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Inhibition and Activation of Bacterial Luciferase Synthesis

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Luciferase synthesis is repressed when bioluminescent bacteria are inoculated into fresh medium but is induced after the cells have grown in the medium for some time. In minimal medium, an activator which leads to induction of the enzyme is released into the medium by the bacteria. Complete medium contains a dialyzable and quite stable inhibitor which leads to repression of luciferase. The bacteria remove the inhibitor from the medium and also produce activator, thus allowing synthesis of the enzyme. Two unidentified nonluminescent strains of bacteria were unable to remove the inhibitor. Two different bioluminescent strains, *Photobacterium fischeri* and *P. fischeri* strain MAV, produce specific activators that are ineffective with cells of the other strain. The two activators are different with respect to heat stability, but both are small molecules. The activators can be assayed on the basis of their ability to counteract the inhibitor. Identification of the inhibitor and the activators may allow the bioluminescent system to be linked to other metabolic processes of the cells.

When bioluminescent bacteria from an exponentially growing and brightly glowing liquid culture are inoculated into fresh medium, logarithmic growth continues with no lag. Luciferase synthesis, however, is repressed, and induction does not take place until the culture has reached a characteristic cell density (8). Kempner and Hanson (5) showed that a conditioned medium, showing no lag in light production, can be prepared by removing the bacteria from a culture in which induction has taken place and that the conditioning is caused by the removal of an inhibitor contained in fresh complete medium. Nealson et al. (8), however, argued that in minimal medium, where a similar phenomenon occurs, the conditioning is due to the formation of an activator or inducer.

This paper confirms both the presence of an inhibitor in fresh complete medium and that of an activator in conditioned minimal medium. The conditioning of complete medium is shown to involve not only the removal of an inhibitor but also the formation of an activator. Finally, some of the properties of the activators from two luminescent strains were studied.

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MATERIALS AND METHODS

Photobacterium fischeri (ATCC 7744) and *P. fischeri* strain MAV were previously described (4). The minimal medium consisted of NaCl, 30 g; Na_2HPO_4, 3.7 g; KH_2PO_4, 1 g; $(NH_4)_2HPO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; glycerol, 2 ml; and 1 liter of distilled water. The complete medium consisted of minimal medium plus 5 g of tryptone (Difco) and 3 g of yeast extract (Difco).

Conditioned media were prepared as follows. The cells were grown past the point of luciferase induction with vigorous rotary shaking in side-arm flasks. The cell density was measured with a Coleman Junior II spectrophotometer at 660 nm, and the light was measured with a photomultiplier photometer (3, 6) equipped to hold the side arms of the flasks. When the cultures had reached the desired cell densities, they were centrifuged for 10 min at 12,000 $\times$ *g*, and the supernatant fluids were filtered through sterile membrane filters (Millipore Corp.).

Evaporation of media was carried out below 45 C with a Buchler model PF-9GN rotary evaporator. Ultrafiltration was carried out at 4 C with an Amicon model 50 filter with UM-2, UM-10, and PM-10 membranes.

The various media were inoculated to an optical density (OD) of 0.01 or less with centrifuged cells from fully induced cultures of which in vivo and in vitro light measurements should have been equal (8). The cultures were grown with vigorous shaking in 4 ml of medium at 25 to 28 C in screw-cap culture tubes (16 by 124 mm) to which 1-cm Pyrex cuvettes

had been fused. The OD values of the cultures were determined with a Beckman DU spectrophotometer at 660 nm with a Corning 2-61 filter placed between the sample and the phototube to exclude interference by the bioluminescence. An OD of 0.1 corresponds to 10^8 cells/ml in complete medium and to 4×10^8 cells/ml in minimal medium. The light produced was measured with an apparatus similar to that described above.

RESULTS AND DISCUSSION

The results are summarized in Table 1, which presents the optical densities at which cultures of *P. fischeri* and strain MAV, when inoculated into the media described in the first column, show the rapid rise in light production that indicates induction of luciferase. The middle columns show the conclusions drawn from the data, namely the presence or absence of inhibitor and either *P. fischeri* or strain MAV activator in the media described in the first column. The way in which the conconclusions were reached will be discussed.

In complete medium, induction took place at OD 0.3 to 0.4 with strain MAV (Fig. 1, curve 1) and at OD 0.1 to 0.15 with *P. fischeri* (Table 1). Smaller amounts of tryptone and yeast extract in the complete medium led to induction at lower OD values. Inoculation into a conditioned medium (5) led to no lag, irrespective of the OD to which the medium was inoculated (Fig. 1, curve 2). When complete medium was conditioned by MAV, evaporated to dryness at less than 45 C, reconstituted to its original volume with complete medium lacking salts (conditioned, evaporated, and reconstituted medium), and then inoculated with cells of strain MAV, the light again dimmed until induction took place at OD 0.17 (Fig. 1, curve 3). This dimming showed that

TABLE 1. *Effect of inhibitor and activator on optical density (OD) of cultures at the point of luciferase induction*[a]

Medium	Initially present in medium			OD at point of induction after inoculation with	
	Inhibitor	MAV activator	*P. fischeri* activator	*P. fischeri*	MAV
Complete	+	−	−	0.1–0.15	0.3–0.4
Complete, conditioned by MAV	−	+	−	0.03–0.05	No lag
Complete, conditioned by MAV, ultrafiltered	−	+	−		No lag
Complete, conditioned by MAV, dialyzed against fresh complete medium	+	−	−		0.3–0.4
Complete, conditioned by MAV, boiled	−	−	−		0.03–0.05
Complete, conditioned by MAV, evaporated and reconstituted with water	−	+	−		No lag
Complete, conditioned by MAV, evaporated, and reconstituted with complete medium lacking salts	+	+	−		0.17
Complete, conditioned by MAV, boiled. evaporated, and reconstituted with complete medium lacking salts	+	−	−		0.3–0.4
Complete, after growth of nonluminescent bacteria (2 strains)	+	−	−		0.2–0.25
Complete, conditioned by *P. fischeri*	−	−	+	No lag	0.03–0.06
Complete, conditioned by *P. fischeri* and ultrafiltered	−	−	+	No lag	
Complete, conditioned by *P. fischeri* and boiled	−	−	+	No lag	
Complete, conditioned by *P. fischeri*, evaporated, and reconstituted with complete medium lacking salts	+	−	+	0.03	
Minimal[b]	−	−	−	No growth	0.03–0.05
Minimal[b], conditioned by MAV	−	+	−		No lag
Minimal[b], conditioned by MAV, boiled	−	−	−		0.03–0.05

[a] The point of luciferase induction was taken as the point at which the rapid increase in light production took place. "No lag" indicates that light production increased continuously after inoculation. "MAV" is *P. fischeri* strain MAV (4).

[b] Arginine hydrochloride (0.5 g/liter) was added.

addition of the complete medium lacking salts led to deconditioning of the medium. The evaporation process itself had no effect since evaporation and reconstitution with water still resulted in a conditioned medium (Table 1). These experiments show that the complete medium contains an inhibitor which leads to repression of luciferase synthesis, in agreement with the conclusions of Kempner and Hanson (5).

Cells of strain MAV in minimal medium pass through a lag in luciferase synthesis as with complete medium, but the onset of the rise in light production occurs at a much lower cell density, OD 0.03 to 0.05 (Fig. 1, curve 4). Conditioned minimal medium shows no lag (Fig. 1, curve 5). Changes in the concentration of the components of the minimal medium cause no change in the point at which induction takes place unless the ionic strength is changed (K. Nealson, *personal communication*). The minimal medium, therefore, contains no inhibitor, and the conditioning must be due to the production of an activator which leads to induction of the enzyme, as suggested by Nealson et al. (8). The activator produced by MAV in minimal medium is labile to heating at 100 C for 5 min, since a heated conditioned medium again shows a lag (Fig. 1, curve 6).

Induction of luciferase might be expected to proceed by a similar mechanism in both minimal and complete medium from which the inhibitor has been removed. Any MAV activator produced by the bacteria that is present in the conditioned complete medium should be destroyed by heating, leaving the solution free of both activator and inhibitor. Such a medium should then be similar to minimal medium which also contains neither activator nor inhibitor. When conditioned complete medium was heated at 100 C for 5 min and inoculated with cells of strain MAV to OD 0.01 or less, the light dropped at first but increased again at OD 0.03 to 0.05, just as in minimal medium (Fig. 1, curve 7). Furthermore, a boiled conditioned, evaporated, and reconstituted medium behaved just like fresh complete medium, as expected, since both media should have contained only inhibitor (Table 1). These experiments indicate that conditioned complete medium contains an activator which leads to induction of luciferase synthesis.

Nealson (Ph.D. thesis, University of Chicago, 1969) found essentially no difference between a conditioned and a boiled conditioned medium when inoculated with cells of strain MAV to an OD of 0.02 to 0.03. This is too high an OD, since induction takes place at OD 0.03 to 0.05. To observe the dimming phase, the initial OD should be below 0.01. Also, the cells to be inoculated should be centrifuged, since otherwise the activator in the medium will be enough to allow luciferase to be induced in the inhibitor-free medium.

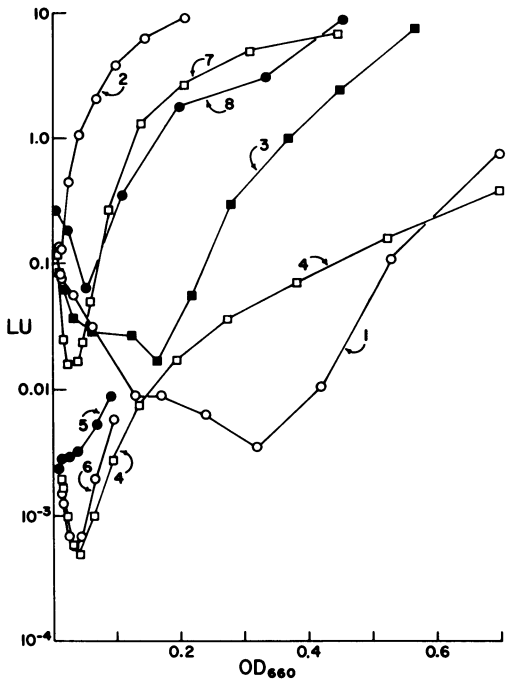

FIG. 1. *MAV was inoculated into the following media, and light output of the resulting cultures was followed as a function of cell density: curve 1, complete medium; curve 2, conditioned complete medium (OD 1.3); curve 3, conditioned complete medium evaporated and reconstituted with complete medium lacking salts; curve 4, minimal medium; curve 5, conditioned minimal medium (OD 0.15); curve 6, boiled conditioned minimal medium; curve 7, boiled conditioned complete medium; curve 8, complete medium conditioned by P. fischeri (OD 0.44). One light unit (LU) equals 2 × 10^{10} quanta/sec (3). Values in parentheses indicate the OD values of the cultures from which the conditioned media were prepared.*

Heating complete medium conditioned by *P. fischeri* to 100 C for 5 min had no effect, showing that the *P. fischeri* activator is heat stable and therefore different from the MAV activator, though both activators pass through ultrafiltration membranes. A medium conditioned by MAV, when inoculated with *P. fischeri*, showed a lag in light production followed by induction at OD 0.04, as if the medium had contained neither activator nor inhibitor (Table 1). Similarly, a medium condi-

tioned by *P. fischeri*, when inoculated with cells of strain MAV, showed a lag followed by induction at OD 0.05 (Fig. 1, curve 8). These experiments show that both strains remove the inhibitor but that the activator from one strain does not lead to induction in the other strain. The activators are strain specific. This specificity is interesting since the luciferases are also structurally distinct (4).

A conditioned, evaporated, and reconstituted complete medium should contain both activator and inhibitor. As mentioned above with MAV, such a medium led to induction at OD 0.17 compared to 0.32 in complete medium, showing that activator can counteract the action of the inhibitor (Fig. 1, curves 1 and 3). Mixing the reconstituted media with complete medium led to induction at intermediate optical densities. With *P. fischeri*, a reconstituted medium led to induction at OD 0.03 compared to 0.15 for the complete medium, indicating that the *P. fischeri* activator has a more powerful effect on *P. fischeri* than the MAV activator on MAV or that *P. fischeri* produces more activator than MAV. These experiments also conclusively demonstrate the existence of the activators in the conditioned complete media.

Supplementing complete medium with only 1% of conditioned medium led to a definite lowering of the OD of induction. This sensitive assay for activator was used to show that denser cultures of *P. fischeri* contain more activator (Fig. 2).

Preliminary work with another strain of luminescent bacteria, strain 22 (obtained from K. Nealson), has shown that it also removes the inhibitor but produces an activator that is different from the other two, whereas two unidentified strains of marine nonluminescent bacteria neither produced activator nor were able to remove the inhibitor (Table 1). These experiments, and the fact that neither activator nor inhibitor has any effect on the growth rate of the luminescent bacteria, suggest that the activator and inhibitor are directed specifically towards the luminescent system.

Kempner and Hanson (5) unsuccessfully tested a large series of chemicals for their ability to mimic the action of the inhibitor. They showed that the inhibitor is dialyzable, stable to boiling at *p*H 1 or 13, not extractable with $CHCl_3$, and not precipitable with $AgNO_3$. It is also stable to H_2O_2 and H_2/Pt and it passes through ultrafiltration membranes. The following chemicals did not mimic the action of either inhibitor or activator when tested

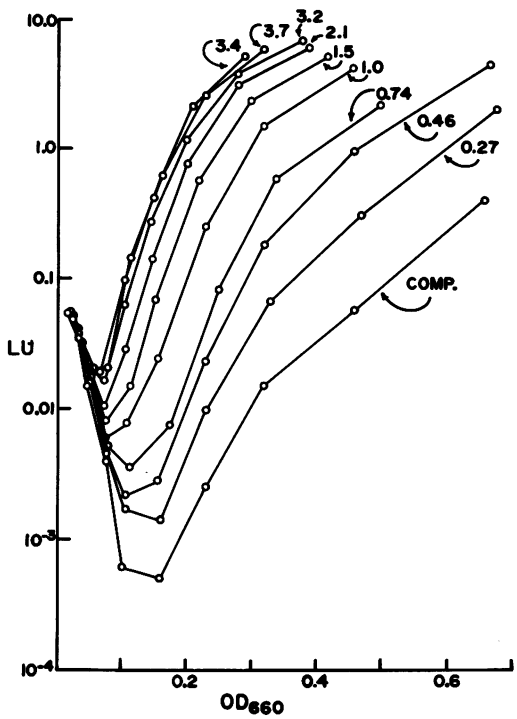

FIG. 2. *Assay for Photobacterium fischeri activator. P. fischeri was inoculated into 3.96 ml of complete medium plus 0.04 ml of complete medium conditioned by growth of P. fischeri to the OD values indicated. Light output was followed as a function of the OD of the resulting cultures. The shorter lag times indicate higher concentrations of activator in the conditioned media prepared from the denser cultures.*

with MAV: many mineral salts, amino acids, nucleosides, vitamins, glycolysis and Krebs cycle intermediates, and sugars; acetic acid, decanoic acid, tetradecanol, *p*-aminobenzoic acid, β-alanine, 3-methylhistidine, putrescine, spermidine, spermine, glucosamine, and chromic acid cleaning solution. 3′,5′-Cyclic AMP does exert an effect on the luminescent system which will be described in a forthcoming publication, but it does not mimic inhibitor or activator.

The mechanisms of action of the inhibitor and the activators were not investigated. For this reason, the terms repressor and inducer were avoided. Also, there is no evidence that either the inhibitor or the activators are single chemical species.

As yet, no reason for the selective advantage of the luciferase system to the bacteria having it has been elucidated (2). The arginine effect (1, 7, 8) and the identification of the inhibitor and the activators may allow the luciferase

system to be related to other metabolic pathways of the cells and allow reasonable suggestions to be made about the importance of the system to the bacteria.

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ADDENDUM IN PROOF

It should be pointed out that the activators discussed in this paper can be considered to be bacterial pheromones (Law and Regnier, Annu. Rev. Biochem. **40**:533-548).

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