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Temperature-Sensitive Mutants of Bioluminescent Bacteria

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ABSTRACT **Mutants of a marine luminous bacterium, in which the ability to emit light is conditional upon temperature, have been isolated. The mutants obtained fall into three classes, which are readily distinguishable by both *in vivo* and *in vitro* criteria. In one class an altered, more temperature-sensitive luciferase is produced; in a second, the luciferase is actually not produced at the higher temperature. The third is defective in the production of an aldehyde-like factor that is a known requirement in the *in vitro* reaction.**

Temperature-sensitive mutants possess lesions whose phenotype is conditional upon temperature (1, 2, 3). In the luminous bacteria, mutations that prevent bioluminescence do not appear to be deleterious to growth and survival (4, 5); consequently, colonies that are temperature-sensitive with respect to bioluminescence can be readily and specifically detected by visual inspection at the restrictive temperature. Mutant cells can then be grown and analyzed equally well at both permissive and restrictive temperatures. Taking advantage of these features, we have isolated and characterized mutants having temperature sensitivity with respect to the luminescent system.

The bioluminescent bacteria possess a specific luciferase which, *in vitro*, catalyzes an oxidation of reduced flavin mononucleotide ($FMNH_2$) by molecular oxygen with the concomitant emission of light (6). The photon yield is greatly increased (by a factor of 100 or more) if a long-chain aliphatic aldehyde (e.g., decanal) is present. The way in which aldehyde increases the photon yield *in vitro* is not understood (7); moreover, no *in vivo* factor analogous to this aldehyde has been demonstrated. The control of luciferase synthesis is also not understood; it is evidently subject to some special control, for the enzyme is synthesized only at a particular time, in a relatively short burst during the period of exponential growth (8). The factor(s) responsible for this control have not been identified. The cells also exert control over the availability of reducing power to luciferase in an unknown way.

These and other aspects of the biochemistry and physiology of this luminescent system can be studied especially well using conditional mutants. Three classes of such mutants have been identified: those in which an altered, more temperature-sensitive luciferase is produced; those in which the aldehyde or aldehyde factor is apparently not produced at the higher temperature; and those in which luciferase synthesis is conditional upon growth at the permissive temperature.

Abbreviations: TSL, mutants producing thermally-sensitive luciferase; TSAS, temperature-sensitive aldehyde-stimulable mutants; TSLS, temperature-sensitive luciferase-synthesis mutants.

MATERIALS AND METHODS

The marine luminous bacterium used in this work is designated as strain MAV (9). Mutants were induced by treatment of a washed suspension of cells (5×10^9 cells/ml) with MNNG (*N*-methyl-*N*-nitro-*N*-nitrosoguanidine) at a concentration sufficient to kill approximately 90% of cells in 40 min (5). After this treatment the mutagen was removed by dilution; the mutagenized cells were plated onto a complex agar medium (see next paragraph) and incubated at 36°C for 12 hr. Wild-type colonies give good luminescence when incubated under these conditions. For the isolation of mutants, colonies exhibiting suboptimal luminescence were picked, streaked on the complex medium, and incubated at 22°C. Colonies that emitted near-normal luminescence when grown at 22°C but were dark when grown at 36°C were designated "temperature-sensitive in luminescence" and were passed through single-colony isolations at 22°C and retained for study.

Complex medium was prepared by the addition of 5 g of Difco Bactotryptone, 1 g of Difco yeast extract, and 2 ml of glycerol to 1 liter of sea water. For solid medium, 12 g of Difco Bacto-Agar was added per liter.

The experiments with liquid cultures were carried out in a temperature-controlled shaker at 25.9 ± 0.1, 29.9 ± 0.1, or 36.1 ± 0.1°C, designated hereafter as 26, 30, and 36°C, respectively. Cultures were grown with 75 ml of complex medium in 250-ml flasks, inoculated with 0.5 ml of culture grown at 36°C for 12–15 hr. Care was taken to keep the shaking rate constant from experiment to experiment.

Light intensity was measured with a photomultiplier photometer (10), and is expressed in quanta/sec. Measurements on the cells during growth were made on samples removed from the flasks at the times indicated. The samples were discarded after use. Cell density was determined at 660 nm in the Coleman Jr. spectrophotometer with a red filter, and is expressed in relative units of optical density. Well-aerated cells emit continuously at a nearly constant intensity and the *in vivo* bioluminescence was measured with 1 ml of such a sample. When aldehyde stimulability *in vivo* was being measured, 0.1 ml of a decanal solution was added to the 1 ml of cells being assayed. The decanal solution was a 1:5 dilution into sea water of an aldehyde sonicate prepared by ultrasonic treatment of 50 μl of decanal in 10 ml of distilled water. If cells are stimulable, luminescence characteristically

rises to a peak value within a few seconds and then slowly declines. The peak was taken as the measure of luminescence with aldehyde.

For the determination of extractable luciferase activity, a 5-ml sample of culture was centrifuged at 2°C. The pellet was resuspended in 2.5 ml of 0.05 M phosphate buffer (pH 6.5), 0.01 M in EDTA, lysed by sonication, and centrifuged to remove cell debris. For characterization of the luciferase from the mutants, the enzyme was partially purified from a more concentrated sonicate (6 ml of the above buffer per gram wet weight of cells) by precipitation between 45% and 70% saturation of ammonium sulfate at 4°C. The precipitate was resuspended and dialyzed against 0.1 M phosphate buffer (pH 6.5)–0.5 mM dithiothreitol.

The luciferase content of cells was determined by the initial maximum light emission in the *in vitro* assay (8) in which the sample is rapidly mixed with reduced flavin in the presence of oxygen and aldehyde at 22°C. Reaction mixtures contained 1 ml of 0.2% bovine serum albumin in 0.1 M phosphate buffer (pH 6.5), 50 μl of a decanal sonicate, diluted as necessary (usually about 1:15), to give the optimum peak intensity in the assay, and luciferase (either the cell lysate or the partially purified luciferase) in a volume not exceeding 50 μl. The reaction was then initiated by injecting from a syringe 1 ml of 50 μM catalytically-reduced flavin. In the assay, the initial maximum light intensity is proportional to the amount of luciferase present; the amounts used in these experiments ranged between 0.001 and 15 μg.

Luciferase was denatured in 5 M guanidine·HCl–10 mM dithiothreitol at 22°C for 30 min (11). This process dissociates the protein into its two subunits, which differ in both charge and molecular weight (9, 12). Renaturation was achieved by dilution into buffer (1/40) at pH 6.5 at 11°C. The lowered concentration of guanidine·HCl presumably permits the peptide chains to refold and the subunits to reassemble into a native molecule having a molecular weight of approximately 79,000. Measurements of thermal denaturation were made by diluting the luciferase (1/40) into temperature-equilibrated assay buffer.

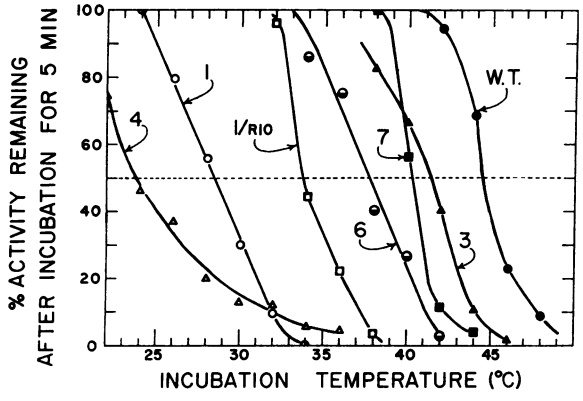

FIG. 1. Thermal stability of the luciferase from several of the mutants of the TSL class. Thermal inactivation was studied at pH 7.0 in buffer equilibrated to the temperature shown on the abscissa prior to the addition of enzyme. Luciferase extracts were adjusted to give equal initial activities upon dilution into the buffer. W. T. is wild-type luciferase. The different mutants of the TSL class are referred to by numbers (3, 4, 6, and 7), the luciferase from a bright revertant of TSL-1 is labeled 1/R-10.

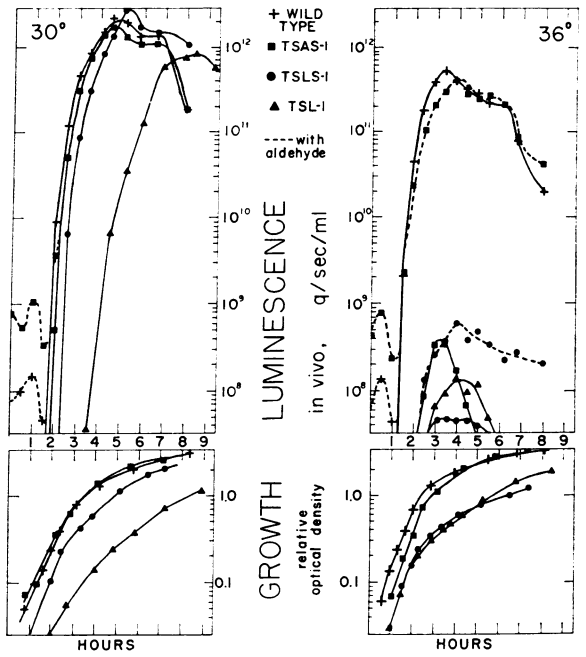

FIG. 2. Luminescence of growing cells at the permissive (26°C for TSL-1, 30°C for all others) and restrictive temperatures for conditional luminescence mutants. Luminescence is measured in quanta/sec per ml of culture at the time after inoculation indicated on the abscissa. Aldehyde stimulability was tested in all experiments; in those cases where a positive response was obtained, the level of luminescence reached with added *n*-decanal is indicated by a dotted line. Growth rates for the cells at the two temperatures are shown in the lower panels.

RESULTS

Types of temperature-sensitive mutants

One class of temperature-sensitive mutants, designated TSL, includes those with a more thermally-sensitive luciferase. The luciferase of the mutant selected for description, TSL-1, is among the more temperature-sensitive of the eight isolates studied (Fig. 1). Its *in vivo* luminescence is near the wild-type level at 26°C, but 1000 times lower at 36°C, growth being good at both temperatures (Fig. 2).* *In vitro* assays indicate that the amount of extractable luciferase activity for the mutant grown at 36°C is approximately 1/300 that of the mutant grown at the permissive temperature (Table 1). However, the concentration of luciferase in extracts of cells grown at 36°C, as determined by antibody assay, was 10% that of the wild type grown at the same temperature, which indicates that inactive luciferase is being produced.

The fact that the lesion that prevents activity at 36°C is in the luciferase molecule can be deduced from the sensitivity of the isolated enzyme to thermal inactivation. TSL-1 luciferase is 50% inactivated after incubation for 5 min at 28°C, while a similar inactivation of the wild-type luciferase requires a temperature of about 45°C (Fig. 1). A second dis-

* Since TSL-1 has a lower optimum temperature for luminescence than does the wild type, 26 and 36°C were established for it as permissive and restrictive temperatures, respectively; 30 and 36°C were used for wild type and other mutants. At all temperatures TSL-1 is morphologically altered to a filamentous form which tends to aggregate, resulting in a lower apparent optical density in liquid culture.

TABLE 1. *Ratios of the values at the restrictive to those at the permissive temperature for in vivo luminescence and extractable luciferase activity*

Strain	*In vivo* peak luminescence (36/30°C)[a]	*In vivo* peak luminescence with added decanal (36/30°C)[a]	Extractable luciferase[b] activity (Imax with decanal) (36/30°C)[a]
Wild type	2.5×10^{-1}	2.5×10^{-1}	0.92×10^{0}
TSAS-1	2.2×10^{-4}	2.4×10^{-1}	1.2×10^{0}
TSLS-1	8.0×10^{-6}	1.6×10^{-4}	6.6×10^{-4}
TSL-1	4.0×10^{-4}	4.0×10^{-4}	2.9×10^{-3}

[a] This ratio is 36/26°C for TSL-1.

[b] Except for TSL-1 at 36°C, extractable activity remains constant for 1–2 hr after peak luminescence induced with decanal. These determinations were made on extracts of cells taken 1 hr after peak luminescence, except for TSL-1 at 36°C, which was taken 0.5 hr after the peak.

tinguishing feature of TSL-1 luciferase is its inability to recover activity after treatment with 5 M guanidine·HCl followed by dilution into buffer at 11°C. After such treatment the wild-type luciferase typically recovers at least 45% of its original activity, whereas TSL-1 recovers only about 0.1% (Table 2). Most of the TSL mutants show a reduced ability to renature compared with the wild type, but TSL-1 is the most renaturation-deficient member of the TSL class. With regard to the enzymatic characteristics of TSL-1 luciferase, those examined appear essentially the same as the wild type (Table 2).

A second class of mutants includes those bacteria which, though normally dark at 36°C, will emit at nearly wild-type intensity (10–80%) upon the addition of a long-chain aldehyde such as decanal. Nine were isolated; they are designated temperature-sensitive aldehyde-stimulable mutants (TSAS). TSAS mutants thus possess luciferase, but its activity is not being expressed at 36°C. In TSAS-1, luminescence is normal at 30°C and is not stimulated by aldehyde (Fig. 2). However, at 36°C without added aldehyde, luminescence is less than 10^{-3} that of the wild type. The addition of decanal brings the luminescence to within 80% of the wild-type level. TSAS-1 produces luciferase in nearly equal quantities at 30 and at 36°C (Table 1). As judged by both its enzymatic properties and its denaturation and renaturation characteristics (Table 2), the TSAS-1 luciferase itself appears to be unaltered.

Members of the third class (7 isolates) are designated as temperature-sensitive luciferase-synthesis mutants (TSLS). Little luminescence is observed (10^{-3} of the wild-type intensity) in cultures grown at 36°C, even with added aldehyde (Fig. 2). Extracts of TSLS-1 cells grown at 36°C show equally low levels of both luciferase activity (Table 1) and reaction to anti-luciferase. No alterations in the luciferase extracted from TSLS-1 grown at 30°C were detected (Table 2). TSLS-1 and several other TSLS mutants also are highly filamentous in form when grown above 30°C.

Effects of temperature shifts

In liquid culture the three classes of mutants are distinctive with regard to their behavior following a temperature shift. Fig. 3 shows the effects on luminescence of a shift from permissive to restrictive temperature early in the synthesis phase.† Luminescence in the wild type reached about the intensity it would have reached had the cells been grown at 36°C throughout (see Fig. 2). In contrast, shifting TSL-1 caused a precipitous drop in luminescence, and added aldehyde had no effect. This very rapid drop is comparable to the rate of inactivation of TSL-1 luciferase at 36°C *in vitro*, and indicates that luciferase within the cells has a similar thermal sensitivity.

TSAS-1 cells behave very differently (Fig. 3). After a temperature shift from 30 to 36°C there is no further increase in luminescence, but the existing luminescence declines quite slowly, at a rate comparable to the rate of decay of luminescence that occurs in the wild type several hours after reaching its maximum. Moreover, luciferase continues to be synthesized, as shown both by the aldehyde-stimulable luminescence and by the *in vitro* luciferase activity.

With TSLS-1 both the *in vivo* luminescence and the extractable luciferase continue to increase for some time after the shift (Fig. 3), though at a rate below that of the wild type. A hypothesis consistent with these observations is that the temperature-sensitive species is neither the luciferase nor a molecule directly involved in its synthesis, but rather a species concerned with the control of synthesis.

The effects of the converse temperature shift, that is, from the restrictive to the permissive temperature, also show that the three mutant classes are distinctly different, especially

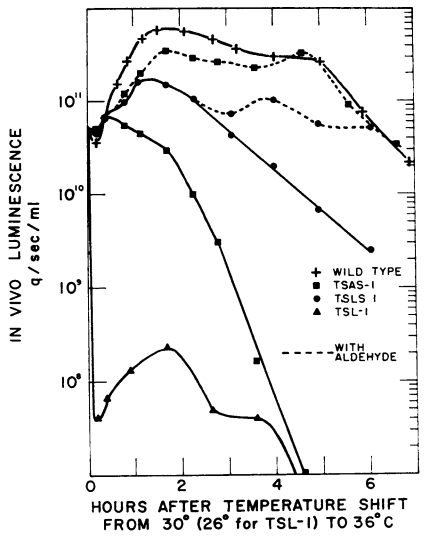

FIG. 3. Effects of a shift from the permissive to the restrictive temperature on the luminescence of growing cells. The shift occurred early in the luciferase synthesis phase, when less than 5% of maximum luminescence had been reached. When *n*-decanal stimulated luminescence, the level reached is indicated by a dotted line.

† Nealson *et al.* (8) have shown that the appearance of luminescence during a particular period of growth in liquid cultures (see Fig. 2) is attributable to *de novo* synthesis of luciferase at that time. Although the factors that initiate and terminate luciferase synthesis have not been identified, the effect of a temperature shift is dependent upon the point in the luciferase synthesis phase at which the shift is made.

TABLE 2. *Characteristics of the luciferases isolated from temperature-sensitive mutants grown at their permissive temperature*[a]

Strain	Luciferase assay with $FMNH_2$[b]				% Renaturation after denaturation in 5 M guanidine·HCl	Temperature for 50% inactivation of activity after 5 min at pH 7.0 (°C)
	I_{max},C10 / I_{max},C12	*k*, C10 (sec^{-1})	*k*, C0 (sec^{-1})	*k*, C12 (sec^{-1})		
Wild type	8.7	0.25	0.13	0.031	47	44.5
TSAS-1	8.8	0.26	0.15	0.030	48	44.5
TSLS-1	8.0	0.23	0.13	0.030	42	44.5
TSL-1	8.0	0.25	0.14	0.031	0.1	28.5

[a] These are the only characteristics examined to date.
[b] C10 = decanal in assay; C12 = dodecanal; C0 = no aldehyde, 20 mM NH_2OH; all at 22°C.

with regard to kinetic aspects. The experimental results of these studies will be presented in a separate paper.

Reversion studies

Revertants of the three mutants were sought by subjecting each to a second mutagenesis with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine, plating at 36°C, and selecting colonies that emitted light. Since one is looking for light emission against a dark background, it is possible to detect revertants with far greater sensitivity and less ambiguity than was possible in the original production of dark mutants. The three mutants differ in their rate and character of reversion to visibly luminescent forms.

With TSL-1, about 0.001% of the survivors of the mutagenesis gave appreciable luminescence at 36°C. Of the five studied, one is near the wild-type intensity of light emission and the other four are quite dim. In all five, the luciferase is thermally more stable than the parent, but in none is it as stable as that of the wild type. The explanation for the bright revertant appears to be not only that it has a somewhat more stable luciferase (TSL-1/R-10, see Fig. 1), but that it also is an overproducer of luciferase at all temperatures. Actually many extremely dim revertants have been isolated that are overproducers of TSL-1 type luciferase. Such overproducers apparently occur rather frequently.

With TSAS-1, about 0.03% of the survivors after mutagenesis showed increased luminescence at 36°C, but in none of the 18 examined is the intensity >10% that of the wild type. Aldehyde stimulability is retained; the reason for the partial reversion is not yet known.

TSLS-1 evidently reverts far more readily and completely. In one experiment 13 bright colonies were found among 5000 survivors examined, and in all of these the luminescence was equal to or near that of the wild type.

DISCUSSION

The temperature sensitivity of TSL-1 can be explained entirely on the basis of its altered luciferase. The fact that a mutation in luciferase that results in a more temperature-sensitive molecule also alters the renaturability of the protein molecule is probably not fortuitous, since any alteration in the primary structure of a protein would be expected to influence the steps leading to higher orders of structure. While this consequence is demonstrable *in vitro* as a deficiency in renaturability, it is perhaps interesting that there is no evidence of any such deficiency *in vivo*. For example, the *in vivo* rate of formation of active (e.g., folded) luciferase appears normal and unaffected by the presumed alterations in the primary structure of the luciferase. For example, TSL-1 makes near wild-type amounts of active active enzyme *in vivo* at 26°C, yet after denaturation in 5 M guanidine less than 0.1% of the activity is regained *in vitro* at 26°C or below.

The most satisfactory explanation for the temperature-sensitive lesion in TSAS-1 is that it alters a protein involved in the synthesis of the "natural aldehyde". If, instead, the "natural aldehyde" itself were temperature-sensitive, one would expect the luminescence to decline more promptly after a shift from the permissive to the restrictive temperature. The converse shift (restrictive to permissive) indicates that, like luciferase, the "aldehyde" can be synthesized only during a particular phase, which suggests that the synthesis of the two is under coordinate control (8). It should also be noted that the difference between the permissive and nonpermissive temperatures is only 6°C in these experiments; actually, temperatures only 4°C apart (31 and 35°C) give virtually identical results. Such a high apparent activation energy for the process is consistent with the hypothesis that a protein is the temperature-sensitive molecule.

With TSLS-1 the precise cause for its inability to produce luciferase at high temperature is unknown, but the lesion appears to involve a protein concerned with the control of luciferase synthesis (13). The fact that every TSLS mutant examined so far (seven) shows a high degree of aldehyde stimulability at the restrictive temperature suggests again that the synthesis of luciferase and that of "natural aldehyde" are under coordinate control.

Compared to mutants previously isolated, temperature-sensitive mutants offer unique advantages, not only for the study of bacterial bioluminescence, but also for the investigation of several other basic problems. Temperature sensitivity permits one to distinguish TSLS mutants from those in which luminescence is prevented by a lesion in the luciferase gene itself. The TSLS mutants will be valuable in the study of the control and timing of luciferase synthesis, addressed as well to more general questions concerning proteins involved in the control of the synthesis of specific proteins. Conditional TSAS mutants are especially valuable because cells that are identical genetically will have or lack the "natural aldehyde" when grown at temperatures as little as 4°C apart. Combined with studies of appropriate revertants, this should make it possible to discover the identity of the "natural aldehyde". Finally, TSL mutants have several special uses. The luciferase in cells grown at a permissive temperature can be rapidly and specifically inactivated *in vivo* without otherwise adversely affect-

ing the cells, so that the physiology and biochemistry of its function can be investigated. The isolated temperature-sensitive luciferase will be of particular interest, not only for the study of the luciferase reaction, but also for the study of more general problems related to protein structure: conformation, thermal stability, and renaturation. For such studies we expect that it will easily be possible to obtain tens or even hundreds of different luciferase molecules that are altered in thermal stability but essentially unchanged enzymatically.

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