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Use of Controlled Luciferase Expression To Monitor Chemicals Affecting Protein Synthesis

JORMA LAMPINEN,[1,2]* MARKO VIRTA,[1] AND MATTI KARP[2]

Department of Biochemistry and Pharmacy, Åbo Akademi University, FIN-20521 Åbo, Finland,[1] and Department of Biochemistry and Biotechnology, University of Turku, FIN-20520 Turku, Finland[2]

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In this article, we present a new bioluminescent test system for the screening of chemical compounds with an inhibitory effect on protein synthesis. The test is based on the measurement of real-time in vivo light production by *Escherichia coli* strains expressing different luciferase genes. The eukaryotic *luc*GR gene from *Pyrophorus plagiophthalamus* was found to be the best of three types of luciferase genes tested. Chemicals with known inhibitory effects on protein synthesis were used as test chemicals together with some general toxicants. The incubation of a test chemical with cells was performed either prior to or after the induction of protein synthesis, and the difference in the results of the two methods distinguishes the possible influence on protein synthesis from direct metabolic inhibition. Using lyophilized bacteria, the test is performed in less than an hour without any bacterial cultivation, which makes the test suitable for rapid and sensitive screening of chemicals or environmental samples. Compared with the standardized 50% inhibitory concentration calculation method of the bioluminescent cytotoxicity test, the more direct approach of calculation developed in this study proved to be more convenient than and as reliable as the standard method.

Among many different luminous groups, two luminescent systems are perhaps best known: the bacterial system, illustrated by *Vibrio harveyi* and *Vibrio fischeri*, and the firefly system, represented by the North American firefly (*Photinus pyralis*). The enzyme responsible for light production is called luciferase, and in bacterial systems, it is a dimeric protein with two nonidentical subunits (*lux*A and *lux*B) (7, 18). All bacterial luciferases catalyze a reaction that involves the oxidation of a long-chain aldehyde and $FMNH_2$ and produces blue-green light (15). The genes of the synthetic pathway for the aldehyde substrate are located on the same operon with two luciferase genes, but only the luciferase genes are essential for light production (30) if aldehyde substrate is added exogenously. All naturally luminescent *Vibrio* strains also produce a low-molecular-weight autoinducer controlling the expression levels of luciferase, which causes cell density-dependent bioluminescence (29).

Another type of luminescence mechanism and luciferase are found in insects, for example, in the North American firefly (*P. pyralis*) and in the luminous click beetle *Pyrophorus plagiophthalamus*. The firefly luciferase catalyzes oxidation of the substrate D-luciferin in the presence of ATP, yielding oxyluciferin, AMP, PP_i, and light (28). The click beetle luciferases are able to produce light of four different colors, the emission maximum ranging from 547 to 593 nm. The corresponding four genes have been cloned and expressed in *Escherichia coli* (40).

Bioluminescent bacteria have been used for in vivo monitoring of the toxicity of environmental samples (3), and commercial assays based on these bacteria are also available (for example, Microtox; Microbics Co., Carlsbad, Calif.). The main problems are the low temperature optimum (15°C) and high NaCl concentration, which remarkably reduces the sensitivity to almost any chemical (16). Other in vivo toxicity assays based on recombinant strains have also been developed to avoid those troubles (24, 26).

We have previously described a set of different vectors for luciferase expression in well-known host organisms such as *E. coli* and *Bacillus subtilis* (24–26). These luminescent organisms have been shown to perform in bioluminescence toxicity tests similarly to the wild-type *V. fischeri* (3), but the test can be performed at higher temperatures (25 to 30°C) and lower salinity (down to 0%). In addition, the changes in luminescence can be controlled easily. The clear difference between the bacterial and insect luciferases has been crucial. Insect luciferases seem to be more sensitive indicators of any toxic effect in almost all cases. This is probably a result of the direct use of the central metabolite ATP in the enzyme reaction instead of $FMNH_2$, which means that the insect luciferase reaction reflects the intracellular state more directly than that of the bacterial luciferase (22, 26).

In this study, we have used the efficient regulation of luciferase synthesis for testing chemicals affecting protein synthesis. We have previously showed that recombinant bacteria can be used as biosensors in luminescent cytotoxicity tests (24, 26, 39). All earlier recombinant strains carried a constitutive luciferase expression system and the selectivity based on the host bacteria. Luciferase genes were used only as a convenient reporter for the detection of general metabolic inhibition of the cell. The assay system presented in this paper uses a different approach, since the selective sensitivity to protein synthesis inhibitors is based on regulation of protein synthesis during the test. In this assay, the detection of toxicity depends directly on the inhibitory effect on the synthesis of the reporter protein. This was achieved by placing three different luciferase genes under the control of the strong bacteriophage λ leftward promoter. Under this promoter, luciferase is not synthesized when bacteria are grown at low temperatures (<36°C), but the synthesis can be very efficiently switched on by a brief heat shock, resulting in accumulation of recombinant protein to up to 25% of total cellular protein (36). The system was used to compare test procedures in which luciferase is already present when a

* Corresponding author. Mailing address: Department of Biochemistry and Pharmacy, Åbo Akademi University, P.O. Box 66, FIN-20521 Åbo, Finland. Phone: 358-21-2654027. Fax: 358-21-2654745. Electronic mail address: jorma.lampinen@abo.fi.

chemical is added with the procedure in which luciferase is synthesized after exposure to the chemical. If the chemical has a negative effect on protein synthesis, the results should differ remarkably between the two procedures. The phenomenon was tested with reference chemicals known to affect protein synthesis and with chemicals having other toxic effects. In addition, we created a simplified method for the calculation of 50% inhibitory concentration (IC_{50}) values from luminescence measurements. The IC_{50} is a central value in short-term microbial assays in the estimation of the toxicity of a chemical, and it is similar to the 50% lethal dose (LD_{50}) value used commonly in animal testing (3, 5, 6). The IC_{50} is generally defined as the concentration at which the phenomenon being tested is reduced to 50% of that in the untreated control. The phenomenon can be the activity of a certain enzyme, dye uptake, mobility, etc., or, as in case of LD_{50} in animal testing, mortality. According to this, the IC_{50} is defined for bioluminescent assays as the concentration at which the light production of the sample exposed to the toxic chemical is 50% of that of the unexposed sample. The results obtained with the new approach show excellent correlation with those of the standard protocols.

MATERIALS AND METHODS

Bacteria and plasmids. The bacterial strain used was *E. coli* K-12 strain M72 [Sm^r *lacZ*(Am) Δ*bio-uvrB* Δ*trpE42* (λ*N7*(Am)-*N53*(Am) *cI857* Δ*H1*)] (2). This strain was chosen because it carries a chromosomal insertion of the *cI857* repressor gene that is essential for the temperature-sensitive regulation of all plasmid constructs. Three plasmid constructs with different luciferase genes were used as reporters in bioluminescence tests. The strains were maintained on L agar (LA) plates and grown in Luria-Bertani (LB) (34) medium supplemented with ampicillin (100 μg/ml), with shaking (260 rpm) at 30°C.

The cloning procedures used to construct the plasmids were as follows. To produce pCSS301, pPLcAT110 (37) was digested with *Xba*I and treated with calf intestinal phosphatase (Pharmacia, Uppsala, Sweden). The *P. plagiophthalamus* luciferase gene (*luc*GR) was removed from plasmid plucGRtac (40) as a 1.6-kb *Bsp*HI fragment, and cohesive ends were removed with mung bean nuclease as described before (34). The two DNA fragments were ligated, transformed by electroporation (23) into *E. coli* K-12, and plated to LA plates supplemented with ampicillin (100 μg/ml). To produce pPHOLU, pPLcAT10 (37) was digested with *Cla*I and *Hin*dIII, and plasmid pCGLS11 (12), carrying the *lux*AB genes of the luminescent bacterium *Photorhabdus luminescens* (formerly called *Xenorhabdus luminescens*), was digested with *Cla*I and partially with *Hin*dIII (a 3.0-kb fragment) and ligated with pPLcAT10. Plasmid pCSS118 was produced by a different technique, including two cloning steps. The *lux*AB genes of *V. harveyi* were digested from plasmid pWH102 (14) with *Sal*I and *Pvu*II and ligated with *Sal*I- and *Pvu*II-digested pPLcAT10 to produce plasmid pCSS112. Thereafter, the gene encoding the repressor protein cI857 from plasmid pOU61 (27) was digested with *Bgl*II and *Sal*I, filled in with Klenow enzyme, and introduced into the *Pvu*II restriction site of plasmid pCSS112 to produce plasmid pCSS118. As a result, this plasmid contains the *cI857* allele, which facilitates the regulation of *lux* genes in *E. coli* strains which do not contain the wild-type repressor allele. The resulting plasmids were screened for light production and induction by heat treatment, and plasmid structures were confirmed by restriction analysis and partial DNA sequencing (34).

For lyophilization, a single colony was inoculated into 5 ml of medium and grown overnight; thereafter, the culture was diluted 1:100 with fresh medium and grown at 30°C to an optical density at 600 nm of 1.5. Cells were harvested by centrifugation (5,200 × *g*, 10 min), suspended in the same volume of protective medium containing 15% sucrose, and lyophilized as 0.5-ml aliquots by standard procedures (17, 35). Lyophilisates were tested for effective regulation of the *p*L promoter and efficient induction of luciferase synthesis by comparing the induction factors of lyophilized cultures with those of freshly prepared cultures.

Measurement of activity and induction efficiency. Rich medium (LB), minimal medium (M9-minimal medium) (34), Hanks' balanced salt solution (HBSS) (24), and 100 mM citrate buffer (pH 5.0) were tested for in vivo luciferase activity and heat-induced protein synthesis efficiency. Cells were diluted for the standard activity measurements with an appropriate buffer to the proper cell density (roughly 5×10^5 cells per assay), and 100 μl of the cell suspension was transferred to the wells of a white microplate (Labsystems Oy, Helsinki, Finland). Luciferase synthesis was induced by denaturing the repressor protein cI857 with a 5-min heat shock in a 42°C water bath, after which samples were kept at 30°C for 30 min. After the incubation period, the luminescence reaction was started by adding 100 μl of the substrate solution. As a substrate, 1.0 mM D-luciferin (BioTools Oy, Turku, Finland) in 100 mM citrate buffer (pH 5.0) was used for click beetle luciferase and 0.001% (vol/vol) *n*-decanal sonicated in water was used for bacterial luciferases.

Light emission was measured with a Luminoskan microplate luminometer (Labsystems Oy) connected to a microcomputer. The luminometer is a photometric device with a photomultiplier tube, and the measured unit is a relative light unit corresponding to the number of photons emitted by the sample, presented as millivolts. Maximal light production was used to determine induction factor I as follows: induction factor $I = I_I/I_B$, where I_I is the maximal light emitted by the induced sample and I_B is the maximal light emitted by the noninduced sample. Measurements were performed with four replicates and repeated at least three times, and averages of all replicates were used. In the inhibition measurements, the concentrations causing 50% inhibition were calculated by comparing inhibited samples with uninhibited controls (inhibition, 0%) at the same time point. The relative inhibition of each sample was then calculated and plotted against the concentration. The linear parts of the curves were used for calculation of IC_{50} values with regression analysis. Values for background luminescence were omitted from all measurements and calculations, because both cell controls without substrates and substrate controls without cells were constantly below 1% of the lowest measured values of uninhibited samples.

Reference chemicals and measurements of protein synthesis inhibitors. Four reference chemicals without known direct effects on protein synthesis were selected to detect the bioluminescent response: sodium fluoride (Sigma S1504), nicotine (Sigma N3836), mercuric chloride (Sigma M1136), and phenol (Carlo Erba 108-95-2). They have been used in our earlier work (24, 26); they are also reference chemicals proposed by the Multicenter Evaluation of In vitro Cytotoxicity (4) project, and reliable reference data are therefore available (6, 19, 20, 21). The reference chemicals with known effects on protein synthesis or indirect membrane effects used were tetracycline hydrochloride (Sigma T7660), chloramphenicol (Sigma C0378), cycloheximide (Sigma C6255), and polymyxin B sulfate (Sigma P1004).

Two different procedures were used in the measurements. Actual protein synthesis inhibition efficiency was measured as follows. The chemical was incu-

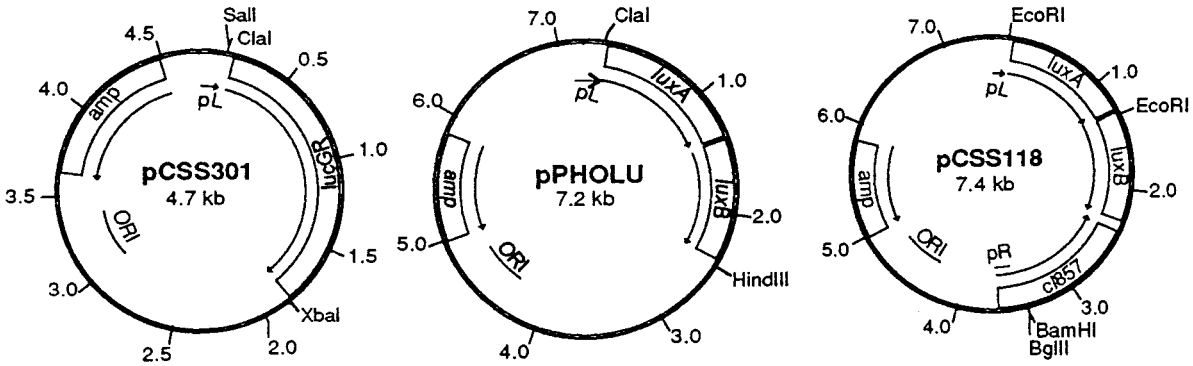


FIG. 1. Structures of inducible luciferase expression vectors. The structures of three different inducible luciferase expression vectors with powerful control of protein synthesis are shown. The essential restriction endonuclease cleavage sites and genetic structures are presented. *luc*GR, eukaryotic luciferase gene from *Pyrophorus plagiophthalamus*; *lux*A and *lux*B, genes for bacterial luciferase subunits A and B, respectively, from *Photorhabdus luminescens* in vector pPHOLU and from *Vibrio harveyi* in vector pCSS118; *amp*, β-lactamase gene; *p*L, bacteriophage lambda leftward promoter; ORI, origin of replication; *cI857*, gene for temperature-sensitive repressor protein cI857; pR, bacteriophage lambda rightward promoter.

TABLE 1. Induction factors of test strains in different media[a]

Strain	Cells	Induction factor	
		LB medium	M9 medium
K-12/pCSS301	Fresh	320 ± 25	130 ± 12
	Lyophilized	340 ± 6	66 ± 5
K-12/pPHOLU	Fresh	41 ± 3	30 ± 3
	Lyophilized	44 ± 2	37 ± 3
K-12/pCSS118	Fresh	260 ± 15	110 ± 17
	Lyophilized	190 ± 13	100 ± 8

[a] The induction factor was calculated as the in vivo luminescence ratio between induced and noninduced samples ($l_{ind.}/l_{nonind.}$).

bated with uninduced cells for 10 min, after which the cells were heat shocked for 5 min to induce luciferase synthesis, the substrates were added, and continuous in vivo measurement was started. In this case, 30 min of contact time was allowed after a 15-min incubation period (method A). The second procedure imitated the standard bioluminescent toxicity test (5) and was performed by inducing luciferase synthesis prior to addition of the reference chemical. In this approach, cells were heat shocked and incubated at 30°C for 15 min, the reference chemical was added, and light emission was measured continuously from that point on (method B). The crucial difference between the methods is that method A measures the ability of the chemical to inhibit protein synthesis and method B measures the ability of the chemical to inhibit general cellular activities. In method B, the inhibition of light emission is independent of protein synthesis, and the method works as a reference control for method A. If the chemical inhibits in vivo light production with higher efficiency in method A than in method B, this is an indication of an inhibitory effect on protein synthesis.

The actual assays were performed as follows. Lyophilized cells were rehydrated with 0.5 ml of buffer, stabilized for 30 min at 30°C, and diluted 1:20 in the same buffer to a final cell density of 5×10^6 ml^{-1}. The cell suspension (90 μl) was transferred to a microtiter plate, and 10 μl of a reference chemical diluted in the same buffer was added. The effective concentration ranges were determined by serial dilutions with a dilution factor of 1:100. The IC_{50} values were then determined by using six concentrations inside the effective range in one measurement with serial dilutions at a dilution factor of 1:5. The concentration ranges used in the final assays were 0.03 to 80 mg/liter (method A) and 3.2 to 10,000 mg/liter (method B) for chloramphenicol, 0.004 to 12 mg/liter (method A) and 2.4 to 7,500 mg/liter (method B) for tetracycline, 0.13 to 400 mg/liter for polymyxin B, 3.2 to 10,000 mg/liter for cycloheximide, 1.5 to 4,600 mg/liter for phenol, 6.7 to 21,000 mg/liter for sodium fluoride, 10 to 32,000 mg/liter for nicotine, and 0.013 to 40 mg/liter for mercuric chloride. Buffer (10 μl) was added to two samples of the cell suspension; one was used as a negative control and the other was used as a positive control to monitor induction of luciferase synthesis. After incubation and induction periods (organization and timing depending on the method used), substrates were added and in vivo luminescence values were measured.

Calculation of IC_{50}. IC_{50}s are calculated by the standard method (5, 10) with correction factors obtained from blank cuvettes. With this correction factor, the natural change in the light emission of a blank sample during the contact time is subtracted from all sample values, and therefore the decrease caused by the toxic agent only can be measured. Calculation is performed according to these standards as follows: correction factor $R_n = l_n/l_0$, where l_0 is light emitted by the blank at time zero and l_n is light emitted by the blank after contact time n. The gamma value is then calculated individually for each sample; it is the measure of decrease and is defined as the ratio between light lost as a result of the toxic effect and light remaining. Therefore, gamma = (light loss)/(light remaining) = $[(R_n \cdot l_0) - (l_n)]/l_n = [(R_n \cdot l_0)/l_n] - 1$, where l_0 is light emitted by the blank at time zero, l_n is light emitted by the blank after contact time n, and R_n is the correction factor. When gamma equals 1, half of the light production has been lost as a result of toxicity, and that is the IC_{50}. The IC_{50} is normally estimated graphically by plotting the gamma values against the corresponding sample concentrations.

We tested whether similar IC_{50}s could be obtained by a more direct approach without any correction factors. We presumed that light emission behavior is completely similar in blanks and in samples, and thus similar kinetic changes in in vivo light production occur in all samples despite the presence or absence of a toxicant. Therefore, the absolute blank and sample values could be used directly to calculate the remaining activities when light emission was measured after exactly the same amount of time as used as contact time for the toxicant. Therefore, remaining activity R (%) = $(l_X/l_B) \times 100$, where l_X is light emitted by sample X after the contact time and l_B is light emitted by the blank after the same contact time. The IC_{50} can then be estimated by plotting these R values against the sample concentrations, and when $R = 50\%$, the corresponding concentration is the IC_{50}.

RESULTS

Plasmid constructions. Plasmid constructs were based on the parental plasmids pPLcAT10 and pPLcAT110 (37) containing the main leftward promoter *p*L from bacteriophage λ. The promoter can be switched on by denaturing repressor protein cI857 upon a temperature shift to 42°C. Three luciferase genes representing both bacterial and insect luciferases were cloned under the regulation of the λ *p*L promoter. The luciferase genes used were *luc*GR from *P. plagiophthalamus* (40), *lux*AB from *P. luminescens* (12), and *lux*AB from *V. harveyi* (14), and the resulting plasmids were named pCSS301, pPHOLU, and pCSS118, respectively (Fig. 1).

Bioluminescence properties of test strains. The induction factor indicates the efficiency of protein synthesis induction

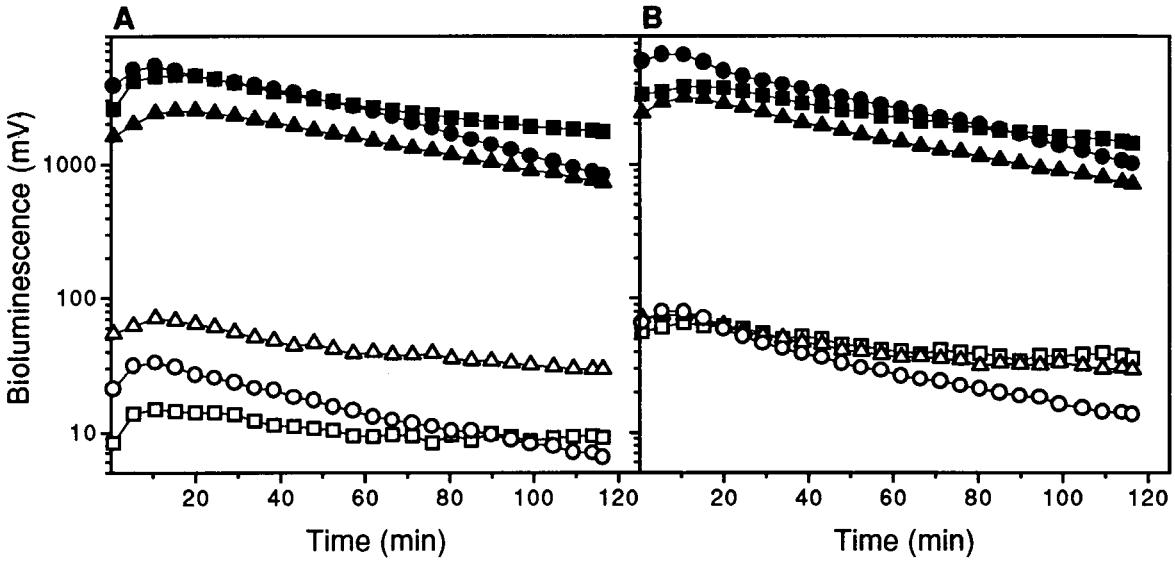


FIG. 2. Efficiency of luciferase synthesis induction: comparison of induced and noninduced samples in two different media. (A) LB medium; (B) M9 minimal medium. Squares, K-12/pCSS301; triangles, K-12/pPHOLU; circles, K-12/pCSS118. Open symbols, not induced; solid symbols, induced. The bioluminescence values presented are actual light emissions from the samples. Standard deviations are not shown because they were covered by the symbols.

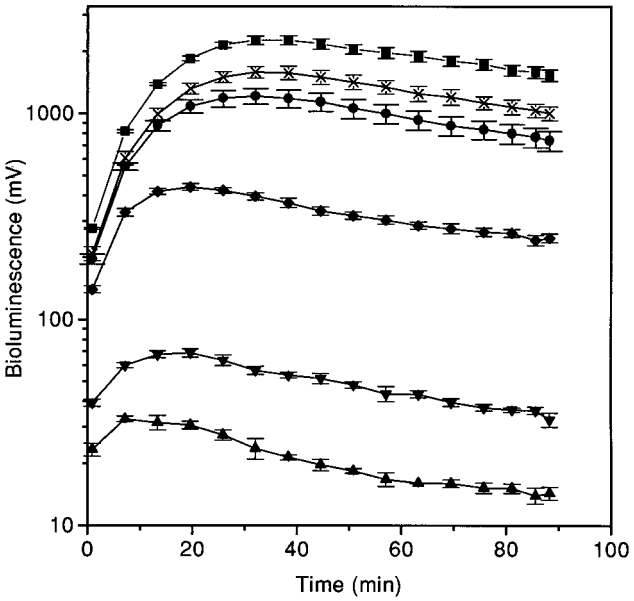


FIG. 3. Typical light emission curves for the bioluminescent sensitivity assay. Different concentrations of chloramphenicol were incubated with K-12/pCSS301 cells in M9 medium prior to protein synthesis induction, and bioluminescence was measured. Chloramphenicol: down triangle 0 (■), 0.13 (×), 0.64 (●), 3.2 (◆), 16 (▼), or 80 (▲) mg/liter. The bioluminescence values presented are actual light emissions from the samples.

and operation of regulatory elements. The induction factors were measured in four different media (LB medium, M9 minimal medium, HBSS, and citrate buffer) without any toxicants to test which would yield sufficient levels of light. In the measurement, about 5×10^5 cells were diluted into 100 μl of each buffer, protein synthesis was induced by a 5-min heat shock, substrate solution was added, and luminescence was measured continuously from that point on. Similar uninduced samples were used as negative controls. The induction factors, as measured by in vivo light emission, were determined for freshly cultivated and lyophilized cell samples, and the results are summarized in Table 1. The results of inductions in HBSS and citrate buffer were omitted from Table 1 because the induction factors were absolutely too small (10 to 35). The time needed for efficient luciferase synthesis could also be determined together with the induction factors, and the results are shown in Fig. 2, which shows the actual light production curves of lyophilized samples used for calculation of the values in Table 1.

When luciferase synthesis is induced at the beginning of the assay, steady, stable light production is not normally observed. The changes in light emission during the assay period were tested with a reference chemical, and typical curves are presented in Fig. 3, where original light emission values from an assay are plotted against time. In this assay, the lyophilized cells were diluted into M9 medium, different concentrations of chloramphenicol were added, the samples were incubated for 20 min at 30°C, protein synthesis was induced, the substrates were added, and light production was measured immediately.

Calculation of IC_{50}s. We calculated IC_{50}s at different time points by using the approaches described above for two different toxicants: chloramphenicol and nicotine. These assays followed assay method A, and changes similar to those in Fig. 3 in light production were observed. The results, calculated according to the two protocols, the standard and our own, are presented in Fig. 4. The latter protocol simplifies the measurements and was therefore used in later assays.

Measurement of protein synthesis inhibition. Only two of the media produced sufficient induction levels for actual assays. Therefore, assays in LB medium and M9 minimal medium were performed with the two described procedures and three chemicals: tetracycline, phenol, and sodium fluoride. The procedures differed in the duration of contact time with the

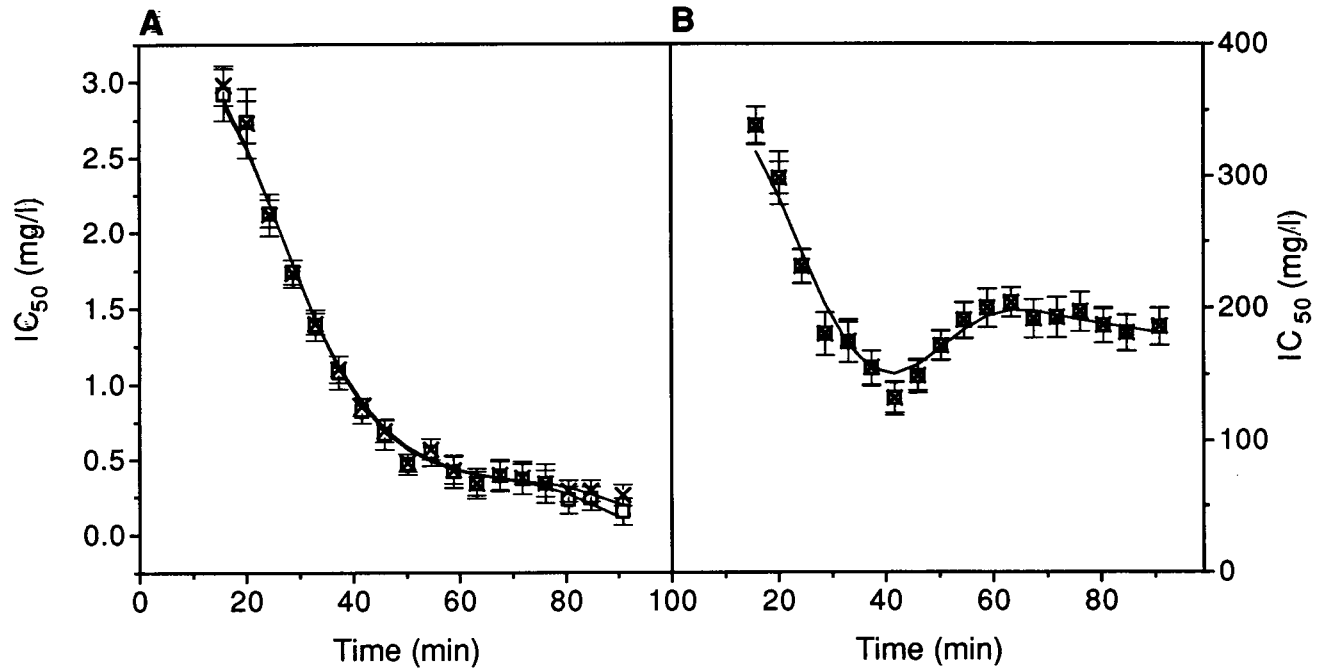


FIG. 4. Comparison of different calculation methods. IC_{50} curves of some sensitivity assays with (A) chloramphenicol and (B) nicotine. IC_{50}s were calculated by standard calculation methods (■) and by the direct comparison method described here (×).

TABLE 2. IC_{50}s in LB medium[a]

Method	Strain	IC_{50} (mg/liter)		
		Tetracycline	Phenol	Sodium fluoride
A	K-12/pCSS301	0.66 ± 0.1	400 ± 27	76 ± 8.2
	K-12/pPHOLU	0.37 ± 0.03	1,100 ± 21	250 ± 120
	K-12/pCSS118	0.07 ± 0.01	1,100 ± 23	1,400 ± 40
B	K-12/pCSS301	10,400 ± 74	200 ± 10	71 ± 5.4
	K-12/pPHOLU	470 ± 38	1,000 ± 57	3,200 ± 160
	K-12/pCSS118	2,000 ± 20	1,100 ± 55	1,800 ± 76

[a] IC_{50}s were measured with 30 min of contact time at 30°C. Each value represents the mean of three independent measurements, each including four replicates. Method A: test chemical added, incubation for 10 min, induction for 5 min, incubation for 15 min, measurement of luminescence. Method B: induction for 5 min, incubation for 10 min, test chemical added, incubation for 30 min, measurement of luminescence. Similar IC_{50}s for the same chemicals in M9 minimal medium are presented in Table 3.

chemical and the time of induction of luciferase synthesis. Light production was always measured continuously from the end of the induction period, and the IC_{50} for the test chemical was calculated from all results. The 30-min IC_{50}s were measured and calculated as described above, and the results from measurements in LB medium are shown in Table 2 and in M9 medium are presented in Table 3.

Two different procedures were used to measure the IC_{50}s of the reference chemicals. The schedules of induction and chemical addition are shown in Table 2. All IC_{50} curves are presented in Fig. 5, and the IC_{50}s with 30 min of contact time are shown in Table 3. On the left side of Fig. 5 are shown IC_{50} curves for when the test chemical and cells are incubated prior to the induction of luciferase (method A), and on the right side are shown curves for when the toxicant was added after the induction of protein synthesis (method B).

DISCUSSION

Bioluminescence properties of test strains. Lyophilized cells were used to ensure high precision and reproducibility. The induction factor values (Table 1) are important parameters in the evaluation of the strains and media for the assay protocols. Protein synthesis was not properly induced in HBSS or citrate buffer, probably because of the shortage of nitrogen source. Only LB medium and M9 minimal medium resulted in sufficiently high levels of protein synthesis to be useful in measurements. The induction factors varied remarkably among three test strains. Plasmid-bearing strains are alike in their regulation and overall structures. For example, the bacterial luciferase subunits *lux*A and *lux*B of *V. harveyi* and *P. luminescens* have high homology at the amino acid level. Between the α subunits, the identity is 85.4% and the similarity is 92.1%; between the β subunits, they are 60.1 and 77.1%, respectively; and for the entire luciferase enzymes, they are 72.8 and 84.2%, respectively. The origins of these bacterial luciferase genes are, however, totally different than that of the insect luciferase gene, and the original hosts all differ from the host bacteria used. Therefore, their expression in *E. coli* was expected to be somewhat different.

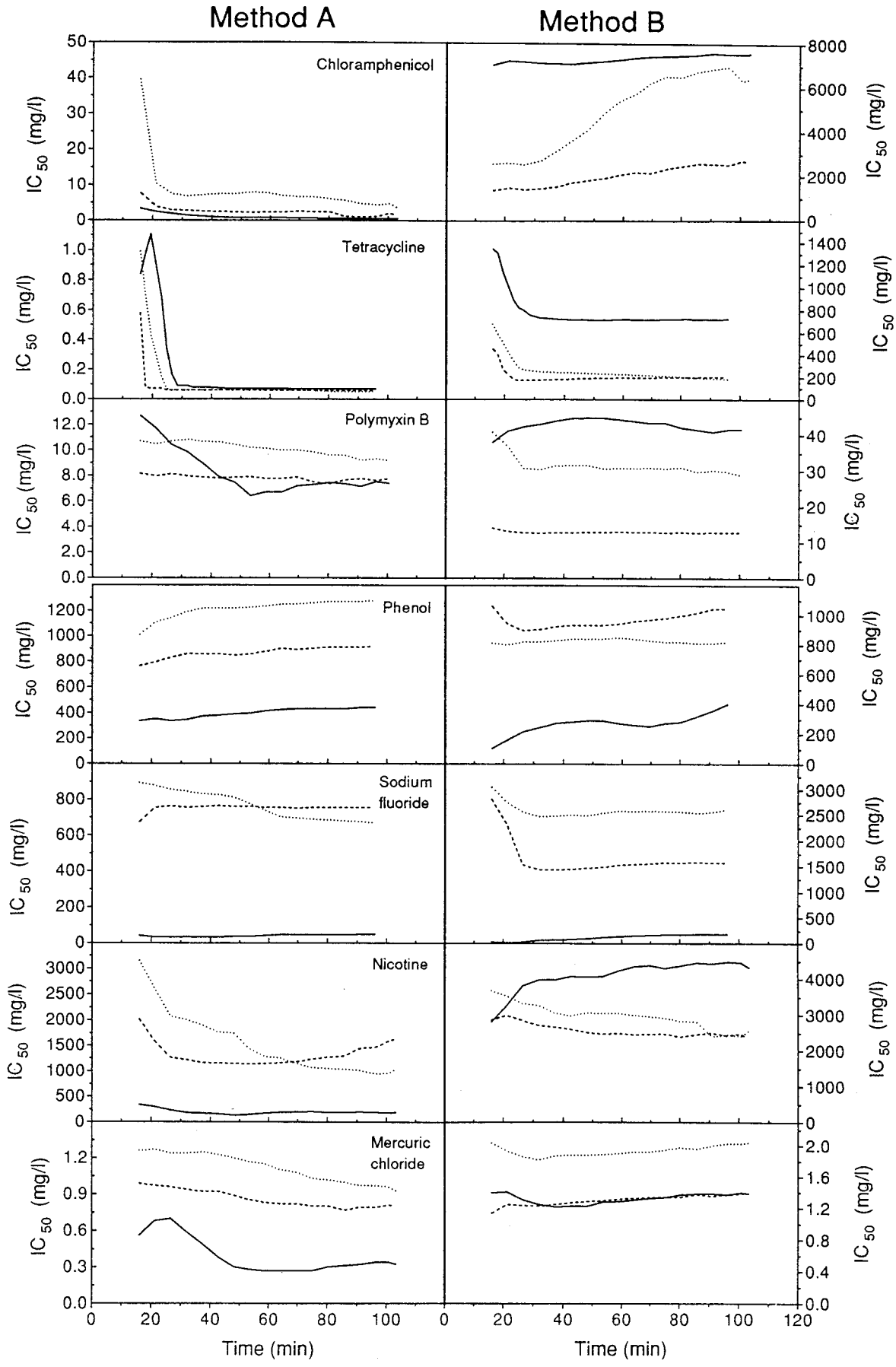
The suitability of the gene structure, measured as codon usage, was checked by using the GCG Program Package (Genetics Computer Group Inc., Madison, Wis.) and the *D*-squared calculation method of Grantham et al. (13). The codon frequencies of the luciferase genes were compared with those of highly expressed genes in *E. coli*. In this method, the *D*-square value gets smaller as the patterns of codon usage become more similar. The *D*-square value for highly expressed *E. coli* genes is 0 when their own frequency table is used as a control. The calculated *D*-square values for luciferase genes were 4.4 for the click beetle luciferase, 3.8 for the *V. harveyi* luciferase, and 7.1 for the *P. luminescens* luciferase. This clearly shows that the genetic structure of the *P. luminescens* luciferase genes is far less suitable for expression in *E. coli* than that of the click beetle or *V. harveyi* luciferases, because the codon usage differs a great deal from that preferred by *E. coli*. This might well be the reason for the fairly low inductions achieved with pPHOLU, but it remains to be solved in the future. Both other constructs were induced rather well in both media. The induction factors of lyophilized and fresh cells were fairly similar in all constructions, although slightly weaker induction was observed with the lyophilized cells. This is probably due to the short incubation period of only 20 min at 30°C after rehydration of the bacteria.

The curves in Fig. 2 show clearly that large amounts of luciferase were synthesized during the 5-min induction period, the highest light production is achieved within 20 min after the beginning of the induction, and the induced levels were tens of times above the uninduced levels. Afterwards, light production remained at a rather constant level for at least 2 h. The curves in Fig. 2 are independent of whether the bacteria have been induced, which results in almost constant induction factors throughout the measurement period. As seen in Fig. 3, light production first increased after induction and thereafter decreased almost linearly. There are probably two reasons for this. Luciferase synthesis is triggered by the heat pulse from an extremely low basal level, and light production rises until the full efficiency of protein synthesis is achieved. Thereafter, a

TABLE 3. IC_{50}s of reference toxicants[a]

Method	Strain	IC_{50} (mg/liter)							
		Protein synthesis inhibitors				General toxicants			
		Chloramphenicol	Tetracycline	Polymyxin B	Cycloheximide	Phenol	Sodium fluoride	Nicotine	Mercuric chloride
A	K-12/pCSS301	1.4 ± 0.1	0.09 ± 0.01	9.8 ± 0.4	$>10 \times 10^3$	350 ± 19	34 ± 3.4	190 ± 16	0.6 ± 0.03
	K-12/pPHOLU	6.8 ± 0.4	0.06 ± 0.01	10.8 ± 0.5	$>10 \times 10^3$	1,200 ± 38	840 ± 34	1,900 ± 49	1.2 ± 0.1
	K-12/pCSS118	2.8 ± 0.3	0.06 ± 0.01	8.0 ± 0.6	$>10 \times 10^3$	860 ± 38	760 ± 55	1,200 ± 65	0.9 ± 0.1
B	K-12/pCSS301	7,100 ± 420	730 ± 36	40 ± 2	$>10 \times 10^3$	240 ± 9.4	63 ± 8.4	4,000 ± 160	1.3 ± 0.1
	K-12/pPHOLU	2,700 ± 240	250 ± 17	31 ± 2	$>10 \times 10^3$	820 ± 56	2,500 ± 170	3,100 ± 230	1.8 ± 0.1
	K-12/pCSS118	1,500 ± 110	170 ± 14	12.8 ± 0.9	$>10 \times 10^3$	900 ± 66	1,400 ± 130	2,700 ± 150	1.2 ± 0.1

[a] See Table 2, footnote *a*.


Method A
Method B
Chloramphenicol
Tetracycline
Polymyxin B
Phenol
Sodium fluoride
Nicotine
Mercuric chloride
IC50 (mg/l)
Time (min)

linear decrease in light output was observed, presumably due to the decrease in the concentration of the substrates as a result of very intense luciferase reaction. Another possibility is that the increase followed by a decrease is due to a pulse of luciferase synthesis and a linear decay because of the instability of the luciferase enzyme.

Calculation of IC_{50}s. There is no difference between the calculation methods, since completely equal IC_{50} values are obtained with both (Fig. 4). This demonstrates that the changes in light production are similar in all samples, and therefore the presence of the toxicant does not have an effect on this phenomenon. Only the level of light production is altered, and therefore it proved to be possible to ignore changes in light production and use light emission values from one contact time. Clearly, there is no need to measure the initial light emission values of each sample, because the same results are achieved with the single endpoint measurement.

Measurement of protein synthesis inhibition. In M9 minimal medium, measurements proved to be slightly more sensitive than in LB medium (Tables 2 and 3), although the former did not produce as high induction efficiencies as the latter (Table 1). This difference between media is probably explained by high levels of proteins, polypeptides, etc., in LB medium, which can bind toxic chemicals and therefore reduce the bioavailability of the toxicant. Since the difference in the sensitivity of the tests is not great, both media can be used for these assays.

The reference chemicals (phenol, sodium fluoride, nicotine, and mercuric chloride) that do not have any inhibitory effect on protein synthesis were selected because of the existing IC_{50} data. With these chemicals, the inhibition of light emission does not reflect directly the inhibition of protein synthesis but the general inhibition of cellular activities. The chemicals were used to confirm the hypothesis that the assay is especially sensitive only to protein synthesis inhibitors. With these chemicals, the IC_{50}s were expected to be similar in the two different methods, induction either before or after exposure, and similar to the values reported previously. The IC_{50}s determined by different researchers and assays for these chemicals vary from 20 to 2,000 mg/liter for phenol, 10,000 to 30,000 mg/liter for sodium fluoride, 40 to 8,000 mg/liter for nicotine, and 0.4 to 3 mg/liter for mercuric chloride (6, 19–21). As seen in Table 3, our results with both protocols agree rather well with these values. Sodium fluoride has a much lower IC_{50} than reported by other researchers, but this phenomenon has also been seen in our earlier experiments (26). In the case of sodium fluoride and nicotine, there is a slight difference between the methods, which suggests that the toxic function of the chemicals might be somehow related to protein synthesis. Because the effect is much weaker than with the known protein synthesis inhibitors, the main toxic effect of these chemicals presumably is metabolic inhibition somehow connected to protein synthesis. The two other chemicals did not behave this way, and therefore the toxicity mechanisms affect something other than protein synthesis.

Four chemicals that are known to inhibit protein synthesis, chloramphenicol, tetracycline, polymyxin B, and cycloheximide, were used as reference chemicals. The chemicals were used to confirm the hypothesis that the assay detects protein synthesis inhibitors with much higher sensitivity than other microbial assays. Their IC_{50}s are up to several tenfold lower with method A than with method B. This is most clearly seen with the common antibiotics chloramphenicol and tetracycline, for which the IC_{50}s were in some cases thousands of times higher when measured with method A (Table 3 and Fig. 5). The IC_{50}s measured with method B were somewhat higher than those reported by some others (9, 32), but their measurements were based on cell-free systems. Tetracycline hydrochloride inhibits protein synthesis by interfering with the binding of aminoacyl-tRNA to the ribosomal A site of 16S rRNA (31), and concentrations of about 30 μM (14 mg/liter) should produce 50% inhibition in *E. coli* (9). Chloramphenicol has been reported to inhibit bacterial protein synthesis by blocking the peptidyl transferase reaction of the 50S subunit, and the IC_{50} has been reported to be about 20 μM (6.4 mg/liter) (32). The in vivo measurement that we used determines only that portion of the chemical that is available for biological functions, so the difference is to be expected because of the membrane permeability barrier, binding to cell organelles, and detoxification. The results are, however, in rather good agreement with those obtained with chloramphenicol in in vivo assays: Calleja et al. (6) determined the 50% effective concentrations to be between 500 and 3,000 mg/liter in five different ecotoxicological tests, and in a French multicenter study involving six laboratories, Fautrel et al. (11) measured LD_{50}s for chloramphenicol with rat hepatocytes to be in the range of 1,800 to 2,500 mg/kg.

Polymyxin B sulfate was selected as a reference chemical because it has a strong disruptive influence on the cell membrane, and therefore an undirect effect was to be expected. An effective concentration has been shown to be in the range of 1 to 3 mg/liter (38). We assumed that it could also be assayed more sensitively with method A than with method B. This hypothesis proved to be to some extent correct, but the difference between the methods was much smaller than with chloramphenicol or tetracycline. The difference is, however, clear and significant, and therefore the assay system can also be useful in the measurement of membranolytic compounds.

Cycloheximide was selected as a reference chemical because it has been reported to be one of the most efficient inhibitors of protein synthesis in eukaryotic cells. This chemical affects all three phases of synthesis, initiation, elongation, and termination. Fifty percent inhibition is obtained with a 0.1 μM (28 μg/liter) concentration (CHO cells), and for total inhibition, about 2 μM (560 μg/liter) is needed (33). In our assays, the chemical showed unexpected behavior (the data are excluded from Fig. 5 because it was impossible to calculate the curves): even concentrations of up to 10 mg/liter did not have any significant effect on light production, and therefore on luciferase synthesis, in prokaryotic surroundings. This result is highly significant compared with the results of Fautrel et al. (11), who reported that the LD_{50} of cycloheximide was 2 to 4 mg/kg. This is a clear reminder of the fact that the response to a certain chemical can be totally different in eukaryotic and prokaryotic cells, and prokaryotic tests alone can never produce reliable information on the true effect on humans or animals.

Figure 5 and Table 3 clearly show the efficiency and specificity of this sensitive assay. The overall differences between the luciferase genes are not so clear in this assay system as with the earlier recombinant assays using constitutive luciferase expres-

FIG. 5. IC_{50} curves of reference chemicals with respect to contact time. IC_{50} curves with different measurement protocols were plotted. Method A: test chemical added, incubation for 10 min, induction for 5 min, incubation for 15 min, continuous real-time measurement of luminescence. Method B: induction for 5 min, incubation for 10 min, test chemical added, incubation for 30 min, continuous real-time measurement of luminescence. Symbols: ——, K-12/pCSS301; · · · · · ·, K-12/pPHOLU; – – –, K-12/pCSS118. Typical variations of these curves ± standard deviations are presented in Table 3.

sion (26). However, differences similar to those found earlier (26) are seen with chemicals not having an inhibitory effect on protein synthesis. We assayed four such chemicals with two different methods, and the strain carrying the eukaryotic luciferase was clearly the most sensitive in six cases out of eight; only once was it less sensitive. This agrees well with our earlier results with a totally different luciferase expression system (26).

With the known inhibitors of protein synthesis, the eukaryotic luciferase showed clearly the lowest sensitivity when luciferase synthesis was induced prior to exposure to the reference chemical, and the sensitivities of all luciferases were about the same with the opposite assay method. The fact that the sensitivities of different luciferases to known inhibitors are similar in method A is understandable because of the basic principle of the assay. When all luciferase is synthesized after exposure to an inhibitor, the levels of light emission reflect directly the amount of the enzyme, not the enzyme activity or the energy balance inside the cell. The effective concentrations of the inhibitors in this assay were so low that no effects on the general activities inside the cell can be expected. The differences in the luciferase reactions, ATP consumption, etc., are totally masked by the effects on protein synthesis and are therefore insignificant in this assay system. The low sensitivity of the insect luciferase to known inhibitors in method B is a little more difficult to explain, but it is presumably due to the different lifetimes of the luciferases inside the cell.

Different bacterial luciferases have not been compared in sensitivity tests, either with the controlled luciferase synthesis assay or with the classical constitutive assay. Our results do not show any significant differences between the two bacterial luciferases. Although these two bacterial luciferases are very similar in structure, they are rather different in ecological and taxonomic status, one being found in a marine and the other in a terrestrial bacterium. This suggests that there are no great benefits to be achieved by the selection of bacterial luciferases, although that from *P. luminescens* has been generally shown to be far more temperature resistant (8).

The IC_{50}s obtained with method A are, as far as we know, the lowest ever measured with in vivo assays. This clearly shows the advantage of the controlled assay, in which the enzyme is not synthesized until after exposure to the test chemical. In this way, chemicals affecting protein synthesis can be indicated in the pharmacological or biochemical concentrations. For example, in molecular biology, these antibiotics are commonly used for maintenance of selection pressure, and normal working concentrations are 30 mg/liter for chloramphenicol and 10 mg/liter for tetracycline (34). These concentrations can be easily determined in less than an hour with this in vivo assay without any plate counts or other classical methods. This system can be easily applied to sensitivity testing of antibiotics or other compounds that might have a direct or indirect effect on protein synthesis. A rather similar assay system, but using constitutive expression of bacterial luciferase, for the assessment of antimycobacterial activity with bioluminescent *Mycobacterium smegmatis* has been published (1). The approach described in this communication is widely applicable to screening of various chemicals as well as to studies in which inhibitor molecules of protein synthesis are studied.

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