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Myristic acid stimulation of bacterial bioluminescence in "aldehyde" mutants

(fatty acids/bacterial luminescence/*Beneckea harveyi*/*Photobacterium fischeri*)

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ABSTRACT **The involvement of long chain aldehyde in bacterial luminescence was known both from its being required for light emission in the *in vitro* reaction with pure luciferase and from its ability to stimulate luminescence *in vivo* in a certain class of dark "aldehyde" mutants. We have found that the luminescence of some (but not all) of such aldehyde mutants is also stimulated by long chain aliphatic fatty acids, with a marked specificity for myristic (tetradecanoic) acid. This stimulation has been demonstrated in aldehyde mutants of two species of luminous bacteria, *Beneckea harveyi* and *Photobacterium fischeri*. The responses, both in intensity and yield, are proportional to the amount of added tetradecanoic acid over a 1000-fold range, down to 10 pmol ml^{-1}. Unsaturated long chain fatty acids are potent inhibitors of the tetradecanoic acid stimulation, but they do not effect the *in vivo* luminescence of wild-type bacteria.**

The finding that long chain aldehydes are involved in bacterial bioluminescence came from the discovery of a factor in extracts of hog kidney cortex that would stimulate the luciferase reaction *in vitro* (1). Its identification as palmitaldehyde (2) was followed by the isolation of "dark" (but luciferase-containing) mutants that responded to exogenously added aldehyde, becoming luminescent *in vivo* (3–5). Although there were differences in the specific responses to different chain length aldehydes, all straight chain aldehydes longer than six or eight carbons (depending on the species of bacteria) were capable of stimulating effectively, both *in vitro* (6–8) and *in vivo* (3, 9).

However, no other compound—ketone, alcohol, acid, or other—has previously been found to substitute for aldehyde in the bacterial reaction, either *in vivo* or *in vitro*. The one report (10) of a compound active as a substitute for aldehyde in the *in vitro* reaction has not been confirmed (11).

In the course of experiments concerned with changes in the ATP pool during the induction and synthesis of the bioluminescence system, we found that some of the aldehyde mutants, independently isolated, differed from one another (23). On the basis of the idea that the mutants might differ with regard to the biosynthetic step being blocked and thus with respect to the accumulating precursor, we tested the effects of long chain fatty acids. In one class of aldehyde dark mutants the *in vivo* bioluminescence was, indeed, stimulated by fatty acid, with a marked chain length specificity for myristic (tetradecanoic) acid.

MATERIALS AND METHODS

Bacterial Strains. *Beneckea harveyi*, MAV 392 (12) is designated as a wild type. Two mutants were derived from MAV after mutagenesis with nitrosoguanidine: (*i*) TSAS-F1, temperature-sensitive aldehyde synthesis; and (*ii*) M17, a dim mutant that emits light with exogenous aldehyde (5, 13). Two mutants of *Photobacterium fischeri* were also used, Y-1-A1 and MJ-1E, which are dim unless exogenous aldehyde is added.

Media and Conditions for Growth. The medium designated as a complex medium consists of artificial sea water (ASW) to which 5 g of peptone (Difco), 3 g of yeast extract, and 0.02 M 3-(*N*-morpholino)propanesulfonic acid (Mops) at pH 7.3 were added per liter. ASW contains (g/liter) NaCl, 17.55; KCl, 0.75; $MgSO_4 \cdot 7H_2O$, 1.23; $CaCl_2 \cdot 2H_2O$, 1.45; $FeSO_4 \cdot 7H_2O$, 0.028; and $K_2HPO_4 \cdot 3H_2O$, 0.075.

Growth was carried out in liquid culture with shaking at 25°. Cell density was determined in a Klett–Summerson photometer (filter number 66) a density of 100 Klett units was equal to about 5×10^8 cells ml^{-1}.

Bioluminescence Determination. *In vivo* luminescence of aliquots placed in a scintillation vial was measured in a photomultiplier photometer (14) and expressed in quanta sec^{-1} ml^{-1}, using the standard of Hastings and Weber (15).

Effects of Fatty Acids and Aldehydes on Luminescence. All fatty acids and aldehydes (≥99% pure; Sigma) were dissolved in absolute ethanol and kept in the dark at −18° until used. To test their effect on luminescence, 1–10 μl of the proper lipid concentration was injected into 1 ml of the bacterial culture, and the resulting bioluminescence was recorded with time.

RESULTS

Fig. 1 compares bioluminescent responses as measured by the initial maximum intensity following the addition of tetradecanoic acid or decanal to cells of two types of aldehyde mutants of *Beneckea harveyi*, recorded at different times during the growth of the bacteria in liquid culture. The luminescence of both mutants is stimulated upon addition of aldehyde, but only one mutant (M17) responds to the long chain fatty acid. These experiments also illustrate the inducible nature of the bioluminescent system (16, 17), consideration of which will be of importance in the interpretation of some later experiments. During the first 100 min, exponential growth of the cells occurs with no concomitant synthesis of the bioluminescent system; during the second 100 min, the period of induction, luminescence rises rapidly along with extractable luciferase, while the growth rate remains about the same.

Fig. 2 shows that the intensity of the bioluminescence response of aldehyde mutants to fatty acids is much less with chain lengths both longer and shorter than tetradecanoic acid, and that this is so for fatty acid-responsive mutants of two species, *B. harveyi* (M17) and *P. fischeri* (Y-1-A1). On the other hand,

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Abbreviations: ASW, artificial sea water; Mops, 3-(*N*-morpholino)-propanesulfonic acid.

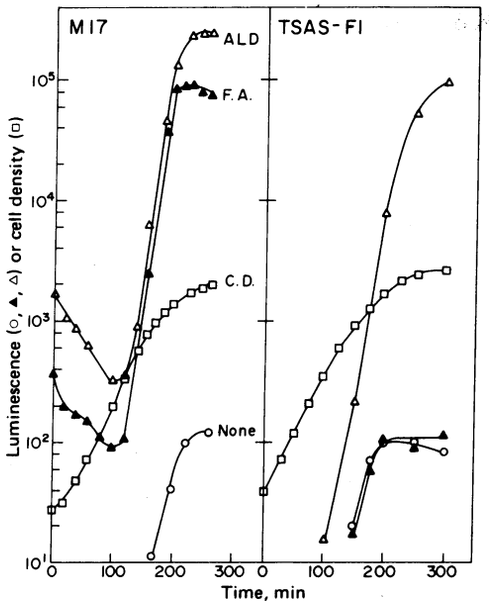

FIG. 1. Responses of two mutants of *B. harveyi*, M17 and TSAS-F1, to decanal and tetradecanoic acid. Cells were grown in complex medium with shaking at 36°. At different times during growth, 1-ml samples were taken for luminescence determination with no additions (○) or in the presence of 50 μM decanal (△) or 5 μM tetradecanoic acid (▲). Ordinates: cell density (□) in Klett units (multiply values by 10^{-1}), and luminescence (multiply values by 10^7 to give quanta sec^{-1} ml^{-1}).

the intensity responses of these same mutants to different chain length aldehydes are about the same, within an order of magnitude (Fig. 3). Similar distinctions between fatty acid and aldehydes are found if the photon yield is determined in relation to the chain length (Table 1).

There is a striking effect of pH upon the response to tetradecanoic acid (Fig. 4). At values above pH 6.5 the response

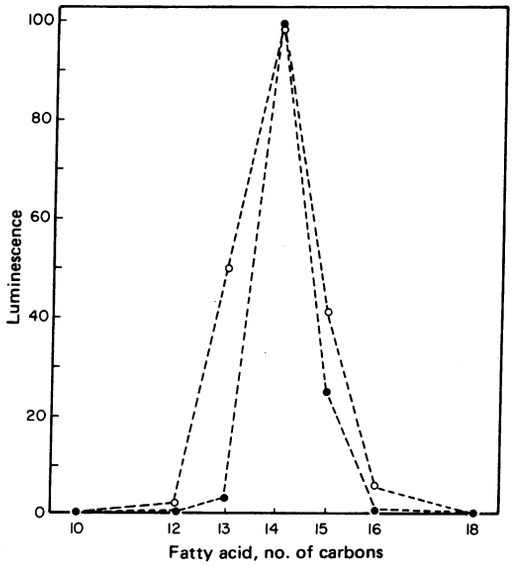

FIG. 2. Effect of different chain length fatty acids upon the luminescence (in relative units) of dim aldehyde mutants of both *B. harveyi* (M17) (○) and *P. fischeri* (Y-1 ald⁻) (●). Cells were grown in liquid complex medium to a density of 180 Klett units at 25°. A sample of each of these fatty acids in ethanol was added to 1 ml of the cells in a vial to give a final concentration of 1 μM; the maximum light intensity, reached within a few seconds, was recorded.

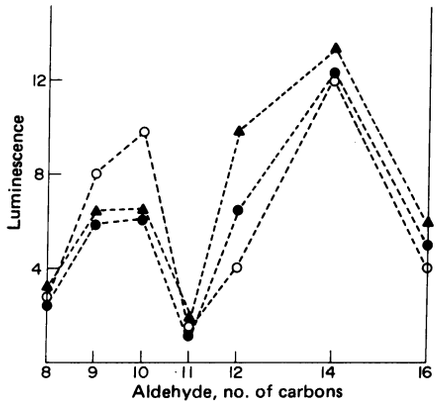

FIG. 3. Effect of different chain length aldehydes upon the luminescence (in same units as Fig. 2) of dark aldehyde mutants. All strains were grown in complex medium to a density of 150 Klett units at 25°. Aldehydes from ethanolic solutions were added to 1-ml cell cultures to give final concentrations of 1 μM and the initial peak intensities were recorded. (○) *B. harveyi* M-17; (▲) *P. fischeri* MJ-1; (●) *P. fischeri* Y-1-A1.

declines abruptly, and similarly so for fatty acids of different chain lengths. Because this pH is far from the pK for the acid (~4.8), this effect is attributed to negative cell surface charges that are acquired at the higher pH values. The responses to aldehydes are unchanged over this pH range.

The response of aldehyde mutants to tetradecanoic acid is linear over a considerable (>1000-fold) range of concentrations,

Table 1. Response of M17 cells to different fatty acids, fatty acid derivatives, and aldehydes

Lipid	Relative maximum luminescence	Total quanta × 10^{-9}
Ethanol	<0.0001	<1
Decanoic acid	0.3	100
Dodecanoic acid	2.0	400
Tridecanoic acid	56	6,000
Tetradecanoic acid	100	15,000
Pentadecanoic acid	45	5,000
Hexadecanoic acid	5.5	150
Octadecanoic acid	0.1	<1
Myristoleic (*cis*-9) acid (14:1)	8.5	2,000
α-Hydroxymyristic acid	12.1	ND
Myristyl alcohol	<0.0001	<1
Methyl myristate	<0.0001	<1
Palmitoleic (*cis*-9) acid (16:1)	2.5	450
Linoleic (*cis*-9, *cis*-12) acid (18:2)	0.01	<1
Octanal	2.7	38
Nonanal	8	600
Decanal	10	700
Undecanal	1.5	50
Dodecanal	4	120
Tetradecanal	12	200
Hexadecanal	4	250

M17 cells were grown in a complex medium to an optical density of 180 Klett units. The culture was diluted 1:20 in ASW buffer at pH 6.5 in a final volume of 1 ml, and the luminescence in the presence of different fatty acids and aldehydes at 1 μM (all dissolved in ethanol) was determined. The activity with tetradecanoic (myristic) acid was defined as 100. ND, not determined.

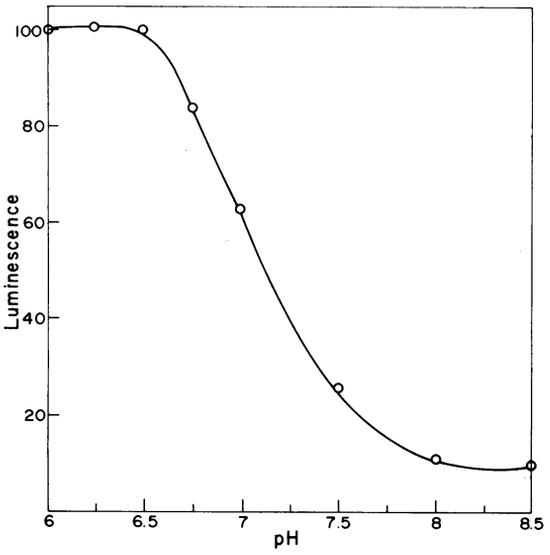

FIG. 4. Effect of pH on the luminescence response to tetradecanoic acid. M17 cells were grown in complex medium to a cell density of 180 Klett units. The culture was centrifuged in the cold and the cells were resuspended in ASW containing imidazole·HCl (0.05 M) buffer at the different pH values. The maximal luminescence in the presence of 1 μM tetradecanoic acid was recorded. Ordinate: luminescence in relative units.

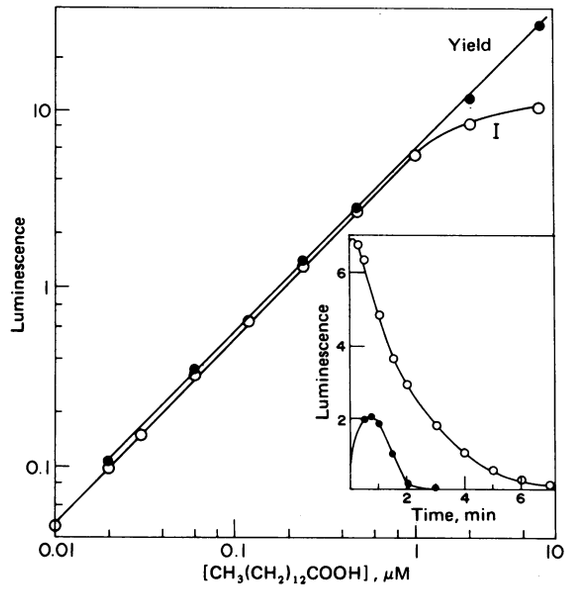

FIG. 5. Effect of concentration of tetradecanoic acid on bioluminescence. *B. harveyi* M17 cells were grown at 25° in complex medium to a density of 180 Klett units. The culture was then diluted 1 to 20 in ASW/Mops buffer at pH 6.5 and luminescence of 1-ml aliquots was determined in the presence of different amounts of tetradecanoic acid. Luminescence intensity without additions, 1×10^7 quanta sec^{-1}. Total quanta (yield) was obtained by integration of the full time course of the reaction, which with small amounts of acid lasted only a few minutes (see *Inset*), but with large amounts took hours. Intensity (*I*) units are in quanta sec^{-1} $ml^{-1} \times 10^9$; total light yields are in quanta ml^{-1} 5×10^{12}.

(*Inset*) *B. harveyi* (M17) cells were grown in a complex medium at 25° to a density of 190 Klett units, an aliquot (0.1 ml) was diluted into 0.9 ml of ASW/Mops buffer at pH 6.5. Tetradecanoic acid (○) or tetradecanal (●) in ethanol (0.5 nmol of each) was added and the luminescence response with time was recorded. The yields were 2.2×10^{13} and 4.4×10^{12} quanta with fatty acid and aldehyde, respectively.

with regard to both initial maximum intensity and total quanta. This is shown for *B. harveyi* (M17) in Fig. 5. Amounts as low as 5 pmol of tetradecanoic acid have been detected with ease, and smaller amounts could be determined by employing smaller volumes in the assay mix. This response thus provides a potentially valuable bioassay for this compound. Similar results were obtained with *P. fischeri* (Y-1-A1).

The bioluminescence quantum yield of fatty acid and aldehydes *in vivo* can be determined by measuring the total amount of light produced by the addition of small amounts of compounds. In one experiment (Fig. 5, *inset*) the photon yield per molecule was calculated to be about 0.07 for tetradecanoic acid and about 0.015 for tetradecanal. Both the absolute values and their ratios vary considerably, depending upon the physiological state of the cells. With cells harvested at a low cell density, prior to or during the early stage of induction (Fig. 1) the yields as well as their ratios (fatty acid to aldehyde) may be much less than with cells harvested at the peak of luminescence. Or if cells are washed and resuspended in buffer free of nutrient, the yield with fatty acid is lower than with cells resuspended in buffer containing glucose. Finally, if the fatty acid produced in the reaction (18) recycles (19), then higher apparent quantum yields, possibly greater than one, should be obtainable.

Several substituted or unsaturated long chain fatty acids were tested and found to be relatively ineffective in stimulating luminescence (Table 1). Whether or not the small stimulation observed is due to a possible impurity is not certain. But in any event the 14:1, 16:1, and 18:2 unsaturated fatty acids, when added together with tetradecanoic acid, are potent inhibitors of the tetradecanoic acid-stimulated luminescence (Fig. 6). With linoleic acid the percent inhibition is less at higher concentrations of tetradecanoic acid (Fig. 7), indicative of competitive inhibition. The compounds also cause a transient (2 to 4-min) inhibition in aldehyde-stimulated luminescence. The corresponding saturated fatty acids are not inhibitors (i.e., stearic acid does not inhibit tetradecanoic acid stimulation). Even more interesting is the fact that these unsaturated fatty acids are ineffective as inhibitors of the luminescence of the wild-type cells; only the fatty acid-stimulated light emission is affected.

DISCUSSION

Whereas the responses of aldehyde mutants of both *Beneckea* and *Photobacterium* species to long chain aldehydes show relatively low specificity with respect to chain length, their responses to fatty acid are quite specific in this regard. Tetradecanoic acid is at least 20 times more active than other naturally occurring (even-numbered) fatty acids, and the 10-carbon acid is less effective than tetradecanoic acid by a factor of 10^{-3}. The fact that the fatty acid response remained undiscovered so long certainly relates in part to this fact.

The striking activity of tetradecanoic acid, including the relatively high bioluminescence quantum yield that it provides, suggests the possibility that it is the natural precursor of the aldehyde. On the other hand, some doubt is cast by the fact that the apparent quantum yield with fatty acid is higher than with aldehyde and the fact that the unsaturated fatty acids inhibit the tetradecanoic acid-stimulated luminescence but not that of the wild-type cells. However, it is possible that transport of fatty acids into the cell could be involved in the inhibition. Luciferase *in vivo* and its aldehyde-generating system might function in a structurally protected site, possibly in a membrane location inaccessible to the unsaturated fatty acids. Evidence suggesting the involvement of membrane polypeptides in the

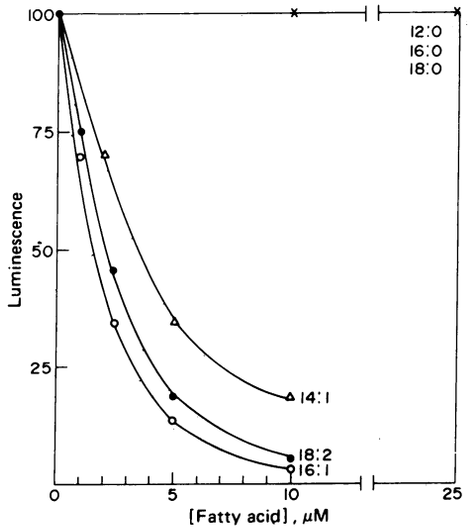

FIG. 6. The effect of unsaturated fatty acids on the stimulation of luminescence by tetradecanoic acid. M17 cells were grown at 27° in a complex medium to final cell density of 200 Klett units. Samples (1 ml) were placed in vials in the presence of a constant amount of tetradecanoic acid (25 μM) and different concentrations of unsaturated fatty acids (myristoleic, 14:1; palmitoleic, 16:1; and linoleic acid, 18:2). The maximal luminescence determined was plotted as percent of the control luminescence obtained with tetradecanoic acid alone (after subtraction of the luminescence obtained by the tested fatty acid at the concentration used; see Table 1).

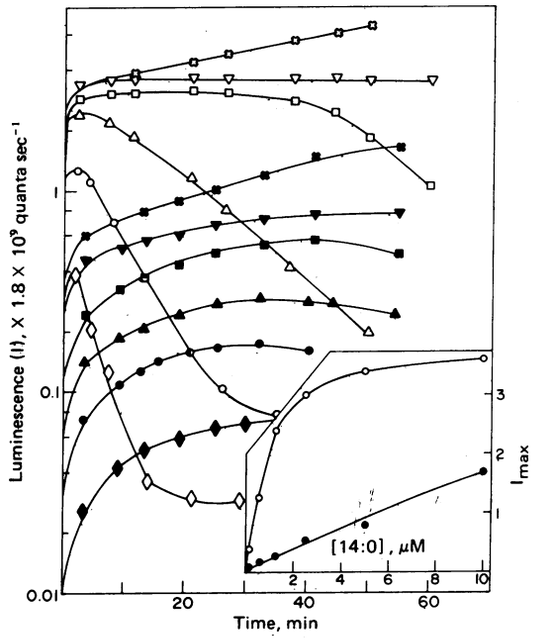

FIG. 7. Time courses of stimulation by tetradecanoic acid (14:0) at different concentrations with and without linoleic acid. M17 cells were grown in a complex medium to a cell density of 190 Klett units at 27°. The culture was diluted 1:20 into ASW, pH 6.5. Aliquots (1 ml) were placed in scintillation vials in the presence of different concentrations (as plotted in *Inset*) of tetradecanoic acid without (open symbols) or with (filled symbols) a constant amount (5 μM) of linoleic acid. The luminescence was determined as a function of time. The *Inset* shows the maximal luminescence (I_{max}) for the twelve different experiments plotted as a function of the tetradecanoic acid concentration with (●) and without (○) linoleic acid.

bacterial luminescence system (20) lends credence to this suggestion, as does the recent demonstration of a glycoprotein with bacterial luciferase activity (21). The one report (22) of the isolation of natural aldehyde (from cells of two species, *Photobacterium phosphoreum* and *Achromobacter fischeri*) also suggests that the 14-carbon aldehyde may be the one functioning *in vivo*. Although the total quantity of extractable aldehyde was very small (enough to sustain luminescence for less than one second), the longer chain aldehydes (12, 14, and 16 carbons) contributed the major percentage. Tetradecanal comprised 63% of the aldehydes extracted in *P. phosphoreum*, while in *A. fischeri* it was 32%. The fact that tetradecanoic acid acts at such low concentrations and results in such high quantum yields with aldehyde mutants indicates that it is functionally significant. How it specifically ties into the cellular reaction is a matter for future investigation.

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