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Mutants of Luminous Bacteria with an Altered Control of Luciferase Synthesis

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Arginine is known to increase the luminescence in vivo and in vitro of the marine bacterium *Beneckea harveyi* growing in minimal medium. Mutants in which this arginine effect is either diminished or absent were isolated as bright clones on a minimal medium after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis. On a minimal medium both with and without added arginine and also on complex medium, these "minimal bright" mutants produce higher levels of luminescence than the wild type both in vivo and in vitro. This is attributed to the production of an increased amount of luciferase, which itself is wild type in terms of its specific activity.

Bacterial luciferase catalyzes a mixed-function oxidation of reduced flavine mononucleotide and a long-chain aliphatic aldehyde, resulting in the emission of light and the production of oxidized flavine mononucleotide and the corresponding carboxylic acid (9–11, 21).

Luciferase is an inducible enzyme, but the inducer (referred to as autoinducer) is produced by the cells themselves and accumulates in the medium during growth. Luciferase is then produced in a relatively short burst during mid- to late-exponential growth (6, 17). The synthesis of luciferase is repressed by added glucose, and this repression is reversible by cyclic adenosine 3',5'-monophosphate (cAMP) (17, 22). In cells grown on a minimal medium the luminescence is very dim and may be stimulated by certain amino acids, especially arginine (4, 14, 18). We have isolated and partially characterized mutants, designated minimal bright (MB) mutants, which are bright on minimal medium without added arginine. These appear to be altered with regard to the regulation of luciferase synthesis.

(This work was taken from a thesis presented by C. A. W. in partial fulfillment of the requirements for the Ph.D. degree, Harvard University, December, 1974.)

MATERIALS AND METHODS

The luminous marine bacterium *Beneckea harveyi* strain 392 (previously designated strain MAV [20]) was used in all experiments. Cells mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were plated on complex medium at 25°C (2). Survivors were replica plated onto both complex and minimal media. MB mutants were selected as colonies that were bright on both media; they occurred at a frequency of about 1 in 2×10^4 of the survivors and were stable with subculturing for over 2 years.

Complex medium contains (per liter): NaCl, 30 g; $Na_2HPO_4 \cdot 7H_2O$, 7 g; KH_2PO_4, 1 g; $(NH_4)_2PO_4$, 0.5 g; $MgSO_4$, 0.1 g; glycerol, 3 ml; yeast extract (Difco), 3 g; and tryptone (Difco), 5 g. Minimal medium is of the same composition as complex medium, but it lacks yeast extract and tryptone (Difco). Solid medium contains 12 g of agar (Difco) per liter. Liquid cultures (40 ml) were grown in 300-ml side-arm flasks in temperature-controlled (25.0 ± 0.1°C) water baths with reciprocal shaking at 190 rpm. Inocula for experiments were taken from midexponential-phase cultures grown in the same medium. Dow Antifoam-A was added to reduce foaming in cultures grown in complex medium.

Culture density was measured with a Coleman spectrophotometer equipped with a red filter and is expressed in the optical density (OD) units of that instrument; one unit corresponds to approximately 3×10^9 and 1.3×10^9 cells/ml in minimal and complex media, respectively. For activity in vivo, light intensity was measured with a calibrated photometer-photomultiplier (13, 16) either by transferring 1.0 ml of cells from the growing culture to a scintillation vial, in which case light intensity is given in quanta per second per milliliter, or by reading the light intensity in quanta per second directly from the side arm of the culture flask.

For the determination of activities in vitro, cells were harvested by centrifugation, frozen, and upon thawing lysed osmotically in a solution of 10^{-2} M disodium ethylenediaminetetraacetate and 10^{-4} M dithiothreitol, pH 7.0. Sonic disruption was used if osmotic lysis was incomplete. Luciferase activity was measured at 23°C by mixing 1 ml of reduced flavine mononucleotide (5×10^{-5} M) with cell extract (~10 μl) containing luciferase and decanal (10 μl of 0.1% [vol/vol] aqueous emulsion) in 1.0 ml of assay buffer (0.1 M phosphate [pH 7.0] with 0.2%

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[wt/vol] bovine serum albumin). The activity is given as the initial maximum intensity in units of quanta per second per milliliter of cell suspension per OD unit. The same assay was used with the purified luciferase.

cAMP levels were determined by means of the competitive binding assay (7), using erythrocyte membranes prepared as described by Dodge et al. (5). Extracts of the bacterial cells were made from cultures in late exponential growth (about 10^9 cells/ml) by extraction in either 0.2 N formic acid (19) or cold 5% trichloroacetic acid. Slightly higher values were obtained with the latter method of extraction.

Autoinducer was prepared from the supernatant portion of cultures after growth at 28°C in either complex or minimal liquid medium to a density of about 1.2×10^9 cells/ml. Cells were removed by centrifugation, and the medium was concentrated 10- to 30-fold at 34°C with a rotary evaporator. After centrifugation, the supernatant fraction was cooled to 0°C and used promptly. The autoinducer assay was carried out in a medium conditioned by the growth of *Photobacterium fischeri*, which removes an inhibitor acting on the luminescence systems of both species (6). *P. fischeri* produces a specific autoinducer, which does not cross-react with that of *B. harveyi*. Cells of *B. harveyi* were inoculated at a low density into this conditioned medium with or without added autoinducer and grown with shaking at 28°C.

Luciferase, prepared as previously described (1, 8), was a gift from T. O. Baldwin and Patricia Dobson. The enzyme was estimated by polyacrylamide gel electrophoresis to be at least 95% pure. Antiluciferase antibodies were raised in rabbits by injecting intramuscularly 2 mg of luciferase in complete Freund adjuvant four times over a period of 9 days. After 1 month, a booster injection (11 mg) was administered and the animals were bled 1 week later. The antigen (luciferase)-combining capacity was determined by quantitative precipitation. Antiluciferase antiserum (100 μl) was added (in triplicate) to different amounts of purified luciferase in phosphate-buffered saline (0.14 M NaCl-0.01 M phosphate [pH 7.2]) in a final volume of 0.2 ml. After 2 h of incubation at 22°C and overnight at 4°C, the precipitates were removed by centrifugation, washed twice, and redissolved in 0.6 ml of 0.05 M KOH. Absorbance at 280 nm was measured on a Cary model 15 spectrophotometer. The supernatant fractions were reserved for assays of luciferase activity.

The luciferase content of cell extracts was assayed immunologically, using antiluciferase antiserum diluted 1:10 into phosphate-buffered saline with 0.05% bovine serum albumin. Purified luciferase (1.9 mg/ml) was diluted 1:100 in the above buffer, and different amounts were added (in triplicate) to 8 μl of diluted antiserum in a final volume of 0.2 ml. After incubation at 23°C for 4 h, the remaining luciferase activity was measured. Similarly, assays were performed with different amounts of extracts of both wild-type and MB-20 cells, prepared from cultures that had reached maximum luminescence in vivo (2.8×10^9 and 13.9×10^{11} q/s per ml per OD, respectively). Normal rabbit serum was used in controls.

RESULTS

Stimulation of wild-type luminescence in minimal medium. Table 1 shows that, with *B. harveyi* growing in a minimal medium, added arginine stimulates the luminescence in vivo and possibly in vitro as well. Figure 1 illustrates the time course of this effect in vivo. Since luminescence is expressed per unit of cell mass, any increase in the growth rate does not contribute to the effect. The apparent K_m for stimulation is about 10^{-5} M, in agreement with Coffey (4) and Nealson et al. (18). Certain compounds that are structurally and metabolically linked to arginine are also capable of stimulating or inhibiting luminescence. Citrulline and argininosuccinate, the immediate precursors of arginine in the biosynthetic pathways elaborated for *Escherichia coli*, significantly increase the luminescence both in vivo and in vitro, as also reported by Coffey (4) for *Achromobacter fischeri*.

Characterization of MB mutants. Wild-type colonies growing on solid minimal medium emit a very dim light. After mutagenesis, brightly luminescent colonies were observed and isolated. Data for six of the brightest clones are presented in Table 2. Compared with the wild type, their luminescence levels in vivo are approximately 100 to 200 times greater when the cells are grown on minimal medium, 10 to 20 times greater on minimal medium with

TABLE 1. *Effects of various compounds on the luminescence of wild-type cells in minimal medium*[a]

Compound added	Luminescence	
	In vivo[b]	In vitro[c]
None	3.8	3.9
Arginine	48.0	5.0
Argininosuccinic acid	32.0	9.8
Citrulline	24.0	12.0
Proline	5.8	5.8
Agmatine	4.4	5.0
Argininic acid	4.7	3.9
Aspartic acid	4.6	5.6
Homoarginine	2.1	2.6
Glycine	1.2	2.3
Ornithine	1.1	1.6
Glutamic acid	0.9	2.5

[a] Protocol as described in the legend to Fig. 1. All determinations were made 60 min after the addition of the compound.

[b] In vivo units = quanta per second per milliliter per OD $\times 10^{-9}$.

[c] In vitro units = quanta per second per milliliter per OD $\times 10^{-10}$. The luciferase activity as initial maximum intensity was assayed as described in the text.

added arginine, and 2 times greater with the complex medium. Comparable, although not identical, differences were recorded for the luciferase activity in extracts of cells of the different mutants grown on the different media. In minimal medium as compared with the wild type, the mutants are stimulated less (in vivo) or not at all (in vitro) by added arginine. Also, all of the MB mutants, when grown in complex medium, are brighter than the wild type both in vivo and in vitro.

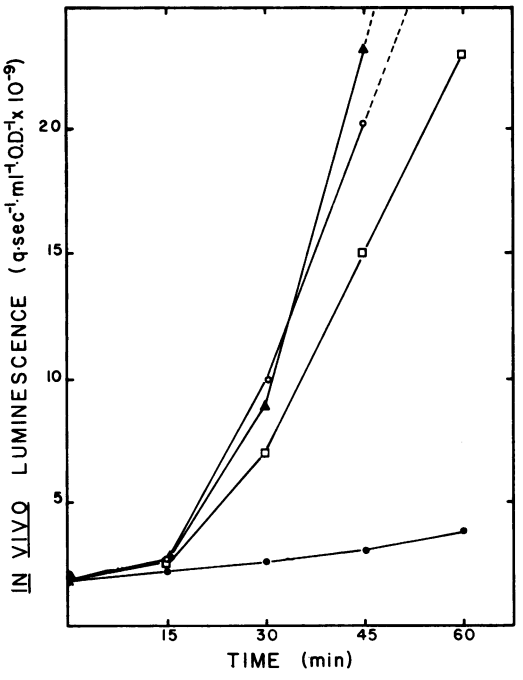

FIG. 1. *Effect of various compounds on the development of bioluminescence in wild-type B. harveyi growing in minimal medium at 25°C. Compounds (final concentrations, 5 × 10^{-3} M) were added to half of the culture at an OD of 0.15, the other half serving as a control. This point corresponds to zero time on the graph. Luminescence per unit of cell mass (OD) (ordinate) is expressed as a function of time after the addition of the compound (abscissa). Symbols: ●, no addition; ▲, arginine; □, citrulline; and ○, argininosuccinate.*

Not all of the clones originally isolated expressed the bright phenotype to the same degree; stable mutants with intermediate luminosity levels on minimal medium were also found. Several were characterized and found to exhibit a more significant stimulation by arginine than did the bright MB mutants. These mutants were not studied further.

Development of luminescence: autoinducer and cAMP. The first three mutants presented in Table 2 were selected for more detailed study. Figure 2 shows the effect of added arginine on the development of luminescence in the wild type and two of the MB mutants growing in the minimal medium. These curves, as well as those shown in Fig. 3, also illustrate the phenomenon of autoinduction (18). Luminescence (and luciferase content) differs at different cell densities. After inoculation in fresh medium to a low cell density, there is an "eclipse" phase, when cell mass increases without a corresponding increase in luminescence. Luminescence, in fact, decreases. Subsequently, luminescence rises more rapidly than cell mass; autoinducer activity can then be found in the medium. When autoinducer is added to another culture in the eclipse phase, luciferase synthesis and the development of luminescence begin without the characteristic delay (6).

The experiments shown in Fig. 3 illustrate with MB-20 the fact that these mutants are similar to the wild type with regard to autoinduction, except with respect to the apparent time at which induction occurs. In all six mutants, similar results were obtained; the eclipse

TABLE 2. *Luminescence of wild type and MB mutants in different media*[a]

Cells	Luminescence					
	In vivo			In vitro		
	Minimal	Minimal + arginine	Complex	Minimal	Minimal + arginine	Complex
Wild type	0.1	1.3	25	0.6	2.5	29
MB-4	12	15	47	14	12	40
MB-20	16	36	68	22	20	73
MB-23	11	21	58	15	11	42
MB-1	15	31	57	11	12	63
MB-3	9	14	47	12	13	36
MB-18	20	29	58	16	16	53

[a] Protocols were as described in the legends to Fig. 2 and 3. Values of luminescence in vivo were obtained at the time of maximum light production. Cells of each culture were then harvested and lysed, and luciferase activities were determined. Units of luminescence = quanta per second per milliliter × 10^{-11}.

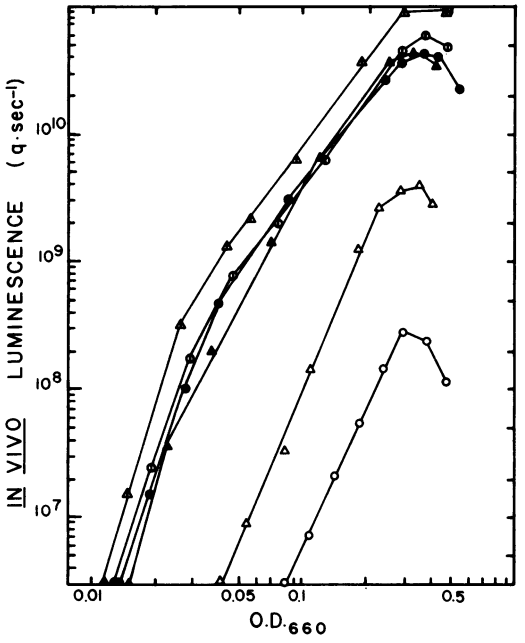

FIG. 2. *Effect of arginine on the development of in vivo luminescence during growth in minimal medium of both wild type and two MB mutants, MB-4 and MB-20. Cells from late-exponential-phase cultures were diluted 200-fold into fresh minimal medium. Arginine (final concentration, 3 × 10⁻³ M) was added at this time. Cell density (abscissa) and in vivo luminescence (ordinate) of the culture were measured with a side-arm flask. Symbols: solid symbols, MB-4; symbols with vertical line, MB-20; open symbols, wild type; circles, no addition; and triangles, arginine.*

periods are shorter in the sense that the onset of measurable luciferase synthesis and the rise of luminescence in vivo occur at somewhat lower cell densities in both minimal and complex media. The curves also illustrate that the mutants are brighter than the wild type in both media, as was shown in Table 2.

Although these observations suggest that the increased luminescence of the mutants could be due to an increased production of autoinducer, this appears to be ruled out. As determined by the bioassay with wild-type responder cells (6), there were no differences in the relative amounts of autoinducer produced by MB-23 and the wild type, regardless of the growth medium (Fig. 4). Moreover, isolated autoinducer added to a wild-type culture in minimal medium fails to stimulate higher maximal levels of luminescence that are characteristic of MB mutants. Thus, the presence of autoinducer alone must not be sufficient for the synthesis of the luminescence system.

The synthesis of luciferase and the luminescence system is subject to repression by glucose and stimulation by cAMP (17). Ulitzur and Yashphe (22) have isolated a mutant requiring added cAMP for bioluminescence. It thus seemed possible that cAMP levels are low in wild-type cells growing in minimal medium, that cAMP production is stimulated by arginine added to that medium, and that the MB mutants are high in cAMP, regardless of the medium. No significant differences, however, exist in the relative levels of cAMP in wild type and MB-20, either with or without arginine (Table 3). In addition, MB mutants 4 and 23 were shown to exhibit typical repression by glucose and its reversal by cAMP, both in complex and minimal media.

Luciferase content of an MB mutant. Whatever the alteration in the control may be, MB mutants have significantly more luciferase activity in cell extracts than does the wild type. This could be due either to luciferase overproduction or to the production of a luciferase with a higher specific activity. Altered degradation

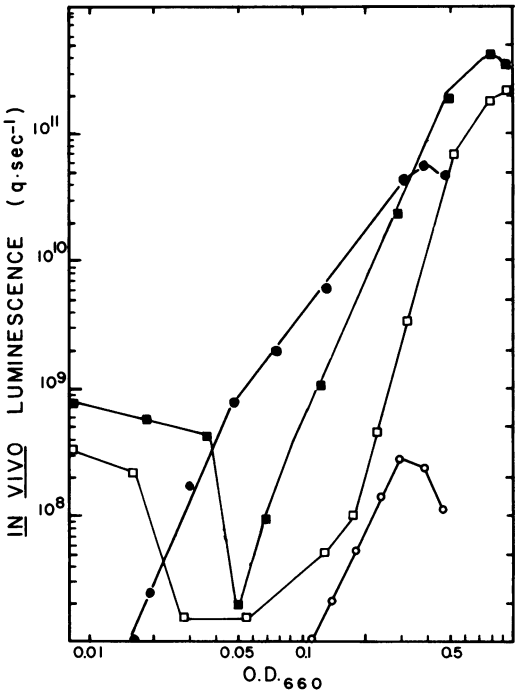

FIG. 3. *Comparison of the in vivo luminescence of the wild type and MB mutant 20 in minimal and complex media. Cells from late-exponential-phase cultures were diluted 200-fold into fresh medium. In vivo luminescence (ordinate) is shown as a function of cell density (OD) of the culture (abscissa). Symbols: solid symbols, MB-20; open symbols, wild type; circles, minimal medium; and squares, complex medium.*

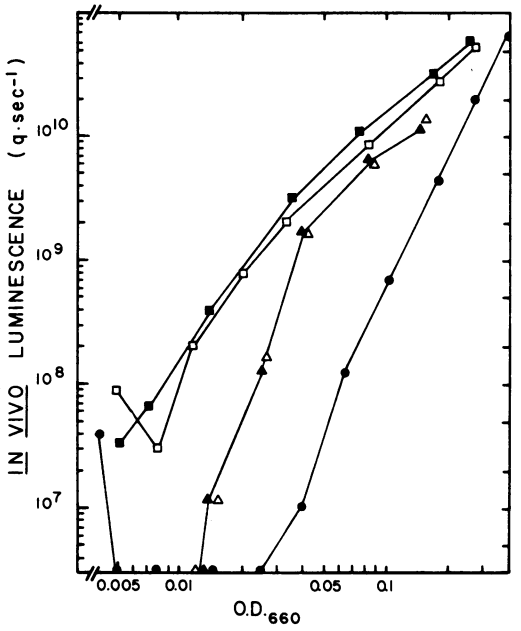

FIG. 4. *Stimulation of in vivo luminescence of wild-type cells by autoinducer isolated from wild-type and MB-23 cultures grown in both complex (squares) and minimal (triangles) media. In vivo luminescence (ordinate) is plotted as a function of cell density (OD) of the culture (abscissa). Symbols: open symbols, MB-23 inducer, 50 μl; closed symbols, wild-type inducer, 50 μl; circles, no addition.*

TABLE 3. *Intracellular levels of cAMP in wild type and an MB mutant*[a]

Cells	Extraction method	cAMP level	
		No arginine	3×10^{-3} M arginine
Wild type	Formic acid	12.5 ± 1.9	11.6 ± 1.9
Wild type	Trichloroacetic acid	17.9 ± 4.6	16.2 ± 4.5
MB-20	Trichloroacetic acid	21.0 ± 3.3	19.6 ± 3.1

[a] cAMP levels in picomoles per 10^9 cells in wild-type *B. harveyi* and mutant (MB-20) cells grown in the absence and presence of 3×10^{-3} M arginine, using two different extraction methods, either formic acid or trichloroacetic acid.

rates could also be involved. The first two alternatives were distinguished by measuring the luciferase content of cell extracts, using an immunological technique.

The amount of purified luciferase precipitated by 100 μl of undiluted antiluciferase antiserum increases with the amount added up to a maximum (~45 μg of luciferase), beyond which less precipitate is formed (Fig. 5a). In the region of luciferase excess, enzymatic activity becomes detectable in the supernatant. This also occurs when the amounts of antiserum and luciferase in the reaction are far lower but in the same relative proportions. An immunological assay of purified luciferase in the nanogram range is illustrated in Fig. 5b. In the region of antibody excess, the luciferase activity after incubation is completely neutralized. In the region of luciferase excess, residual luciferase activity increases with increasing activity added. The slope is approximately unity (0.94), indicating that the antibody present is capable of neutralizing no more than a fixed amount of luciferase and that the excess luciferase is unaffected in terms of its enzymatic activity. The actual amount of luciferase neutralized is determined by extrapolating the linear portion of the curve to the abscissa; from Fig. 5b it can be seen that 0.8 μl of undiluted antiserum would be capable of neutralizing 0.28 μg of luciferase.

Similar assays were performed to determine the relative amounts of luciferase in crude extracts of wild-type and MB-20 cells grown to the point of maximal luminescence per cell in a minimal medium (Fig. 6). The same amount of antiserum (0.8 μl) neutralizes the luciferase in 55 μl of wild-type cell extract and in 31 μl of MB-20 cell extract after a 1:30 dilution. Therefore, the concentration of luciferase in the wild-type cell extract is approximately 5 μg/ml, and in the MB-20 cell extract it is approximately 270 μg/ml, a factor of about 50 higher. This compares with a direct measurement of the relative luciferase activities in the extracts, which gives a value about 40-fold higher for the mutant as

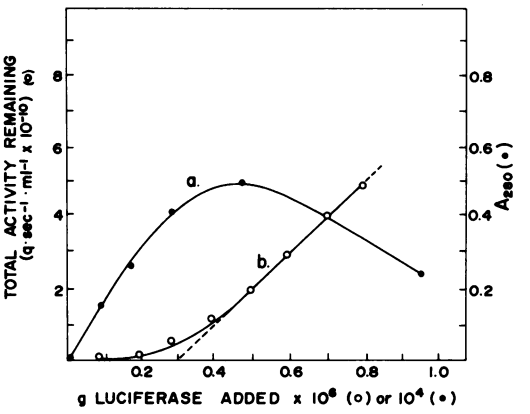

FIG. 5. *Quantitative reaction between purified wild-type luciferase and antiluciferase antiserum. (a) Solid circles: measurement of the amount of the immune precipitate (absorbance at 280 nm [A_{280}], right ordinate) as a function of the grams of luciferase added (grams × 10^4) with 100 μl of antiserum. (b) Open circles: increase of luciferase activity (left ordinate) in the region of luciferase excess in a separate experiment using less (0.8 μl) antiserum and less luciferase (grams × 10^6).*

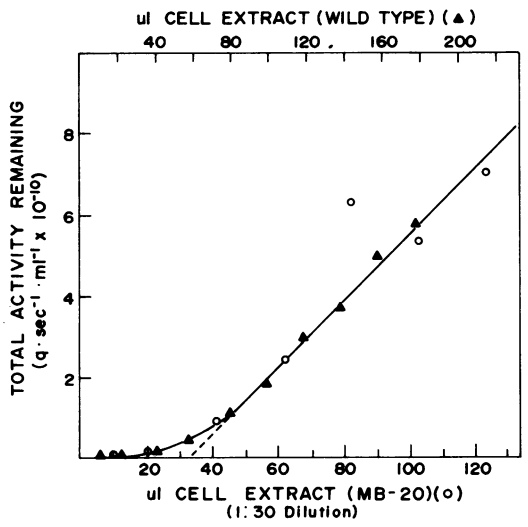

FIG. 6. *Determination of the amount of luciferase in crude extracts of MB-20 and the wild type by quantitative immunological precipitation. By using crude extracts, the amount of luciferase activity inactivated by a fixed amount of antiserum is shown to be the same in wild type and mutant cells, thus indicating that the specific activity of the enzyme is also the same for both.*

compared with the wild type (Table 2). The luciferase specific activities (12) in the extracts can be calculated and are the same for both mutant and wild type, namely, 9.4×10^{13} q/s per mg. This is very similar to that of the pure luciferase, namely, 10.8×10^{13} q/s per mg. All were assayed with decanal at 23°C. An assay of this nature is therefore useful in estimating both the amount of luciferase present in a cell extract and the specific activity of the luciferase. The assay does not depend upon the specific activity of the luciferase in the cell extract, but only upon the immunological similarity of the luciferase in the cell extract to the wild-type luciferase used to raise the antibodies. In the present case, Ouchterlony double-diffusion reactions did not reveal any immunological differences between wild-type and mutant luciferases that would lead to an incorrect estimate of the relative luciferase content of the mutant.

DISCUSSION

We conclude from these experiments that the level of luciferase activity in the mutant does indeed correspond to an actual increase in the amount of luciferase. We postulate that this is due to an increased synthesis of luciferase. An alternative explanation is that the rate of degradation of luciferase is diminished in the mutants. Although this possibility cannot be completely excluded, the isotope labeling experiments of Michaliszyn and Meighen (15) argue against it. Their experiments demonstrated that there is an increase in the rate of incorporation of amino acids into luciferase during the period of induced synthesis and that there is no differential degradation (if any occurs at all) of luciferase in early as compared with late induction phases.

Bacterial luciferase has characteristics of an inducible enzyme, being produced in response to a specific inducer (autoinducer) and in relatively large amounts (>5% of the soluble cell protein is luciferase) (12). Also, luciferase synthesis is subject to catabolite repression (17). The data presented here show that, in addition to the cellularly produced substance(s) (autoinducer), arginine is required for maximum luciferase synthesis in minimal medium. If arginine is omitted from the medium, only a small amount of luciferase is synthesized. Whether arginine acts directly or by way of a metabolite is unresolved, but experiments with inhibitors of protein and messenger ribonucleic acid synthesis have indicated that its effect is manifested at the level of transcription (4, 18).

The molecular nature of the lesion(s) in the MB mutant group is not known. Although the three mutants that were more fully characterized were similar in all those respects studied, they are probably not identical. The results presented here indicate that an increased production of autoinducer or cAMP is not involved. The possibility that increased arginine production is involved was also investigated. Amino acid analyses of the soluble arginine in cell extracts indicated that the arginine pool in the wild type is indeed very low in minimal medium and higher in cells grown in complex medium. The levels were not found to be higher in the mutants. MB mutants 1, 3, 4, 20, and 23 were examined (J. Makemson and J. W. Hastings, unpublished data).

It has been suggested that the structural gene(s) for luciferase may be part of a polycistronic region of the deoxyribonucleic acid (2, 3). The existing data concerning the control of luminescence and the role of arginine in the bioluminescence of these bacteria do not rule out either positive or negative control elements. If negative control is involved, overproduction of luciferase in MB mutants might be due to an alteration of a repressor molecule or an operator sequence associated with its binding. An alteration of a positive control element, e.g., one involving cyclic nucleotides, is less likely since we have demonstrated that MB mutants exhibit neither alterations in their intracellular cAMP pools nor a change in their sensitivity

to glucose repression. An understanding of the molecular basis for sparing the arginine requirement in MB mutants would be facilitated by the use of genetic techniques that are not presently available in this system.

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