growth phase which accounted for the subsequent rise in OD. The cells that then grew were resistant to the specific dye used, but not to the other acridines.

We did not detect induction of auxotrophs by acridine dyes upon replica plating to minimal medium. Approximately 80,000 colonies were tested. However, a rather bizarre result was obtained when cultures were examined for dark mutants. No dark mutants were found in 30 of 35 separate tubes in which the survivors were allowed to grow up after treatment with acridine. Several thousand colonies from each tube were examined. However, dark mutants were present in rather high proportions (0.5 to 1.0%) in the remaining five tubes. These mutants were called acridine darks, or ACR mutants.

All ACR mutants isolated (irrespective of the dye used in their production) exhibited a characteristic altered colonial morphology, appearing much more transparent than the creamy wild-type colonies. No major structural differences were

TABLE 1. *Mutant types and levels of luminescence of mutants obtained after treatment with various mutagens*

Mutagen	Designation[a]	In vivo activity[b]	In vivo activity with aldehyde[b]	Extractable luciferase[b]
Control	MAV	4×10^{12}	4×10^{12}	4×10^{12}
Acridine dyes	ACR-2	4×10^{6}	4×10^{7}	1.8×10^{8}
	ACR-4	2×10^{6}	1×10^{7}	2×10^{8}
	ACR-6	2×10^{6}	1.5×10^{7}	2×10^{8}
	ACR-8	8×10^{6}	6×10^{7}	2.4×10^{8}
ICR-191	ICR-1	1×10^{11}	1.5×10^{11}	2.5×10^{11}
	ICR-1A	1×10^{9}	2×10^{12}	2×10^{12}
	ICR-2A	5×10^{8}	5×10^{11}	2.2×10^{11}
NTG	N-10	2×10^{6}	6×10^{6}	4×10^{7}
	N-2	5×10^{11}	5×10^{11}	7.2×10^{11}
	N-4	1.7×10^{10}	1.7×10^{10}	1.5×10^{10}
	N-6	1.6×10^{9}	1.6×10^{9}	1.5×10^{9}
	N-18	1.2×10^{11}	1.3×10^{11}	1.8×10^{11}
	N-20	4×10^{6}	4×10^{6}	6×10^{6}
	N-21	1.6×10^{9}	1.6×10^{9}	1×10^{9}
	N-14A	2×10^{5}	5×10^{11}	7.2×10^{11}
	N-15A	1×10^{8}	2×10^{9}	3×10^{9}
	N-16A	8×10^{8}	4×10^{11}	7×10^{11}
	N-17A	2×10^{9}	2×10^{10}	3×10^{10}
	N-18A	3×10^{7}	5×10^{11}	1×10^{12}
	N-22A	6×10^{8}	1.8×10^{12}	2×10^{12}

[a] The designation of mutants is first by the mutagen which was used in their production. ACR, acridine dyes; ICR, ICR-191; N, NTG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine). The letter A after the mutant number indicates that it is an aldehyde mutant.

[b] All activities are expressed in quanta per second per milliliter of cells. In vivo light was measured, and all strains were harvested at a cell density of 10^9 bacteria per ml. Luciferase was extracted and assayed as described in the text.

noted between the two types of cells, using either phase or electron microscopy. Although they were quite stable on plates, the ACR dark mutants spontaneously reverted to bright colonies in stationary-phase liquid cultures. In all cases, the restoration of bioluminescence was accompanied by the simultaneous return to the characteristic wild-type morphology, but the resistance to the dye was retained in revertants. Both ultraviolet (UV) light and NTG (Table 2) stimulated reversion, and, as in the case of the spontaneous revertants, both characteristics reverted together. Acridine dyes failed to stimulate reversion.

The exact nature of the lesion responsible for the properties of the acridine mutants has not been identified. A striking feature was that the level of the very dim in vivo luminescence was similar in all ACR darks (about 3×10^{-6} of the wild-type intensity). Similarly, the extractable luciferase activities were the same in different ACR mutants (3×10^{-4} of wild type, Table 1). The revertants were indistinguishable from one another and from the wild type insofar as luminescence and morphology were concerned. Since the ACR mutants reverted to bright when treated with either UV or NTG, it seems unlikely that the original lesions were deletions. The ready reversion of the cells also indicated that they were probably not double mutants and, in addition, that they did not arise simply by the removal or "curing" of an episome. Finally, the fact that a morphological character and bioluminescence mutated and reverted together suggests that a coordinate control may exist over these properties.

TABLE 2. *Nitrosoguanidine induced reversion of dark mutants to brights*

Mutant designation	Strain	Per cent reversion	Approximate no. of colonies examined
Noncomplementing mutants	N-1	4.7	5,000
	N-5	0.5	5,000
	N-12	3.1	5,000
	ACR-2	0.67	5,000
	ACR-4	0.2	5,000
	ACR-6	1.1	5,000
Complementing mutants			
LUC-A$^-$B$^+$	N-4	<0.01	10,000
	N-6	<0.01	10,000
	N-21	<0.005	20,000
LUC-A$^+$B$^-$	N-8	<0.01	10,000
	N-10	<0.005	20,000
Aldehyde mutants	N-2A	0.15	10,000
	N-4A	<0.01	10,000
	N-5A	<0.01	10,000
	N-10A	<0.01	10,000

ICR-191. When ICR-191 was used as a mutagen, killing occurred, followed by the growth of survivors. The survivors were not resistant to ICR-191; thus, it must be presumed that the original mutagen was either detoxified or inactivated.

Mutations to all levels of dimness occurred when cells were mutagenized with ICR-191. All the resulting dark mutants appeared to be of one class—aldehyde mutants. All twenty "dark" clones tested were greatly stimulated by exposure to decanal on plates. However, 20 intermediate intensity "dim" colonies, did not respond to aldehyde; it was presumed that the dims were altered with regard to their ability to produce luciferase or some other factor required for luminescence. They were not studied further.

No explanation is available at this time to account for the differential effect of ICR-191 in mutant production, but it provides a convenient method of obtaining aldehyde mutants.

NTG. A variety of mutant types were isolated after treatment with NTG both with regard to the level of luminescence and the nature of the lesion. The types included those deficient in luciferase activity and those in which in vivo luminescence was greatly stimulated by the addition of aldehyde (Table 1).

Subunit complementation. The luciferase deficient mutants could be divided into two groups, complementing and noncomplementing. Noncomplementing mutants, probably a heterogeneous category, were isolated by using any of the three types of mutagen (Table 3).

If a genetic lesion results in an altered luciferase, and if the luciferase subunits can be affected individually, two types of complementing mutants would be expected, corresponding to a defect in one or the other of the two subunits of luciferase. This might be demonstrable by mixing the crude extracts of the two in the presence of 5 M guanidine hydrochloride, followed by dialysis against buffer to remove the guanidine (9). The enzyme should renature, and some wild-type luciferase should be formed in the cases in which the members of the pair possess defects in different subunits. The activity recovered upon mixing two mutant extracts was compared to the activity recovered in the controls when the extracts were renatured individually (Table 4). Experiments with nonaldehyde NTG mutants revealed two complementing groups designated A and B. By using extracts from one member of each of the two complementing groups (N-10 and N-21) and from one noncomplementing mutant, ex-

TABLE 3. *Enzyme subunit complementation with mutant extracts of photobacterium fischeri strain MAV*

Group to which complementation was obtained[a]	Mutants strains tested	Mutant designation
LUC-A^+B^- [b]	N-3, N-4, N-6, N-16, N-17, N-21	LUC-A^-B^+ mutants
LUC-A^-B^+ [c]	N-8, N-10, N-13, N-14, N-19, N-2A, ICR-44A	LUC-A^+B^- mutants
Both LUC-A^-B^+ [c] and LUC-A^+B^- [b]	N-4A, N-5A, N-10A, N-12A, N-13A, N-19A, N-20A, ICR-1A, ICR-3A, ICR-11A, ICR-20A	Aldehyde mutants
Neither LUC-A^-B^+ [c] nor LUC-A^+B^- [b]	N-1, N-5, N-11, N-12, N-18, N-20, ICR-4, ICR-5, ICR-36, ICR-37, ACR-2, ACR-4, ACR-6, ACR-8	Noncomplementing mutants

[a] The group designations are based on complementation results obtained with purified separated wild-type subunits (Table 6).
[b] Extracts of strains N-8 and N-10 were used as representatives of the group.
[c] Extracts of strains N-6 and N-21 were used as representatives of the group.

TABLE 4. *Complementation in crude extracts*

Controls		Complementation with mutant N-10			Complementation with mutant N-21		
Strain	LU/ml[a]	Predicted LU/ml[b]	Actual LU/ml	Complementation LU/ml[c]	Predicted LU/ml	Actual LU/ml	Complementation LU/ml
Wild type	4.5	2.3	2.5		2.3	2.7	
N-4	0.023	0.012	0.12	10	0.015	0.01	
N-6	0.002	0.0018	0.17	100	0.0035	0.0042	
N-10	0.0015	0.00075	0.0009		0.0032	0.19	50
N-21	0.005	0.0032	0.16	50	0.0025	0.003	
N-2A	1.75	0.88	0.95		0.88	2.1	2.5
N-10A	1.3	0.65	1.4	2.5	0.65	1.9	3

[a] Extracts of each strain were denatured by dilution into guanidine hydrochloride to a final concentration of 5.0 M. Renaturation by dialysis was done for 48 hr. Control values are those obtained when 1 ml of each strain was renatured separately. LU, light units.
[b] Predicted values for the mixtures were calculated from the controls. Predicted = control x + control $y/2$.
[c] Complementation is the ratio of actual to predicted values.

tracts from 37 other mutants were tested in pairwise combination. The results (Table 3) show that many of the mutants tested fit into one of the three groups. However, many were found to fall into an unexpected and as yet unexplained fourth category, one in which the mutant extracts complemented with both classes. These were designated as group AB, and, although we do not know the nature of the lesion which leads to complementation with both group A and group B mutant extracts, it is striking that all are aldehyde mutants. In addition, the quantitative level of double complementation was uniformly low. In none of the experiments was a stimulation greater than threefold observed.

The positive complementation between groups A and B observed in crude extracts was shown to be attributable to the recombination of different subunits as follows. First, the luciferases from two of the complementing strains (N-10, N-21) were purified (Table 5) and again tested for complementation (Table 6). The results were similar to those obtained with crude extracts;

TABLE 5. *Purification of luciferase*

Fraction	Wild type	Mutant N-10	Mutant N-21
Crude lysate (LU[a]/mg)	47.0	10^{-4}	0.067
Purified luciferase (LU/mg)	1,800	4.5×10^{-3}	1.15
Fold purification	40	45	20
Per cent yield	10	0.1	0.25
Total protein in final purified fraction (mg)	280	3	2.4

[a] Light units.

TABLE 6. *Luciferase subunit complementation with purified luciferases*

Renatured components	Relative light units	Complementation
N-10 (purified)	1.0	
N-21 (purified)	1.0	
N-10 + N-21	160	160
Subunit B (from wild type)	4.0	
Subunit A (from wild type)	1.0	
Subunit A + subunit B	100	40
N-10 + subunit B	1.0	
N-10 + subunit A	20	.20
N-21 + subunit B	900	360
N-21 + subunit A	6	6

complementation was observed. Second, the wild type luciferase was purified (Table 5), its subunits were separated, and the individual subunits were used to measure complementation with the purified mutant luciferases. The results were also positive; one of the wild-type subunits exhibited positive complementation with one and only one of the mutant luciferases, whereas the second wild-type subunit was positive only with the other (Table 6).

Reversion studies. Results of attempts to induce reversion with NTG in the various dark and dim mutants are presented in Table 2. A rather unexpected correlation found was that the dark mutants which exhibited positive results in the complementation tests showed no reversion, whereas those which did not complement, including the acridine mutants, reverted well. Among the aldehyde mutants tested, strain N-2A complemented with only one class and reverted. The other aldehyde mutants, which complemented with both classes, did not revert.

Luciferase of the aldehyde mutants. As previously mentioned and shown in Table 1, the aldehyde mutants exhibited a wide range of levels of extractable luciferase activity. Although some possessed levels of activity near that of the wild type, many had much less. If the aldehyde mutants were deficient in biosynthesis of an aldehyde, we might have expected the synthesis of wild-type levels of luciferase. Therefore, we considered the possibility that the aldehyde mutants contained an altered luciferase. The effect of decanal concentration on the in vitro activity for extracts of the wild type and two aldehyde mutants is shown in Fig. 2. The K_m for the luciferase from the wild type was 4×10^{-6} M, whereas the K_m for the luciferase from the two aldehyde mutants of Fig. 2 was 2×10^{-5} M. Extracts of three other aldehyde mutants were tested and found to differ from the wild type in a similar manner. Other dark and dim mutants which did not increase in luminescence on addition of aldehyde to whole cells had the same K_m for aldehyde as the wild type.

A second property of aldehyde mutant extracts which differed from the wild type was that of the rate of decay of light emission of the reduced enzyme intermediate in the absence of added aldehyde. It has been shown that this decay rate does not reflect the utilization of substrate but the lifetime of the enzyme intermediate and is thus truly a property of the enzyme (6). The rate of decay in the absence of added aldehyde differs for various aldehyde mutant luciferases, and these luciferases have decay rates markedly different from that of the wild-type luciferase (Fig. 3). When equal quantities of the extracts from aldehyde mutant N-5A and the wild type were mixed and analyzed in this way, an additive decay curve intermediate between the individual decay rates was observed (*not shown*), indicating that neither a dissociable factor nor subunit interactions were responsible for the differences between the aldehyde and wild-type extracts. The reduced luciferase from two other aldehyde mutants (not shown in Fig. 3) also decayed slower than the wild type reduced luciferase. Luciferase from one (nonaldehyde) dim mutant decayed faster than the wild-type enzyme (Fig. 3); the reduced luciferase from all other nonaldehyde mutants tested decayed at the same rate as the wild-type reduced luciferase.

Physiology of the aldehyde mutants. The induction of luminescence during the bacterial growth cycle in complex medium must be taken into consideration in connection with mutant analysis. As shown in Fig. 1 and 4, the wild type and mutants were similar in the kinetics of in vivo light

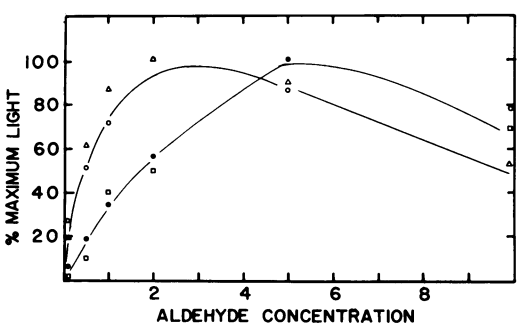

FIG. 2. *Effect of aldehyde concentration on the initial intensity of in vitro luminescence. Molar concentration of decanal used in the reaction is the abscissa value times 10^{-5} M. The reaction mixtures contained 100 μg of protein from a crude extract of the indicated strain. Symbols:* ○, *wild type;* △, *N-10;* ●, *N-5A and* □, *N-10A.*

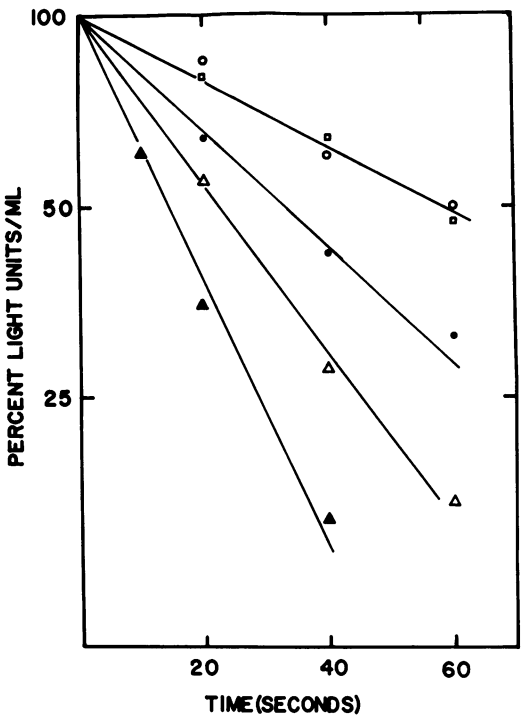

FIG. 3. *Decay rates of the $FMNH_2$ initiated luminescence in the absence of added aldehyde. Crude extracts of several strains were examined for this property. All of the aldehyde mutants examined (○, N-2A; □, N-5A; ●, N-17A) showed decay rates slower than the wild type (△). All nonaldehyde mutants examined showed identical kinetics with the wild type with the exception of ICR-1 (▲) which decayed at a faster rate.*

production and the appearance of luciferase activity. However, the mutants varied widely both in levels of in vivo light and extractable luciferase synthesized in complex medium (Table 1). The pattern was characterized by a period of constant extractable luciferase (a lag phase) followed by a burst of synthesis which occurred at a rate faster than growth. In vivo luminescence was an accurate indicator of the extractable luciferase levels at all times, except during the lag phase when the in vivo activity decreased whereas the luciferase level remained constant (Fig. 4). When an aldehyde mutant was examined in a similar experiment, the pattern of induction of luciferase synthesis persisted, and in vivo light production was low; by adding aldehyde, a stimulation to approximately the in vitro levels of activity occurred. This is in marked contrast to the wild-type cells which did not respond to similar additions of decanal (Fig. 4). The addition of decanal to mutant cells resulted only in a flash of light that decayed exponentially with a half-life of 7 to 10 sec (Fig. 5). The fact that the disappearance of the luminescence was not due to the destruction or utilization of the decanal was shown by the fact that a second addition of decanal did not stimulate the cells and by the fact that fresh aldehyde mutant cells were fully stimulated when added to the cell suspension, the luminescence of which had decayed (Fig. 5).

Luminescence in a minimal medium. An interesting feature of the wild-type cells, which appears related to the results presented above, is that in a minimal medium they synthesized low levels of luciferase yet emitted very little or no light. Like the aldehyde mutants, these cells were stimulated to emit light by the addition of an aldehyde such as decanal (Fig. 6). Similarly, the response to aldehyde was transient; the flash of light decayed exponentially with a half-time of 7 to 10 sec. However, this decline was not due to the disappearance of the added aldehyde. Several minutes were required before the cells could again respond maximally to a second addition of aldehyde (Fig. 6). We have determined that this is the time required for the actual disappearance of the added aldehyde (Fig. 6). Thus, it appeared that in all these respects wild-type cells growing in a minimal medium were similar to aldehyde mutants growing in complex medium; luciferase was synthesized and was in a state ready to emit light but did not do so. The addition of decanal to such cells resulted in a flash of light, but not in a continuous maximal emission,

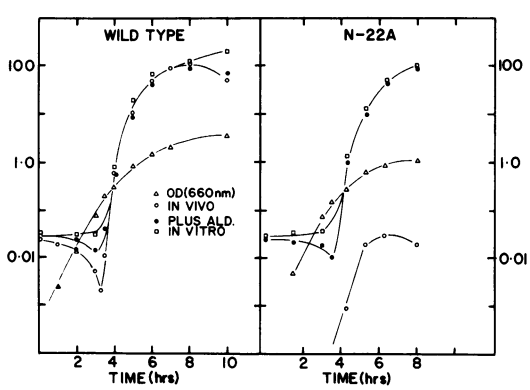

FIG. 4. *Growth and luminescence as a function of time in wild-type and aldehyde mutant N-22A grown in complex medium. Measurements were as described in Fig. 1. △, OD_{660}. No conversion factor is needed for optical density from the units of the ordinate; ○, in vivo luminescence. Units of luminescence are the ordinate number times 2 × 10^{10} quanta/sec; ●, in vivo response to the addition of 1 ml of a 10^{-2} dilution of emulsified aldehyde added to 1 ml of whole cells; □, in vitro luciferase levels, as determined by the standard assay.*

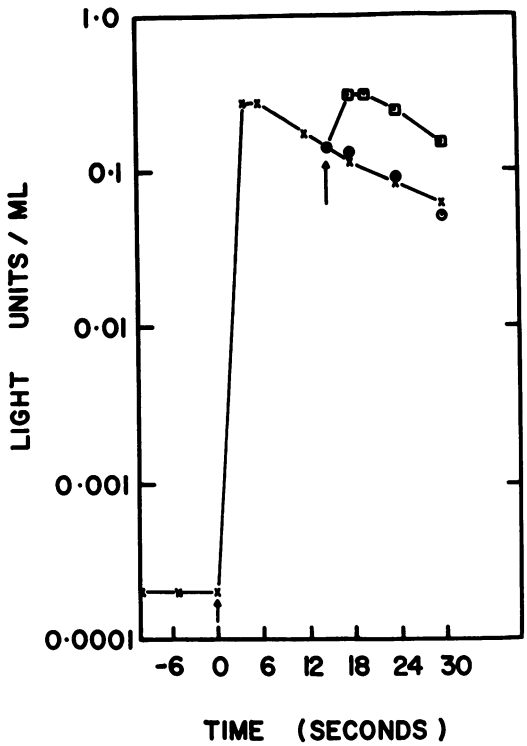

FIG. 5. *In vivo response of whole cells of aldehyde mutant N-5A growing in a complex medium to the addition of decanal. One milliliter of emulsified aldehyde, prepared as indicated in Fig. 4, was added to one ml of a cell suspension of aldehyde mutant N-5A (2 × 10⁸ bacteria per/ml) at zero time (×). A second addition of aldehyde at 15 sec (⊙) had no stimulatory effect. This lack of response to a second aldehyde addition continued for several minutes (not shown). The presence of aldehyde in the cell suspension in which the light emission had declined was demonstrated by the addition of a 1-ml sample of N-5A cells at 15 sec (⊡).*

indicating that the enzyme must be somehow otherwise limited.

Since wild-type cells in minimal and complete media differed not only with respect to the absolute levels of luciferase but also with respect to the efficiency of light emission of the enzyme that was made, one could ask whether it was possible to stimulate the cells growing in minimal medium to produce the component(s) which allowed the expression of the luciferase. Coffey (1) reported that arginine was a specific inducer of luciferase for cells growing in a minimal medium. When we tested various compounds, we also found arginine to have a specific stimulatory effect (Fig. 7), resulting in a 10- to 50-fold increase in specific activity of luciferase compared to cells grown without arginine. The generation time of 150 min was not measurably affected by the addition of arginine in these experiments. Furthermore, after the addition of arginine, there was not only an effect on the synthesis of luciferase but also upon the synthesis of the "cellular aldehyde factor," i.e., nearly all of the luciferase present was expressed, as determined by comparison with the in vitro assay (Fig. 7). Addition of aldehyde to whole cells induced with arginine had no stimulatory effect; this result supports the conclusion that all of the luciferase was being expressed in cells induced with arginine.

Luminescence in such arginine-stimulated (wild-type) cells in minimal medium passed rapidly through a maximum and decline. When aldehyde was added to such cells at a time when the in vivo light had declined after arginine stimulation, a response was again obtained (Fig. 8). We can thus attribute the fall of the in vivo luminescence to the loss of the cellular aldehyde factor, the synthesis or reactivation of which can be stimulated again by the addition of more arginine, with no appreciable effect on the luciferase levels as determined by in vitro assays (Fig. 8). Both this "secondary" arginine stimulation and the initial one were sensitive to inhibitors of both protein (chloramphenicol, puromycin, kanamycin) or messenger ribonucleic acid (mRNA) synthesis (actinomycin D, rifampin). Although these inhibitors blocked the stimulation of the in vivo luminescence by arginine, they did not inhibit the transient stimulation of light in such cells by the addition of decanal (K. H.

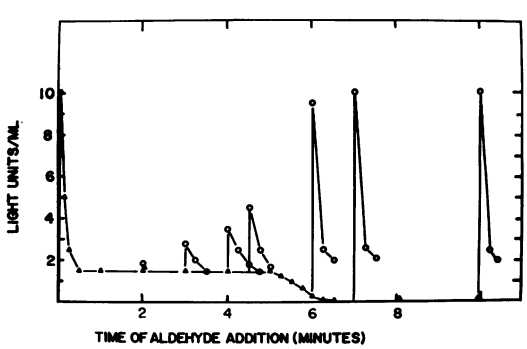

FIG. 6. *Effect of addition of decanaldehyde to whole cells growing in minimal medium. At time zero, 1 ml of aldehyde prepared as described in Fig. 4 was added to a 1-ml sample of wild-type cells growing in minimal medium (△). The response of individual samples to further additions of aldehyde at 2, 3, 4, 4.5, 6, 7, and 10 min (○) increased exponentially with time, reaching a maximum at 7 min. This increase can be correlated with the disappearance of aldehyde from the cell suspension as determined by the addition of fresh cells to the suspension (not shown).*

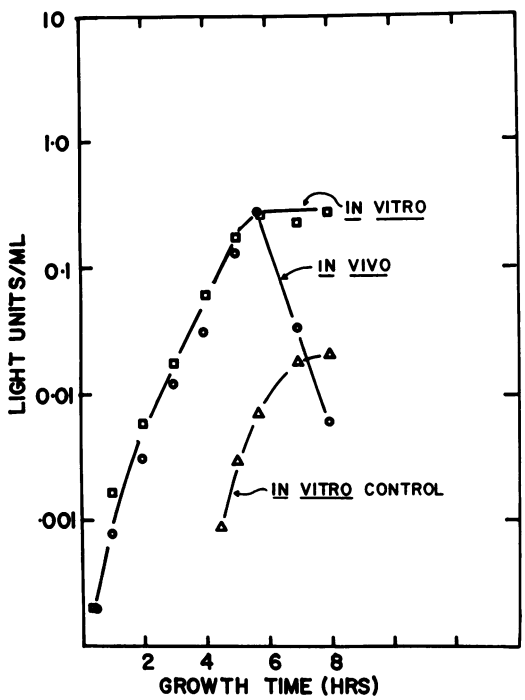

FIG. 7. *Response of wild type P. fischeri growing in a minimal medium to the addition of arginine. The viable bacteria per ml were 4 × 10⁸ and 10⁹ at 0 and 5 hr, respectively, in the presence or absence of added arginine. In vitro values reported represent the extractable luciferase obtained from 1 ml of the culture at the indicated times. Symbols: ○, arginine (100 μg/ml), in vivo light; □, arginine (100 μg/ml), in vitro luciferase; △, no arginine, in vitro luciferase. (There was no significant in vivo light in the absence of arginine).*

Nealson, 1969, Ph.D. Thesis, University of Chicago).

When an aldehyde mutant was grown in a minimal medium, its growth rate, extractable luciferase level, and in vivo luminescence were the same as the wild type. The addition of arginine to these cells stimulated luciferase synthesis but, as expected, did not stimulate light emission (Fig. 9). As in a complete medium, the addition of long-chain aldehyde (decanal or dodecanal) resulted in a typical flash of light.

Thus, studies with both the aldehyde mutants and with wild-type cells grown in a minimal medium indicate that a cellular aldehyde factor is essential for the intracellular activity of luciferase.

DISCUSSION

The isolation and characterization of mutants of *P. fischeri* strain MAV provided several results that were unexpected in terms of present genetic knowledge. First, acridine orange, which was expected to be mutagenic in the bacteria only through the curing of an extrachromosomal haploid gene or genes (10) was effective in the production of dark mutants. Similarly, proflavine and acriflavine treatment yielded dark mutants. Yet, these mutants readily revert to wild type; if a presumed extrachromosomal element that contained the only structural gene for luciferase was eliminated, this would not have been possible. More recently, Sesnowitz-Horn and Adelberg demonstrated that proflavine does induce mutations in *Escherichia coli* but only in diploid regions of the chromosome (15). Therefore, it is possible that *P. fischeri* strain MAV is diploid for the luciferase gene region, however, further studies will be necessary to critically test this possibility. It is worth noting that the ACR mutants were similar in their luciferase levels, colonial morphology, and pattern of reversion to the spontaneous dark mutants reported by Keynan and Hastings (11).

A second interesting fact was that no completely dark mutants were isolated. As noted before, the assay system was very sensitive, capable of detecting an activity as low as 5×10^{-7} of the wild-type level of luciferase; levels that would be recorded as zero in most other

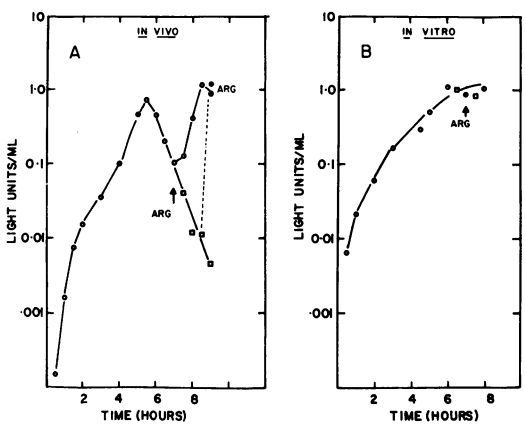

FIG. 8. *Effect of a second arginine addition to wild-type cells growing in minimal medium in vivo (A) and in vitro (B). Cells were induced at time zero with 300 μg of arginine per ml. The viable bacteria per ml were 4 × 10⁸ and 1.7 × 10⁹ at 0 and 7 hr, respectively. In vitro values represent the luciferase obtained from 1 ml of the culture at the indicated times. Symbols: ○, arginine (300 μg/ml) at time zero and 300 μg/ml at 7 hr; □, arginine + 50 μg of rifampin per ml, or arginine + 50 μg of chloramphenicol per ml, or arginine + 50 μg of kanamycin per ml, or no arginine at 7 hr; ●, aldehyde added to the second mixture (□) at 8 hr.*

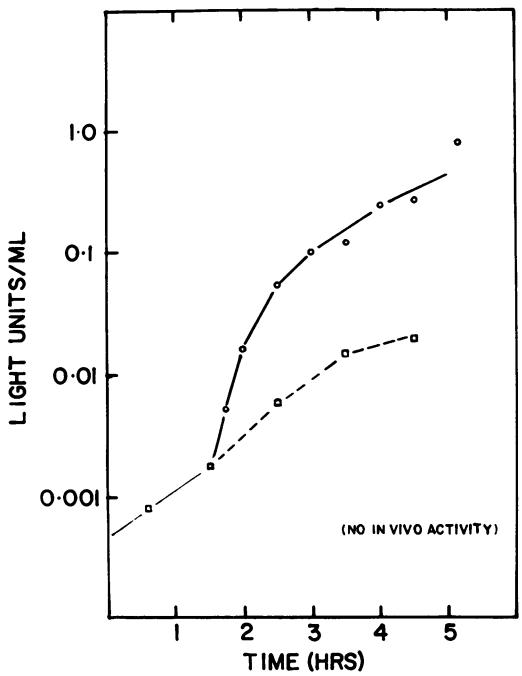

FIG. 9. *Effect of arginine on an aldehyde mutant growing in minimal medium. No in vivo light was detected in cells with or without arginine. The viable bacteria per ml were 5 × 10⁸ and 2 × 10⁹ at 0 and 5 hr, respectively. In vitro values represent the luciferase obtained from 1 ml of the culture at the indicated times. Symbols: □, in vitro activity with no arginine added; ○, in vitro activity with 300 μg of arginine per ml added at 1 hr.*

assay systems. Regulatory gene mutants would be expected to produce some luciferase. However, some mutations in the structural gene, especially deletions or frame-shift mutations that occurred at the beginning of the gene, should have been completely dark unless one assumed that a few revertants to wild type occurred during growth. A sufficiently severe lesion in luciferase would be expected to revert at an extremely low frequency and thereby be dark. It is not understood why completely dark mutants were not found.

The work of Friedland and Hastings demonstrated that bacterial luciferase consists of two nonidentical subunits which can be separated by DEAE-cellulose chromatography in 8 M urea and reassociated to yield full activity (4, 5). More recently, the same group obtained similar results with luciferase from the strain used in this study, strain MAV (9). The results of Friedland and Hastings (4, 5) and those of Schlesinger et al. (14) on alkaline phosphatase complementation in crude extracts led us to attempt the work on subunit complementation. Crude extracts from different mutants were denatured in 5 M guanidine hydrochloride, mixed, and renatured by dialyzing away the guanidine hydrochloride. Complementation was defined as the appearance of more activity in the extract from the mixture of two mutants than would be predicted by the separate renaturation of each of the extracts from the two mutants. The increased activity was presumably due to the association of a nondefective subunit from each of the defective mutants (intercistronic complementation). In fact, it also might be argued that lesions in both of the luciferase subunits were required to produce the dark mutants and that in the complementation experiments authentic wild-type luciferase was not formed but simply improved. For example, class A mutants, although having both subunits altered, would have a qualitatively more effective lesion in the A subunit than in the B subunit. The hypothesis that the complementing mutants that we isolated were defective in both subunits would account nicely for the fact that all the complementing dark mutants failed to revert. However, it is equally interesting to consider the possibility that the stringent structural requirements of the active site of luciferase make reversion an extremely rare event.

When a survey of mutants was taken (Table 3), the results appeared to be straight-forward with two classes of mutants which complemented with each other, but not with members in their own group, and a third class which showed no complementation with either group. This is not to imply that subunit interchange did not take place, only that no stimulation above the predicted level was observed. We designated the complementing classes as LUC-A$^-$B$^+$ (genotype *luc*A$^-$) and LUC-A$^+$B$^-$ (genotype *luc*B$^-$) with reference to the complementation shown with separated wild-type subunits. The LUC-A$^-$B$^+$ class contains those mutants which show activity when renatured with purified A subunits, indicating that they have a defective A subunit, and the LUC-A$^+$B$^-$ mutants, in a similar way have been identified as mutants in the B subunit.

Although the complementation of pure wild-type subunits with each of the purified mutants (Table 6) is qualitatively a verification of which mutants are *luc*A$^-$ and which are *luc*B$^-$, the amount of stimulation observed on mixing the enzyme from strain N-10 with LUC-A$^+$ subunits was only 20-fold, whereas that obtained with the enzyme from strain N-21 and LUC-B$^+$ subunits was 400-fold. Careful experiments on the effect of variation of time and concentration of protein on renaturation are needed before we can say anything further about these apparent discrepancies.

The noncomplementing mutants are as yet undefined and might contain several classes of mutants: i.e., these could be nonluciferase mutants, blocked in luminescence in another part of the in vivo pathway; they could be regulation mutants, producing very low levels of active luciferase; or they could be mutant in both subunits which show no increased activity upon renaturation with either isolated LUC-A^+ or LUC-B^+ subunits.

The aldehyde mutants (those that increase in vivo light in response to added decanal) have shown rather bizarre results in the complementation tests. Positive stimulation in the complementation test occurred upon mixing aldehyde mutant extracts with extracts from strains which were classified as either *luc*A^- or *luc*B^- but with neither the noncomplementing mutant nor with the wild-type extracts. The complementing mutants exhibited a wide range of complementation efficiencies, ranging from 2- to 20-fold, whereas the aldehyde mutants consistently produced low levels of complementation, ranging from 1.5- to 3-fold stimulation above the predicted levels, levels that had to be repeated several times to be considered significant. The aldehyde mutants produced luciferase which required more aldehyde than the wild-type luciferase to give maximal activity (Fig. 2) and were also altered in the rate of decay of the enzyme complex in the absence of added aldehyde (Fig. 3), indicating that the aldehyde mutants had an altered luciferase.

Two questions that bear on the efficiency of conversion of chemical energy to light energy are the following. (i) When and under what conditions is the cellular aldehyde factor produced? (ii) Is the factor a long-chain aliphatic aldehyde? The remainder of this discussion is devoted to these questions. (i) The wild-type strain grown in minimal medium never produced the maximal amount of light that could be produced with the quantity of luciferase that it contained (Fig. 6). However, the wild type fully expressed its luciferase during certain phases of growth either when grown in complex medium (Fig. 4), when arginine was added to minimal medium (Fig. 7), or when aldehyde was added to cells in minimal medium. The response to aldehyde was transitory (Fig. 6). These results indicate that the cellular aldehyde factor was not produced in minimal medium. Furthermore, inhibitors of protein and mRNA synthesis prevented the arginine-induced synthesis or activation of the cellular aldehyde factor. On the other hand, aldehyde mutants were dark unless cells were exposed to aldehyde, even in complex medium. Here also the response to aldehyde was transitory. Their lack of expression of luciferase activity in complex medium (Fig. 5) may be attributed to the fact that they contained luciferase that was so altered (Fig. 2 and 3) that it did not produce high levels of light in the presence of the normal concentration of cellular aldehyde factor. In addition, it is possible that the aldehyde mutants also produced less cellular aldehyde factor due either to an independent mutation or to a polarity effect of the mutation present in the luciferase structural gene. A polarity effect would require that the gene for cellular aldehyde factor be in the same operon as the structural genes for luciferase. (ii) Under all conditions tested in which aldehyde initiates or greatly stimulates light production by whole cells the response is transitory (Fig. 5 and 6). One would certainly expect that the luminescence would remain at its peak level as long as aldehyde were available, yet it does not. It has been suggested that an essential intermediate accumulated in the absence of aldehyde is drained off by some side reaction which utilizes the considerable excess of added aldehyde, i.e., oxidation of cellular NADH. However, if such were the case, then the luminescence of wild-type cells growing in a complex medium should be strongly inhibited by added aldehyde. Although great excesses of aldehyde can indeed inhibit to some (50%) extent, the concentrations normally used to stimulate luminescence caused little (<5%) or no inhibition. Thus, we concluded that the cellular aldehyde factor is not likely to be a long-chain aliphatic aldehyde.

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