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Inhibition of bioluminescence in *Photobacterium phosphoreum* by sulfamethizole and its stimulation by thymine

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In bioluminescent bacteria very few agents have been reported that can selectively inhibit the luminescence. In sensitivity tests with *Photobacterium phosphoreum*, using 55 different antibiotics, it was found that sulfamethizole, an inhibitor of dihydropteroate synthetase and the formation of folic acid, inhibited bioluminescence more than growth. Likewise, in mutants requiring thymine for growth, the luminescence per cell was much less in a medium low in thymine. In neither case could the decreased specific luminescence be attributed to a decrease in the cellular level of luciferase or aldehyde factor; the involvement of additional but unidentified factors in the regulation of in vivo bioluminescence is postulated.

Introduction

The bacterial bioluminescence reaction is classically viewed as a shunt of the electron transport system at the level of reduced flavin. The key enzyme, luciferase, serves as a mixed function oxidase in a reaction in which reduced flavin mononucleotide ($FMNH_2$) and long-chain aliphatic fatty aldehyde are oxidized to FMN, fatty acid and water, with the concomitant emission of light [1,2].

Very few agents are known that are specific for the inhibition of the bacterial luciferase reaction. Among these are 2-diethylaminoethyl-2,2-diphenylvalerate and 2,3-dichloro-(6-phenylphenoxy)ethylamine, which are competitive with flavin and aldehyde, respectively [3]. Pargyline (*N*-benzyl-*N*-methyl-2-propylamine) inhibits luminescence both in vivo and in vitro; its action in the luciferase reaction is competitive with both $FMNH_2$ and aldehyde [4]. In vivo, it may also inhibit aldehyde synthesis. The antibiotic cerulenin clearly and specifically blocks the reduction of fatty acids to the long-chain aldehydes required in the luminescent reaction [5].

As described in this communication, screening with a large number of different antibiotics resulted in the finding that one of these, namely sulfamethizole, also specifically inhibits bioluminescence. However, this effect did not appear to involve a lowering of cellular levels of luciferase, reduced flavin or aldehyde, or substrate competition. The known action of sulfamethizole relates to its inhibition of dihydropteroate synthetase, an enzyme in the pathway leading to the formation of dihydrofolate and the synthesis of thymine [6]. For this reason we generated and isolated mutants requiring thymine for growth and found that in a medium low in thymine the bioluminescence per cell of those mutants was much less. In this case also, the decreased luminescence could not be attributed to a decrease in the amount of luciferase or aldehyde. The nature of the effect of thymine on bacterial bioluminescence remains a question.

Materials and Methods

P. phosphoreum, strain 496 [7] was maintained on slants in a complex medium containing, per liter of distilled water, 3 g fish extract, 3 g yeast extract, 5 g peptone, 3 ml glycerol, 10 or 30 g NaCl, 0.1 g $MgSO_4 \cdot 7H_2O$ and 2 mM phosphate buffer (pH 7) with 15 g agar added for a solid medium [8]. This medium is low in thymine, such that mutants requiring thymine for growth will grow slowly, even slower if the fish extract is omitted.

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Thymine-requiring mutants were isolated following the procedures of Kimchi and Rosenberg [9]. The wild type was inoculated at 10^7 cells/ml into the complex medium at 25 °C with 100 μg/ml thymine added; after 5 h trimethoprim (50 μgm/ml was added). After 24 h, 1 ml was subcultured into the same medium with 100 μg/ml of thymine and 100 μg/ml trimethoprim. After 24 h, the cells were plated for single colonies on a solid complex medium with 100 μg/ml thymine added. The resulting colonies were then replica plated onto complex medium lacking fish extract, with and without added thymine. About 10% were found to grow slowly without added thymine; these were also nonbioluminescent, and were selected for study.

For experimental studies cells were inoculated at a density between 10^6 and 10^7 cells/ml into 100 ml medium in 300 ml Erlemeyer flasks and incubated at 25 °C in a reciprocal-type shaker (50 rpm). Samples were removed as needed for measurements of cell density and light emission, as well as for enzyme activities in cell free extracts.

Cell density was measured by absorbance at 660 nm with a filter to cut off light below 600 nm, thus excluding bioluminescence. These measurements were linear up to an A of 0.6, above which cultures were diluted prior to measurement. An A of 0.1 corresponded to $1.4 \cdot 10^7$ cells/ml, as determined by plating and colony counts.

Luciferase was measured as luminescence in a nonturnover assay initiated by chemically reduced flavin mononucleotide; flavin reductase was measured by light emission initiated by NADH in the presence of excess reductase-free luciferase [10]. Bioluminescence was measured with a photomultiplier photometer and recorded graphically on a chart recorder, as previously described [11]. Intensity values are given in arbitrary units. For measurements of in vivo bioluminescence, 1 ml of cell suspension was mixed with 2 ml of fresh medium (1 or 3% NaCl) in a 3 ml vol. vessel located in front of the photomultiplier window. The dilution per se had no effect on the luminescence within the measurement time (about 2 min).

Results and Discussion

Fig. 1 shows tests of 53 antibiotics, in which their effects on growth and in vivo bioluminescence of cells of *P. phosphoreum* were compared. Antibiotics in the amounts indicated (Table I) were added to the cultures in 3 ml at a density of $5 \cdot 10^7$ cells ml. Incubation with shaking was carried out for 4 h at 20 °C, at which time measurements of cell density and bioluminescence were made. For most antibiotics the inhibition of bioluminescence was accompanied by a proportional inhibition of growth. An exception to this was sulfamethizole (No. 46); luminescence was strongly inhibited at a concentration where growth was not.

This result was confirmed and extended by measurements of the effects at three different concentrations of sulfamethizole on luminescence as related to cell density (Fig. 2). At the highest concentration of the drug (10^{-3} M), the specific luminescence (luminescence per unit cell density) was orders of magnitude less than the control at a given cell density. However, this is not attributable to a reduction in the activities of either luciferase or flavin reductase, as measured in extracts at 5 h and 14 h after the addition of the drug (Table II). At $3 \cdot 10^{-4}$ M sulfamethizole, which does not inhibit

TABLE I

Antibiotics tested and amounts used

The 53 antibiotics tested for effects on growth and luminescence with number assignments and quantities added to 3 ml of medium.

0	No antibiotic	19	furazolidone (20 μg)	37	penicillin (20 U)
1	Aminobenzylpenicillin (30 μg)	20	fusidic acid (30 μg)	38	phenoxyethylpenicillin (40 U)
2	Aminodeoxykanamycin (50 μg)	21	gentamicin (30 μg)	39	phenoxypropylpenicillin (30 μg)
3	Bacitracin (2 U)	22	hetacillin (30 μg)	40	polymixin B (100 U)
4	Carbenicillin (30 μg)	23	josamycin (30 μg)	41	ribostamycin (50 μg)
5	Cefazolin (30 μg)	24	kanamycin (50 μg)	42	spiramycin (30 μg)
6	Cephalexin (30 μg)	25	leucomycin (30 μg)	43	streptomycin (50 μg)
7	Cephaloridine (30 μg)	26	lincomycin (30 μg)	44	sulbenicillin (30 μg)
8	Cephalothin (30 μg)	27	methacycline (200 μg)	45	sulfadimethoxin (400 μg)
9	Clindamycin (30 μg)	28	methylchlorophenyl-isoxazolylpenicillin (30 μg)	46	sulfamethizole (400 μg)
10	Colistin (150 U)			47	sulfamethomidine (400 μg)
11	Demethylchlortetracyclin (200 μg)	29	methyphenylisoxazolylpenicillin (30 μg)	48	sulfamethoxypyridazine (400 μg)
12	Dibekacin (30 μg)	30	midecamycin (30 μg)	49	sulfamonomethoxin (400 μg)
13	Dicloxacillin (30 μg)	31	mikamycin (30 μg)	50	sulfaphenozole (400 μg)
14	Dimethoxyphenylpenicillin (30 μg)	32	minocycline (200 μg)	51	sulfisomezole (400 μg)
15	Doxycycline (220 μg)	33	nitrofurantoin (100 μg)	52	sulfisomidine (400 μg)
16	Erythromycin (50 μg)	34	oleandomycin (30 μg)	53	sulfisoxazol (400 μg)
17	Flucloxacillin (30 μg)	35	oxytetracycline (200 μg)	54	tetracycline (200 μg)
18	Fradiomycin (20 μg)	36	paronomycin (30 μg)	55	tobramycin (30 μg)

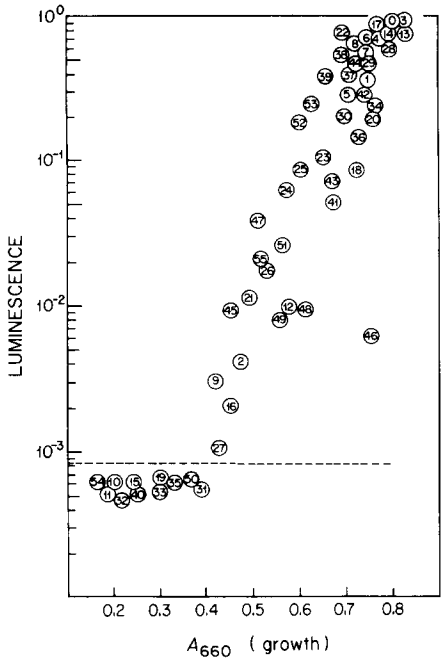

Fig. 1. Plot of growth (A, abscissa) vs. luminescence (ordinate) after growth with 53 antibiotics. Number key is given in Table I. Cells were inoculated in complex medium with 1% NaCl and cultured for 6 h at 20 °C, at which time individual tubes with antibiotics as indicated in Table I were inoculated with 1.5 ml of the culture and 1.5 ml of fresh medium containing 5% NaCl, thus bringing the final NaCl to 3%. Measurements of luminescence and A were made after incubation at 20 °C for 4 h. Dotted line is lower limit of detection of luminescence.

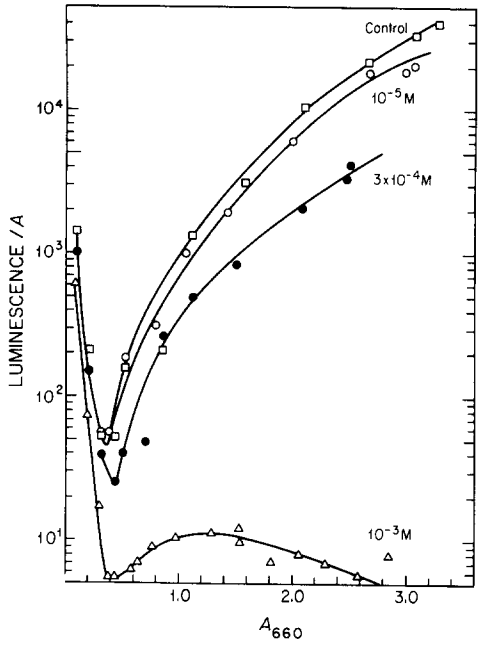

Fig. 2. Effect of sulfamethizole at three different concentrations upon the development of bioluminescence in *P. phosphoreum* at 25 °C. The specific bioluminescence (luminescence/A; ordinate) is plotted as a function of A (abscissa). Cells were inoculated at a density of 10^7 cells/ml in the complex medium without fish extract.

growth, the in vivo bioluminescence per cell is less than 20% of the control at 5 h, while more than 90% of the extractable specific luciferase and flavin reductase activities remain. At 14 h these activities in vitro are somewhat lower, but this is considered to be a secondary effect. The addition of 10^{-3} M thymine to this medium during growth reduced the inhibition by 10^{-3} M sulfamethizole to some extent, but not substantially. Added to the medium at the time of measurement of luminescence, neither thymine nor aldehyde (decanal) was effective in antagonizing the in vivo inhibition.

Because sulfamethizole inhibits a pathway leading to thymine synthesis, we undertook the isolation of thymine-dependent mutants. This was done by incubating cultures in trimethoprim, a drug which inhibits

TABLE II

Effects of sulfamethizole and thymine upon growth (A), luminescence (lum), and enzyme activities (act)

Effect of two concentrations of sulfamethizole on growth (A), luminescence (lum) in vivo ($\cdot10^{-3}$), and enzyme activities in vitro (luciferase and flavin reductase). The induction of luminescence in vivo is far less pronounced here than in the experiments recorded in Fig. 2. This is attributed to differences in the timing of the induction process. Thymine (10^{-3} M) was present in the medium along with 10^{-3} M sulfamethizole in the experiments recorded in the last column. Measurements are also given with thymine (10^{-4} M) and decanal (10^{-5} M; ald) added to the culture at the time of measurements.

Time	Control		Sulfamethizole					
	5 h	14 h	$3\cdot10^{-4}$ M		10^{-3} M		10^{+3} M + 10^{-3} M thymine	
			5 h	14 h	5 h	14 h	5 h	14 h
A	0.5	2.7	0.47	2.7	0.4	1.5	0.37	2.05
Lum/A								
In vivo	2.7	13.3	0.67	2.4	0.11	0.19	0.20	0.30
+ thy	2.7	10.9	0.74	7.6	0.10	0.16	0.21	0.62
+ ald	2.3	12.5	0.73	6.1	0.15	0.17	0.21	0.42
Act/A in vitro								
Luciferase	1.4	1.4	1.3	1.0	1.2	1.2	1.4	1.3
Flavin reductase	5.1	4.5	7.9	1.1	5.4	1.4	4.2	1.1

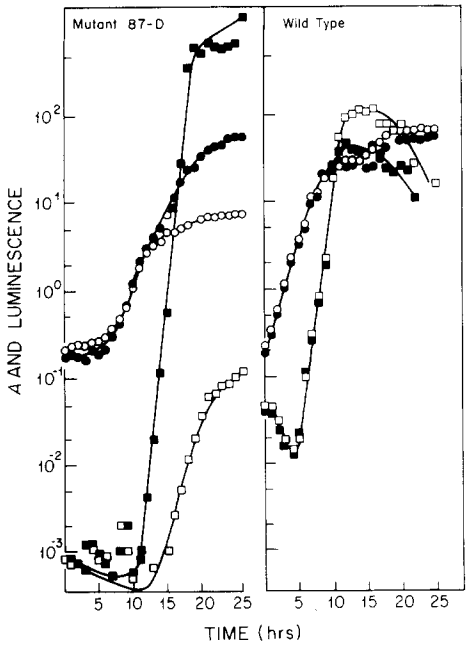

Fig. 3. Effect of added thymine (100 μg/ml) upon the time-course of growth (*A*) and luminescence (ordinate) of the wild-type (right panel) and thymine-requiring mutant 87-D (left panel). Medium and conditions as in Fig. 2. Circles, *A*; squares, luminescence; open symbols, no added thymine; solid symbols, thymine added.

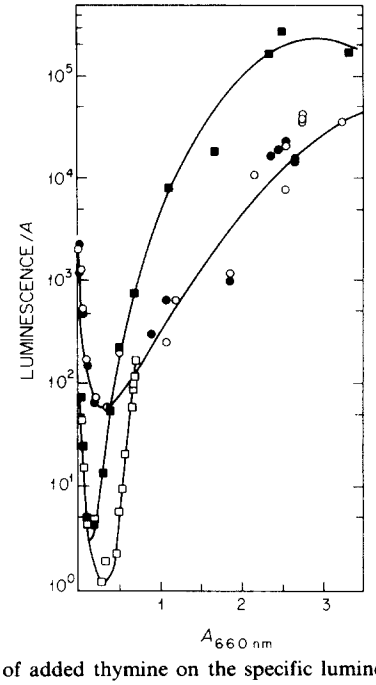

Fig. 4. Effect of added thymine on the specific luminescence of wild type and mutant. Data from Fig. 3. Wild type, circles; mutant 87-D, squares; open symbols, no added thymine; solid symbols, thymine added.

dihydrofolate reductase and blocks the synthesis of tetrahydrofolate [12,13].

Isolates dependent upon thymine for growth were selected and studied. When grown in a medium deficient (but not entirely lacking) in thymine, the development of bioluminescence was severely restricted in relation to cell growth. Time-courses for growth and luminescence with and without added thymine are shown in Fig. 3 for both the mutant and wild type. When specific luminescence (luminescence/*A*) is plotted as a function of *A* (Fig. 4), the large difference between the specific luminescence of the mutant with and without added thymine is evident; no such difference was seen in the wild type.

This stimulation of luminescence in the thymine-requiring mutant is also not associated with increases in luciferase or flavin reductase activities. As shown in Table 3, the addition of thymine to a thymine-deficient medium stimulates in vivo growth and bioluminescence substantially, but has little or no effect on the activities of luciferase or flavin reductase in extracts. Thymine is thus able to exert some effect on the expression of the luciferase system.

TABLE III

Effect of thymine addition to the medium upon growth (A), luminescence (lum) in vivo and extractable enzyme activities in thymine-requiring mutants

Effect of thymine addition upon growth, luminescence and enzyme activities in three thymine-requiring mutants: 64-D, 77-D and 87-D. For the in vivo determinations, the second and third rows represent assays in which thymine (10^{-4} M) and aldehyde (10^{-5} M) were added to the culture at the time of the measurements.

	Mutant strains					
	64-D		77-D		87-D	
	− thymine	+ thymine	− thymine	+ thymine	− thymine	+ thymine
A	0.268	2.50	0.28	0.40	0.33	1.58
Lum/*A*						
In vivo	128	30400	223	1980	41	90500
+ thy	131	32400	217	1880	46	99600
+ ald	150	25600	211	1750	37	85400
In vitro luc/*A*						
Luciferase	16.5	15.0	16.3	18.5	17.8	21.3
Flavin reductase	4.22	5.35	6.5	5.6	8.16	8.72

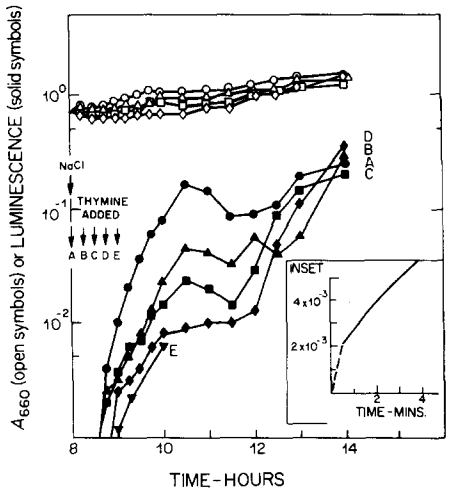

Fig. 5. Growth and luminescence with thymine additions to a thymine-requiring mutant at different times after 2h thymine starvation and step up of NaCl concentration (at hour 8). Cells were grown for 6 h in complex medium with 1% NaCl and added thymine (100 μg/ml). After centrifugation and resuspension for 2 h in 1% NaCl medium without fish extract and no thymine, solid NaCl was added at hour 8 to bring the concentration to 3%, and thymine at the times indicated; subsequent growth and luminescence were recorded. A–E: thymine additions at hour 8 and at 15 min intervals thereafter. The time lag for the appearance of luminescence after thymine addition becomes less, the later is the addition. Ordinates, cell density, A_{660}, and luminescence in arbitrary units. Abscissa, time after inoculation of cultures. Inset shows on an expanded scale kinetics of onset of luminescence following addition of thymine at 60 min (E, at hour 9).

To what might this action be attributed? One possibility is that in cells subject to a growth restriction, the factor responsible for autoinduction is destroyed more rapidly than it is synthesized. However, autoinducer has not actually been demonstrated in this strain of *P. phosphoreum*. Autoinduction has previously been attributed to the shunting of reducing power to luciferase from other cellular functions [11], also in the absence of increases in the levels of either luciferase or NAD(P)H-FMN oxidoreductase activities. A similar mechanism might be involved in the action of thymine. Autoinduction in our *P. phosphoreum* strain contrasts with certain other luminous species and strains, where this increase can be attributed, at least in part, to increases in luciferase and other enzymes associated with the light-emitting system [14,15].

TABLE IV

Effect of thymine on growth and induction of bioluminescence in two thymine-requiring mutants

Effects of different additions to the medium upon growth and luminescence in a thymine-requiring mutant after thymine starvation. Cells were grown for 6 h in complex medium plus 100 μg/ml thymine, then centrifuged and resuspended in medium without fish extract and no added thymine. Additions as indicated were made at hour 8.

Additions	Growth		Luminescence	
	mutant A	mutant B	mutant A	mutant B
3% NaCl	0	25	< 0.03	< 0.03
3% NaCl + thymine	100	100	100	100
3% NaCl + uracil	0	5	< 0.03	0.1
1% NaCl + thymine	80	100	< 0.03	< 0.03

This autoinduction of the luminescence does not occur in cells of *P. phosphoreum* grown in a medium with only 1% NaCl. However, if solid NaCl is added after several hours growth at a cell density of about 10^8 cells/ml, luminescence develops within 30–45 min [8]. With a thymine-requiring mutant, added thymine is required for the occurrence of luminescence in a medium lacking thymine (Fig. 5). An interesting but unexplained observation is that stimulation by thymine can occur even if added much later than the addition of NaCl, and in this case the response to thymine may occur rapidly, within minutes (Fig. 5). Thus the thymine requirement for luminescence may be distinguished from the salt-dependent induction of the system.

In the experiment of Table IV, four cultures were inoculated into 1% NaCl complex medium with thymine added, grown for 6 h, centrifuged and resuspended in medium lacking fish extract and without thymine. At hour 8, solid NaCl was added to three flasks to bring the concentration to 3%, along with thymine in one and uracil in a second. Thymine alone was added to a fourth. Luminescence developed only in the flask with 3% NaCl and thymine; growth was also good in the flask with 1% NaCl and thymine, but no luminescence developed.

Five structural genes (*lux* A, B, C, D and E) are known to be involved in the several species of bioluminescent bacteria studied [16,17]. In *P. phosphoreum* a sixth structural gene (*lux* F), which codes for a 26 kDa protein, has recently been reported to occur as a part of this operon [18]. Transcriptional regulation in *Vibrio fischeri* is known to involve two additional upstream genes, controlling autoinducer (a *lux* I gene product) and its receptor protein (the *lux* R gene product). Transcription is also dependent on cAMP and the cAMP binding protein [19]; it also involves both the Sigma 32 (htpR) protein and *Lex* A protein [20]. Although not yet elucidated in *P. phosphoreum* (or other species), regulation promises to have some similarities, but also some differences from the *V. fischeri* system, as suggested by recent studies in *V. harveyi* [21]. The autoinduction and thymine stimulation of in vivo luminescence reported here occur without concomitant increases in any of the products of the known structural genes of the *lux* operon, indicating that the effects are mediated by a factor or factors not yet identified.

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