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Expression Vectors for the Use of Eukaryotic Luciferases as Bacterial Markers with Different Colors of Luminescence

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An easy way to identify microorganisms is to provide them with gene markers that confer a unique phenotype. Several genetic constructions were developed to use eukaryotic luciferase genes for bacterial tagging. The firefly and click beetle luciferase genes, *luc* and *lucOR*, respectively, were cloned under constitutive control and regulated control from different transcriptional units driven by *P*1, λP_R, and *Ptrc* promoters. Comparison of the expression of each gene in *Escherichia coli* cells from identical promoters showed that bioluminescence produced by *luc* could be detected luminometrically in a more sensitive manner. In contrast, luminescence from intact *lucOR*-expressing cells was much more stable and resistant to high temperatures than that from *luc*-expressing cells. To analyze the behavior of these constructions in other gram-negative bacteria, gene fusions with *luc* genes were cloned on broad-host-range vectors. Measurements of light emission from *Rhizobium meliloti*, *Agrobacterium tumefaciens*, and *Pseudomonas putida* cells indicated that both luciferases were poorly expressed from *P*1 in most bacterial hosts. In contrast, the lambda promoter P_R yielded constitutively high levels of luciferase expression in all bacterial species tested. P_R activity was not regulated by temperature when the thermosensitive repressor *c*I857 was present in the bacterial species tested, except for *E. coli*. In contrast, the regulated *lacI*^{q}-*Ptrc*::*lucOR* fusion expression system behaved in a manner similar to that observed in *E. coli* cells. After IPTG (isopropyl-β-D-thiogalactopyranoside) induction, this system produced the highest levels of *lucOR* expression in all bacterial species tested. As proof of the utility of these constructions, we were able to identify *P. putida* colonies with fusions of either *luc* or *lucOR* to P_R in a mixed population.

Enzymes responsible for light production are called luciferases. The best known organisms capable of producing bioluminescence are marine bacteria that belong to the genera *Vibrio* and *Photobacterium* and the North American firefly, *Photinus pyralis*. A group of highly homologous enzymes with the same chemistry of catalysis as firefly luciferase are the luciferases from a luminous click beetle (43), *Pyrophorus plagiophtalamus*. These click beetles are capable of producing light of at least four different colors, with emission peaks in the range of 547 to 593 nm. The four corresponding genes have been cloned and expressed in *Escherichia coli* (41).

The luminescent phenotype has proved to be a useful tool for microbiologists (for a review, see reference 35). Light emission can be detected in a nondisruptive manner visually, photographically, or with suitable electronic equipment (34). The genes that encode bacterial luciferases, *luxAB*, have been used intensively for monitoring genetically engineered microorganisms (32–34). Eukaryotic luciferase genes have seldom been used for microbial detection; however, some authors have described the use of the firefly luciferase gene for environmental monitoring of genetically engineered microorganisms (31), reporting gene expression (8), and assessing antibiotic susceptibility in *Mycobacterium tuberculosis* (19). In their luminescent reactions, eukaryotic luciferases provide more efficiency and less energy cost than do bacterial luciferases (21). Furthermore, about a 10-fold increase in light production was obtained with eukaryotic luciferases, compared with bacterial luciferase (22). In addition, because of their eukaryotic nature (and thus their presumable absence from all bacteria), the firefly and click beetle genes may provide a unique genotype to bacteria. Therefore, luciferase-tagged bacteria are also suitable to be detected by the most sensitive technique known for bacterial detection, PCR (4, 27). This new genetic material also introduces the possibility of distinguishing bacterial populations not only by its ability to emit light but also by the color of the light emitted. To exploit this difference in luciferase assays, the *lucOR* gene was chosen to develop a new marker gene for bacteria because this luciferase emits light at 595 nm (orange) that can be visually distinguished from that of the firefly luciferase (560 nm). Since the natures of both genes are eukaryotic, they need prokaryotic transcriptional units to be expressed in bacteria. The expression of the marker gene should be high enough to be detected in small colonies but not so high as to create any potential selective disadvantage for the organism, particularly when competing with indigenous organisms. These conditions may be achieved by constitutive expression from moderate promoters or by controlled expression from strong promoters. In this study, the following three systems have been tested for constitutive or regulated expression of *luc* genes: (i) *P*1, or anti-*tet* promoter, from pBR322, which can be repressed by TetR protein and derepressed by subinhibitory amounts of tetracycline; (ii) λ right promoter, P_R, expressed under the control of the thermosensitive repressor *c*I857; and (iii) the *Ptrc* hybrid promoter, repressed by *lacI*^{q} and induced by IPTG (isopropyl-β-D-thiogalactopyranoside). Gene fusions were cloned on broad-host-range vectors to test their ability to produce luminescence in representative gram-negative bacteria. The distinction between two populations of *Pseudomonas putida* by the color of their luminescence is also reported.

MATERIALS AND METHODS

Bacteria and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. *E. coli* XL1-Blue was utilized for plasmid transformation. HB101 was used for studies of luciferase expression.

Culture media and antibiotics. *E. coli* and *P. putida* cells were maintained and

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
E. coli		
HB101	F^- *hsdS hsdM pro leu thi gal lacY recA*, Sm^r	5
XL1-Blue	*recA1 endA1 gyrA96 thi hsdR17 relA1* (*lac*$^-$) [F′ *proAB lacI*^{q}ZΔM15 Tn*10* (Tc^r)]	Stratagene
R. meliloti 1021	Wild type; Sm^r Nal^r Nod^+ Fix^+ in alfalfa	24
A. tumefaciens B6	Wild type; Nal^r; Ti plasmid	16
P. putida 2440	*hsd*R derivative of strain mt-2; Nal^r	30
Plasmids		
Bluescript	Ap^r; α-*lac*; M13 *ori*	Stratagene
pLucOR(BS)	Ap^r; *lucOR* in the *Bsp*HI site of Bluescript	40
pKJB824.17	Ap^r; Tc^r; *c*I857; P_R; ColE1	7
pKK233-2	Ap^r; *Ptrc*; ColE1	1
pKW101	Ap^r; Tc^r; *c*I857; P_R::*luc*; ColE1	13
pJD206	Ap^r; *luc*; M13 *ori*	12
pLOF/Km	Ap^r; *lacI*^{q}; mini-Tn*10* Km^r; *oriT*; *ori* R6K	18
pRK2013	Km^r; *tra*; ColE1	15
pAP2	Tc^r; P_R::*luc*; *oriV*	25
pRK293	Tc^r; Km^r; *oriT*; *oriV*	14
pACR209	Ap^r; Tc^r; *c*I857; P_R::*lucOR*; ColE1	This work
pACR397	Ap^r; Tc^r; *c*I857; *P*1::*lucOR*; ColE1	This work
pLucOR(P1)5	Ap^r; *P*1::*lucOR*; M13 *ori*	This work
pLuc(P1)	Ap^r; *P*1::*luc*; M13 *ori*	This work
pKQ1	Ap^r; *Ptrc*; *lacI*^{q}; ColE1	This work
pEB21r	Ap^r; *lacI*^{q}-*Ptrc*::*luc*; ColE1	This work
pACR3	Tc^r; P_R::*lucOR*; *oriT*; *oriV*	This work
pACR14	Tc^r; *c*I857-P_R::*luc*; *oriT*; *oriV*	This work
pACR18	Tc^r; *c*I857-P_R::*lucOR*; *oriT*; *oriV*	This work
pRKL41	Tc^r; *P*1::*luc*; *oriT*; *oriV*	This work
pRKL31	Tc^r; *P*1::*lucOR*; *oriT*; *oriV*	This work
pEB42r	Tc^r; *lacI*^{q}-*Ptrc*::*luc*; *oriT*; *oriV*	This work

grown in LB (yeast extract [5 g/liter], tryptone [10 g/liter], NaCl [10 g/liter]). For *Rhizobium meliloti* and *Agrobacterium tumefaciens* cells, TY (yeast extract [3 g/liter], tryptone [5 g/liter], $CaCl_2$ [0.84 g/liter]) was used. Selective media were supplemented with ampicillin (100 mg/liter), nalidixic acid (10 mg/liter), or tetracycline (10 mg/liter).

Recombinant DNA techniques. Plasmid DNA isolation, restriction endonuclease digestion, ligation, transformation, agarose electrophoresis, and other standard recombinant DNA techniques followed standard protocols (28). DNA linearized by endonuclease digestion was isolated by Geneclean II (Bio 101, Inc.) according to the instructions of the manufacturer.

Matings. Plasmids were transferred from *E. coli* to other strains with helper plasmid pRK2013 by a triparental conjugation technique (14). Transconjugants were selected in LB (*E. coli* and *P. putida*) or TY (*R. meliloti* and *A. tumefaciens*) solid media supplemented with tetracycline and nalidixic acid.

Construction of gene fusions of *lucOR* and *luc* to *P*1 and P_R promoters. Cloning a *Bsp*HI fragment (1.7 kb) which contained the *lucOR* gene of pLucOR(BS), previously filled in with Klenow DNA polymerase and deoxynucleoside triphosphates, into the *Sma*I site of pKJB824.17 allowed us to obtain different fusions, depending on the *lucOR* open reading frame (Fig. 1). pACR397 expressed luciferase from the promoter of *tetR*, a gene deleted in pKJB824.17, which is also named the anti-*tet or P*1 promoter (3). pACR209 contained a translational fusion, with the first codons of *cro* and the multicloning site of pKJB824.17 joined to the first codon of *lucOR*. The expression of this gene is controlled by the λP_R promoter and the repressor cI857. This repressor is temperature sensitive; thus, cI857 protein did not repress at 42°C. pACR209 is equivalent to pKW101, which was the first expression plasmid of firefly luciferase cDNA (13). To obtain pLucOR(P1)5, a 2.1-kb *Bam*HI segment that carried the *P*1::*lucOR* fusion from pACR397 was isolated and cloned into the same site of Bluescript. pLuc(P1) was constructed by isolating a *Hin*dIII-*Kpn*I 1.7-kb fragment of pJD206 that carried the complete coding sequence of the firefly luciferase gene. This fragment was cloned into the large *Hin*dIII-*Kpn*I fragment of pLucOR(P1)5.

Construction of a fusion for regulated expression of *lucOR* under the control of *Ptrc*. The control of *Ptrc* activity was achieved by cloning an ~1.2-kb *Eco*RI-*Bgl*II fragment from pLOF/Km into an *Eco*RI-*Bgl*II site of *Ptrc* expression vector pKK233-2 to obtain plasmid pKQ1. A *Bsp*HI fragment of pLucOR(BS) with *lucOR* was cloned into a *Nco*I site of plasmid pKQ1 (Fig. 2). The plasmid with the right-sense insertion, pEB21r, was checked by luminometry of clones and restriction analysis.

Construction of broad-host-range vectors with eukaryotic luciferase gene fusions. Two plasmids with potential thermoregulated expression of luciferase were constructed (Fig. 3). Isolation of *Cla*I fragments from pKW101 (partially digested) and pACR209 that contained *c*I857 and translational fusions of λP_R to *luc* and *lucOR* and subsequent cloning into the *Cla*I site of pRK293 created pACR4 and pACR18, respectively. Formation of the unregulated P_R::*lucOR* system of pACR3 was achieved by ligation of a *Hin*dIII fragment from pACR209 into the *Hin*dIII site of pRK293. Plasmid pACR3 was equivalent to pAP2 (P_R::*luc*), which has previously been described and been shown to achieve high luciferase levels in different gram-negative bacteria (25). To observe luciferase expression from *P*1 in other backgrounds, *Bam*HI fragments with *luc* and *lucOR* genes from pLuc(P1) and pLucOR(P1)5 were inserted in the *Bgl*II site of pRK293 to obtain pRKL41 and pRKL31, respectively. The *Sal*I-*Hin*dIII DNA segment that carried *lacI*^{q} and the *Ptrc*::*lucOR* fusion from pEB21r was also cloned in pRK293 digested with *Xho*I-*Hin*dIII. This resulted in the formation of pEB42r.

Light emission measurements. Cultures were grown to late-log phase. Dilutions of these cultures were made with fresh media and left to grow at 29°C to an optical density at 600 nm (OD_{600}) of about 0.2. Cell suspensions were incubated under repression or induction conditions to an OD_{600} ranging from 0.3 to 0.7 for at least 2 h. Cultures were incubated at 29°C, except for experiments with the λP_R promoter in which incubations at 37 and 42°C were also performed. To test tetracycline-mediated induction from *P*1, tetracycline was added to the suspension at a final concentration of 1 μg/ml. After 1 h of preinduction, tetracycline was added again at 10 μg/ml and incubated until luciferase activity was measured. For *Ptrc* derepression, cultures were incubated for 2 h with 1 mM IPTG.

Light emissions from bacterial colonies that contained *luc* genes were detected either by plating cells onto a nitrocellulose filter or by blotting colonies onto filter paper. Filters were moistened with 500 to 700 μl of substrate solution (1 mM D-luciferin–0.1 M sodium citrate [pH 5]). After diffusion for a few minutes, light-emitting colonies were detected in the dark by dark-adapted eyes or photographically either by contact with Kodak OG-1 X-ray film, according to the method of Wood and DeLuca (44) or by reflex camera with Kodak Gold 400 ASA color film.

RESULTS

Expression of *luc* and *lucOR* under the control of the *P*1 promoter in *E. coli*. In order to compare the luminescence generated from *luc* and *lucOR* gene products, two plasmids that carried transcriptional fusions to *P*1, pLuc(P1) and pLucOR(P1)5, respectively, were constructed (Fig. 1). Light measurements from *E. coli* cells with pLuc(P1) or pLucOR(P1)5 revealed the production of different colors; higher values were obtained in luminometric assays of cells which expressed *luc* (Table 2). In cell extracts, the maximal intensity of light production by firefly luciferase was about sevenfold higher than that obtained with click beetle luciferase. Nevertheless, in intact cells, the differences in favor of *luc* were only twofold compared with *lucOR*.

When light activity was studied by a long assay with intact cells, luminescence produced by *lucOR* remained at high levels longer than that from *luc*-producing cells (Fig. 4). In repeated determinations of luciferase activity for 24 h after luciferin addition, the levels of light emission from *E. coli*(pLucOR(P1)5) were comparable to those obtained during the initial minutes. In contrast, a decrease to less than 5% of the initial light emission was found in cells that expressed firefly luciferase (Fig. 4A).

Bioluminescence emitted from cells grown on nitrocellulose filters showed equivalent results (Fig. 4B). The luciferase activity from *E. coli*(pLucOR(P1)5) was detected photographically after 16 h, whereas it was not detected from *E. coli*(pLuc(P1)). Visual observation during the first minutes of reaction indicated that the intensity of light emission from

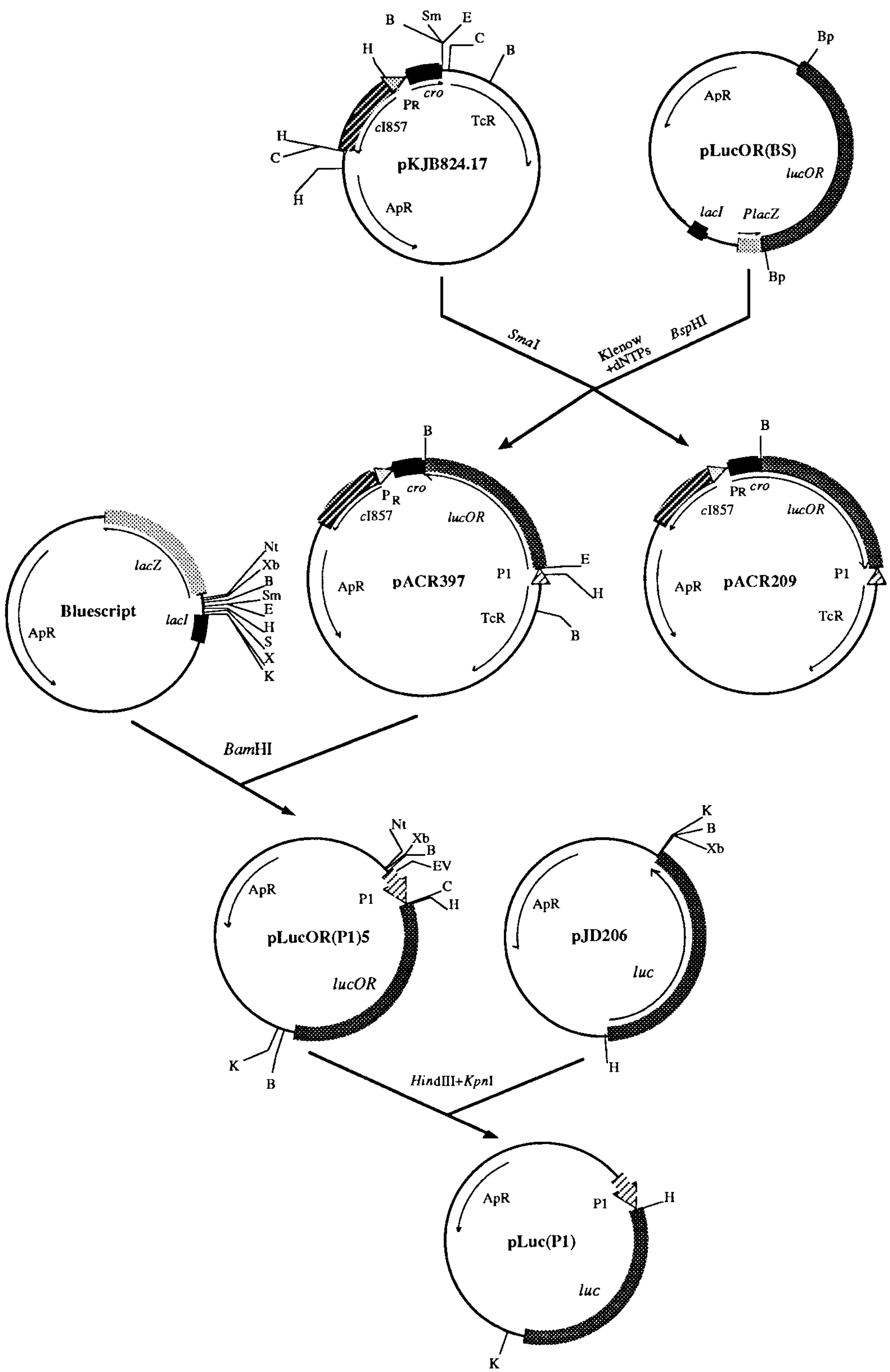

FIG. 1. Strategy for the construction of gene fusions with *luc* and *lucOR* to *P*1 and P_R promoters. Abbreviations (indicating cleave sites for restriction enzymes): B, *Bam*HI; Bp, *Bsp*HI; C, *Cla*I; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; Nt, *Not*I; P, *Pst*I; Sc, *Sca*I; Sm, *Sma*I; X, *Xho*I; Xb, *Xba*I.

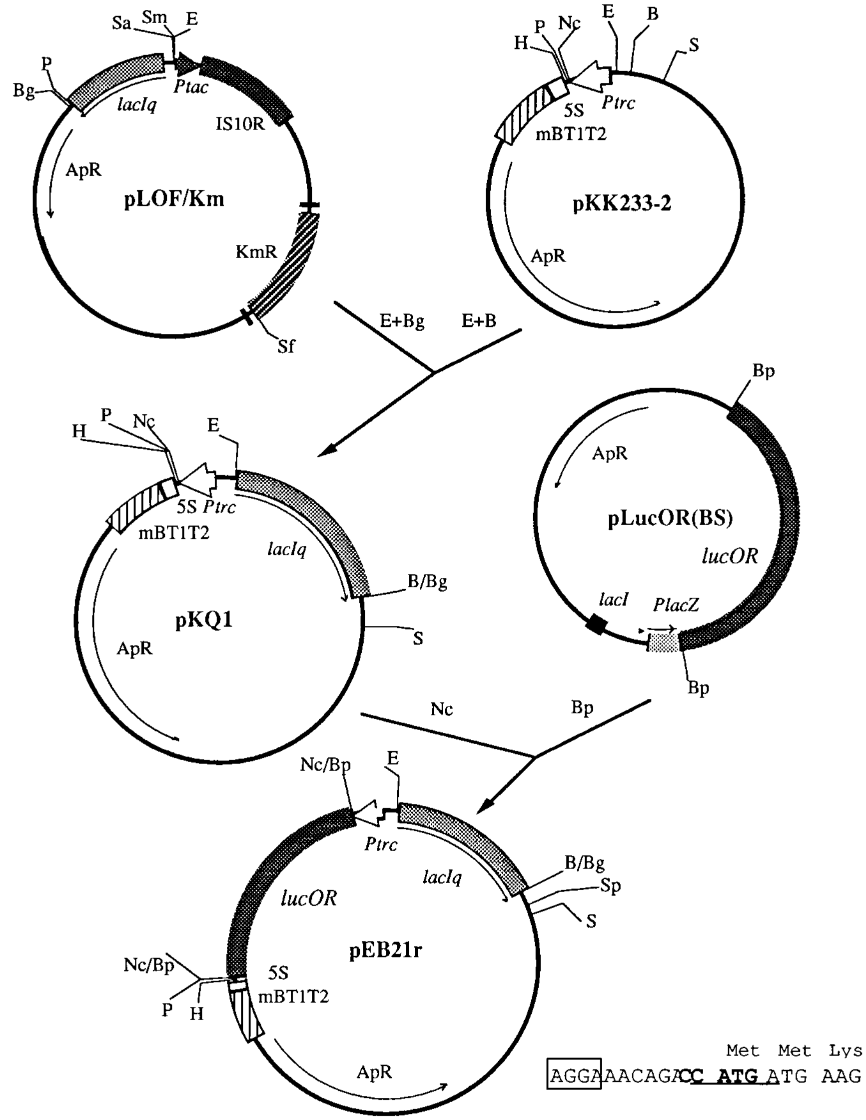

FIG. 2. Construction of a fusion for regulated expression of *lucOR* under the control of *Ptrc*. mBT1T2, transcriptional terminators. Nc/Bp, junction of compatible *Nco*I and *Bsp*HI termini. The resulting construction cannot be digested by either enzyme. The DNA sequence of pEB21r close to the translation initiation site of *lucOR* is indicated. The Shine-Dalgarno sequence is indicated by a box. The sequence that corresponds to part of the *Bsp*HI site is underlined; the portion of *Nco*I nucleotides in the junction is in boldfaced type.

lucOR was at least as high as that from firefly luciferase. These differences in luminometric measurement may be explained by the fact that the efficiency of luminometric light detection is higher when the wavelength is shorter (37). Therefore, the same number of photons emitted by the click beetle luciferase can be expected to yield fewer light units than those from firefly luciferase.

Expression of *luc* and *lucOR* under the control of the P_R promoter in *E. coli*. To test thermoregulated expression of *luc* and *lucOR* under the control of the λP_R promoter, quantitative assays of luciferase activity were carried out with pKW101 and pACR209 (Table 2). Luminescence from *E. coli* cells that carried these plasmids proved to be highly regulated by temperature: induction levels of 9- (*luc*) and 30-fold (*lucOR*) were observed when the temperature was shifted from 30 to 37°C. Further induction was obtained after incubation at 42°C: a 120- to 500-fold increase in light emission was observed for *E. coli* cells with P_R::*luc* and P_R::*lucOR*, respectively. Induction rates were higher when the *lucOR* gene was used as the reporter, probably because firefly luciferase is more sensitive to high temperatures than LucOR is (see below). When extracts were prepared from cells with *lucOR*, light emission was lower and erratic, especially from cells grown at or above 37°C. The cause of this behavior remains unknown.

Expression of *lucOR* driven by a $lacI^q$-*Ptrc* system in *E. coli*. In order to obtain higher and better regulated luminometric values in bacteria that expressed *lucOR*, the strong promoter *Ptrc* was used. Plasmid pEB21r was constructed (Fig. 2). Luciferase activities in *E. coli* cultures in the absence and presence of IPTG were measured. Quantification by luminometer showed that in vivo luciferase activity was superior to that obtained by other constructions (Table 2).

Expression of *luc* and *lucOR* fusions in other gram-negative bacteria. The plasmids for luciferase expression described

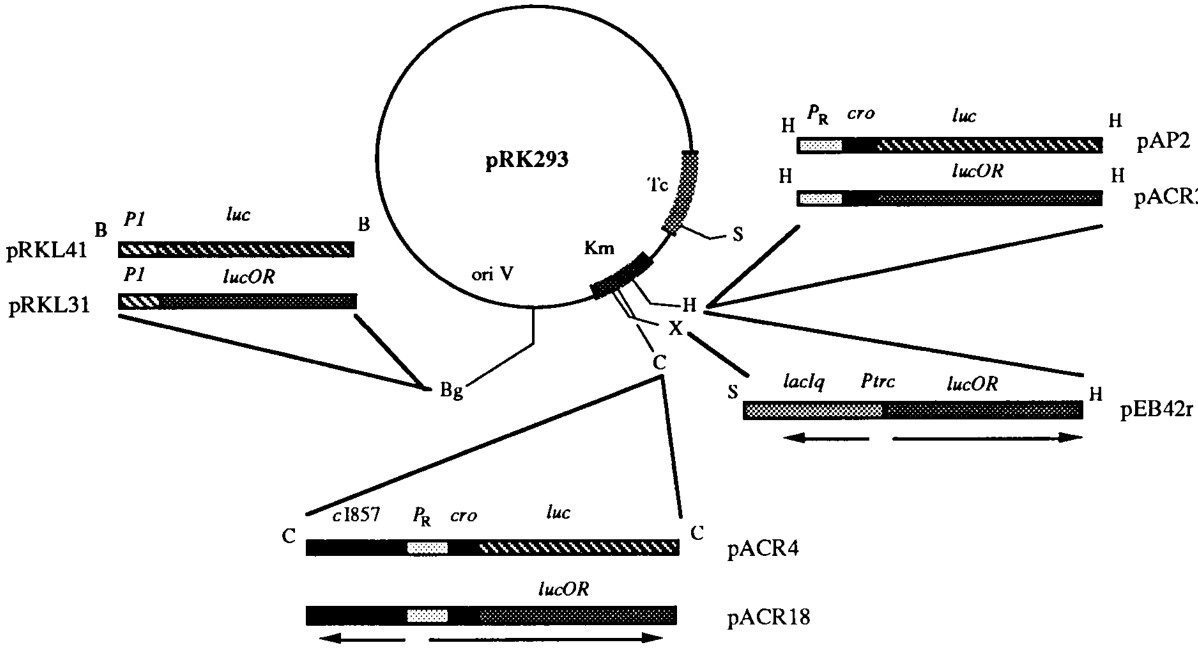

FIG. 3. Plasmids constructed to study the expression of eukaryotic luciferases under the control of different promoters in gram-negative bacteria. All of the plasmids made were based on the RK2 derivative pRK293. Fragments with the represented fusions of *luc* and *lucOR* were cloned in the sites of pRK293 indicated. The names of resultant plasmids are given next to the corresponding cloned fragments. B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; S, *Sal*I; X, *Xho*I.

above cannot replicate in many gram-negative bacteria. For the analysis of marker genes in other backgrounds, such as *Rhizobium* and *Pseudomonas* spp., the broad-host-range plasmid pRK293 was used as the vector for cloning gene fusions (Fig. 3). When *P*1::*luc* and *P*1::*lucOR* fusions were carried on an RK2-derived vector, their luciferase activities in *E. coli* cells were lower than those in ColE1 replicons (Table 3). Since a *tetR* gene is present in pRK293 (14), repression of *P*1 may occur. Although the tetracycline resistance system of pRK293 (*tetR*/*tetA* of RP1) is not strictly the same as that of pBR322, Klock et al. (20) reported that the TetR protein of RP1 can bind to heterologous *tet* operators. The addition of tetracycline did not produce any significant increases in luciferase activity. These lower values may be due to either the lower copy number of the RK2-derived plasmid or inefficient derepression of the promoter.

TABLE 2. Luciferase activities from broken and intact *E. coli* cells that contained gene fusions with *luc* and *lucOR* to *P*1, P_R, and *Ptrc* promoters

Plasmid	Gene fusion	Condition	Luciferase activity (RLU/OD_{600})[a] In vitro	In vivo
pLuc(P1)	*P*1::*luc*	Tc[b]	34,875	8,176
pLucOR(P1)5	*P*1::*lucOR*	Tc[b]	5,390	3,519
pKW101	*c*I857-P_R::*luc*	29°C	215	36
pKW101	*c*I857-P_R::*luc*	37°C	1,955	199
pKW101	*c*I857-P_R::*luc*	42°C	33,451	4,363
pACR209	*c*I857-P_R::*lucOR*	29°C	<2	2
pACR209	*c*I857-P_R::*lucOR*	37°C	28	68
pACR209	*c*I857-P_R::*lucOR*	42°C	530	1,120
pEB21r	$lacI^q$-*Ptrc*::*lucOR*	−IPTG	17,000	3,950
pEB21r	$lacI^q$-*Ptrc*::*lucOR*	+IPTG	130,000	37,375

[a] For determinations of luciferase activity from intact cells (in vivo) in liquid cultures, 50 μl of suspension was placed in a tube and 0.15 ml of 1 mM luciferin–100 mM citric acid (pH 5.0) was mixed with it. The time course of light emission was recorded for 1 min in an LKB 1250 luminometer equipped with a chart recorder. Alternatively, to measure luciferase activities from cell extracts (in vitro), 0.9 ml of cell suspension was mixed with 0.1 ml of buffer to give 0.1 M potassium phosphate (pH 8.0)–2 mM EDTA–1 mg of bovine serum albumin–5% glycerol (final concentration). The mixture was sonicated twice on ice for 30 s; 0.15 ml of 25 mM glycylglycine (pH 7.8)–10 mM $MgCl_2$–5 mM ATP–0.1 mM D-luciferin was then added to 50 μl of each sonicated sample. Specific enzymatic activity was reported as a peak height (in relative light units [RLU]) relative to cell mass estimated by measurements of the OD_{600} of the culture.

[b] No differences in luciferase activity were found in derepression assays with or without subinhibitory amounts of tetracycline.

The luciferase activities from *P*1 fusions in *R. meliloti* cells were even lower than those in *E. coli* cells. Conversely, the highest levels of luciferase expression with *P*1 were achieved in *P. putida* cells. In all backgrounds, the presence of tetracycline increased luciferase expression, but it was never more than twofold.

As shown with ColE1 replicons, constructions based on *c*I857-P_R showed strict regulation of luciferase expression in *E. coli* cultures by temperature. Cultures grown at 42°C gave values of luciferase activity that were 30- to 300-fold higher than those for cultures grown at 30°C. When light emissions from the other gram-negative bacteria tested were measured, thermoregulation seemed to be absent. Luciferase activities from bacteria that expressed either *luc* or *lucOR* at 30°C were even higher than those from such bacteria at higher temperatures. Their levels of light emission were similar to those obtained with constitutive constructions (pAP2 and pACR3). Therefore, there was a lack of repression, suggesting that the *c*I857 gene was weakly expressed in *R. meliloti*, *A. tumefaciens*, and *P. putida* cultures. Nevertheless, the levels of luminescence expressed constitutively from P_R in these bacterial genera were even higher than those expressed in *E. coli* cultures, showing the effectiveness of λP_R in a wide range of bacteria (Table 3).

When *luc* and *lucOR* were constitutively expressed, luciferase activity decreased at higher temperatures. The loss was smaller for *lucOR*-expressing cells. Similar observations were

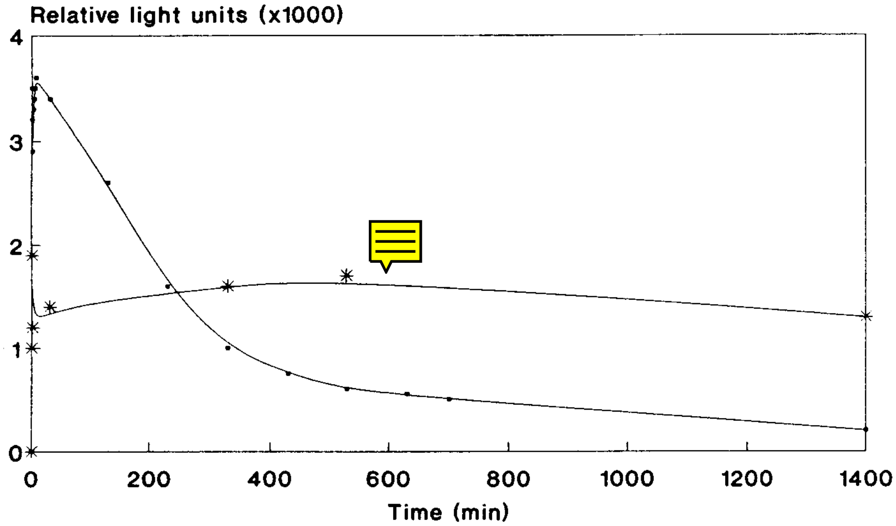

FIG. 4. Kinetics of the in vivo activities of firefly and click beetle luciferase expressed in *E. coli*. (Left) Kinetics of light emission from intact cells of *E. coli* that contained plasmids pLuc(P1) and pLucOR(P1)5 in liquid cultures. Luciferase activity was determined continuously for 24 h after luciferin addition. The ordinate indicates relative light units determined in an LKB luminometer equipped with a chart recorder. The abscissa indicates the time from the addition of luciferin to the cell samples. (Right) Evolution of in vivo luminescence from *E. coli* cultures that expressed *luc* and *lucOR*. Cultures of *E. coli*(pLuc(P1)) (left) and *E. coli*(pLucOR(P1)5) (right) over a nitrocellulose filter were laid on an LB-agar ampicillin plate. After a 1-day incubation at 29°C, the filter was taken and left to dry for 5 to 15 min. It was wet with a 1 mM luciferin–100 mM sodium citrate (pH 5) solution and left to diffuse for 5 min. The bioluminescence emitted was photographed with a reflex camera after a 10- to 15-min exposure. Luminescence at 15 min (A), 1 h (B), 5 h (C), and 16 h (D) after luciferin addition.

made for cells that contained *P*1::*luc* and *P*1::*lucOR* fusions (data not shown), suggesting that the decrease might have been caused by luciferase thermosensitivity rather than P_R differential expression.

To test the ability of constitutive fusions to tag different populations of bacteria with two colors of luminescence, we carried out the experiment described in the legend to Fig. 5. Colonies of one *P. putida* strain with different plasmids (pAP2 or pACR3) were distinguished by the color of luminescence. Yellow colonies were assumed to bear pAP2, and the red-orange ones were assumed to bear pACR3. Even overlapping colonies with two different colors were identified.

Regulated expression of *lucOR* driven by a *lacI*^{q}-*Ptrc* system in *E. coli*, *R. meliloti*, *P. putida*, and *A. tumefaciens* cells. High levels of luciferase expression were obtained with plasmid pEB42r (Fig. 3 and Table 3). In the presence of IPTG, luciferase activity in *E. coli*(pEB42r) cultures was about 20-fold higher than in the absence of inductor. Significant increases in *lucOR* expression were also observed for *R. meliloti* (18-fold), *P. putida* (37-fold), and *A. tumefaciens* (20-fold) with the addition of IPTG. These results provide evidence that *lacI*^{q} may be actively expressed in a range of gram-negative bacteria. *Ptrc* was also effective in all the bacterial hosts tested; upon induction, it also yielded the highest levels of luminescence, compared with those of other constructions. With *Ptrc* and the other promoters tested, *A. tumefaciens* showed the lowest luciferase activities of all the species tested. This might indicate less efficient translation of luciferase transcripts.

DISCUSSION

Several gene fusions of known promoters to luciferase genes *luc* and *lucOR* have been constructed for use as marker genes in gram-negative bacteria. The luciferase genes of *Pyrophorus plagiophtalamus* have been poorly employed as a biological tool for bacteria. Preliminary studies have been carried out with *lucGR* and *lucOR* genes in *E. coli* (41–43) and *Bacillus subtilis* (22), showing promising qualities as reporter genes. Expression of *luc* and *lucOR* under identical transcriptional control allowed some comparisons between the better known firefly system and the click beetle luciferase system. Among the differences found in this study were the following. (i) Light emission kinetics of firefly luciferase differed significantly from that of LucOR. This indicated that the turnover of each enzyme is probably different. (ii) In vivo luminescence emitted by *E. coli* cells with *lucOR* was maintained at a constant level for longer periods than that from *luc*-expressing cells. (iii) Temperatures of ≥37°C affected the levels of active firefly luciferase more severely than those of click beetle luciferase. One major potentially useful difference for bacterial identification was observed when the population that expressed each gene could be differentiated by the color of luminescence.

The three transcriptional units tested in this study have been employed previously for the expression of either prokaryotic or eukaryotic proteins in *E. coli* cells (11). The *P*1 promoter was used for constitutive expression of *luxAB* genes in *Bradyrhizobium japonicum*, allowing the detection of luminescence in

TABLE 3. In vivo luciferase expression in *E. coli*, *R. meliloti*, *A. tumefaciens*, and *P. putida* with genetic constructions based in firefly and click beetle luciferase genes

Plasmid	Fusion	Condition	Luciferase activity (RLU/OD_{600})[a]			
			E. coli	*R. meliloti*	*A. tumefaciens*	*P. putida*
pRKL41	*P*1::*luc*	−Tc	57	37	50	529
		+Tc	53	55	68	907
pRKL31	*P*1::*lucOR*	−Tc	117	32	174	4,211
		+Tc	260	38	264	11,833
pAP2	P_R::*luc*	30°C	8,564	33,891	4,431	13,233
		37°C	3,444	23,664	4,692	11,719
		42°C	3,177	12,259	ND	ND
PACR4	*c*I-P_R::*luc*	30°C	69	24,462	387	3,468
		37°C	297	22,350	278	3,114
		42°C	2,363	14,038	ND	ND
pACR3	P_R::*luc*	30°C	2,564	8,254	1,220	16,884
		37°C	2,311	8,161	720	11,062
		42°C	1,681	6,830	ND	ND
pACR18	*c*I-P_R::*lucOR*	30°C	3	4,094	328	14,067
		37°C	89	5,154	408	3,157
		42°C	904	2,665	ND	ND
pEB42r	*lacI*^{q}-*Ptrc*::*lucOR*	−IPTG	1,744	2,678	452	4,190
		+IPTG	47,219	55,670	5,527	154,561

[a] At 42°C for incubation, drastic inhibition of growth and metabolic activity occurred for *A. tumefaciens* and *P. putida*; this did not allow measurements of luciferase activity to be taken. ND, not detected. RLU, relative light units.

soybean nodules occupied by this bacteria (23). In this study, constitutive expression of luciferase from *P*1 was considerable when *P*1::*luc* and *P*1::*lucOR* fusions were borne on ColE1 plasmids. In contrast, luciferase activity decreased when both fusions were borne on an RK2 derivative plasmid. This reduction may partially be due to the presence of a *tetR* gene in the parental plasmid, pRK293. However, the addition of tetracycline did not considerably affect light emission. This decrease may also be caused by different copy numbers for each type of vector. ColE1 replicons have 20 to 50 copies per cell (3), compared with less than 10 copies for the RK2 plasmid (3, 29, 30). The lack of an efficient Shine-Dalgarno sequence near the initiation codon of both *luc* genes also results in low luciferase levels (data not shown). The levels of luciferase from *P*1 were low in most genetic backgrounds. Therefore, the recognition of this promoter may be more effective in *P. putida* cells than in

FIG. 5. Bioluminescence emitted from *P. putida* colonies with P_R::*luc* and P_R::*lucOR* fusions. Pure *P. putida* cultures with pAP2 and pACR3 were incubated overnight in LB with tetracycline at 30°C. Equivalent volumes of cultures were mixed. Adequate dilutions of mixture were plated onto nitrocellulose filters lying on TY-tetracycline plates. After 30 h of incubation at 29°C, the filter was moistened with 500 to 700 μl of luciferin solution. (A) *P. putida* colonies with plasmids pAP2 and pACR3; (B) bioluminescence emitted by colonies and photographed with a reflex camera after 30 min of exposure to the lens at a distance of 24 cm.

the other backgrounds tested. We have investigated the effect of a Shine-Dalgarno sequence placed 7 bp from the first ATG of *lucOR*; luciferase expression from *P*1 increased about 10-fold in *E. coli* cells (data not shown). Thus, the efficiency of *luc* and *lucOR* expression from *P*1 in the other backgrounds might be also improved.

Strongly regulated promoters, easy and inexpensive to induce, such as the *c*I857-P_R system, may be very useful for microorganisms of potential environmental release. This system may be silenced in the natural environment, avoiding the use of nutrients or energy in marker synthesis. In the presence of the *c*I857 repressor gene, P_R is known to be an excellent regulated promoter (11). This was confirmed when the P_R::*luc* fusion was expressed in *E. coli* in either ColE1 or RK2 replicons. When genetically engineered microorganisms had to be detected, induction of the marker gene could be carried out, allowing identification on solid medium plates or in enrichment broths. However, in the presence of the *c*I857 gene, P_R was not efficiently regulated in some gram-negative bacteria. Winstanley et al. (39) also observed notable differences in the expression of *xylE* when the constructions *c*I857-P_R::*xylE* and *c*I857-P_L::*xylE* were transferred to different gram-negative bacteria, such as *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Aeromonas hydrophila*, etc. The absence of thermoregulation in these other bacteria is likely due to inefficient production of an active repressor. The *c*I857-P_R system has also been tested in *B. subtilis*, and the expression of staphylokinase was also constitutive (6). In this gram-positive bacterium, *c*I857 expression signals proved to be inefficient. We suspect that the same problem occurred with bacteria in our study. Therefore, the well-regulated *c*I857-P_R system could be adapted for a wide range of bacteria by providing the repressor gene with appropiate transcription signals. Nevertheless, constitutive expression of either *luc* or *lucOR* from P_R could be achieved efficiently in all of the bacteria tested. In a previous work, P_R expressed constitutively high levels of luciferase activity, which were easily detected by any of the known methods of luminescence detection (25). The use of constitutively expressed markers might be disadvantageous because of the risk of a deleterious metabolic burden. However, we have recently shown that *R. meliloti* tagged in monocopy with a P_R::*luc* fusion was apparently not affected in terms of its maximal growing rate, its survival in sterile soil, or its capacity to nodulate plants (9).

As a regulated system, *lacI*^{q}-*Ptrc* provided equivalent results for all the bacterial species tested. Efficient expression of *lucOR* from *Ptrc* was also observed. Furthermore, the response to IPTG in some cases (*P. putida*) was higher than that in *E. coli*. These results indicated the production of an active *lacI*^{q} repressor, with similar responses to the inducer. The *Ptac* promoter has been shown to be active in a broad range of gram-negative bacteria (2, 17) and even in gram-positive bacteria such as *B. subtilis* (26). *Ptrc* is nearly identical to *Ptac* but is more similar to the consensus sequence of σ^{70}-dependent promoters than *Ptac* is, because the -35 and -10 *Ptrc* regions are separated by 17 bp, in contrast to the 16 bp of *Ptac*. Thomas and Franklin (36) have suggested that the similarity of a promoter to the consensus sequence -35 to -10 allows efficient expression in a broad-host-range vector. They described *Ptac* as an example of this kind of promoter. According to this hypothesis, efficient expression of *lucOR* from *Ptrc* should be observed in all the bacteria tested. A slight disadvantage of this system may be that background expression is relatively high.

Among the constructions developed in this study, we have outlined three of them for use as bioluminescent marker cassettes, constitutive P_R::*luc* and P_R::*lucOR* fusions and the regulated *lacI*^{q}-*Ptrc*::*lucOR* system. The high levels of luciferase activity obtained with these fusions may allow sensitive detection of tagged bacteria by various methods (with the use of luminometers, or photographic films, etc.). Thus, using the P_R::*luc* and P_R::*lucOR* fusions, we tested the ability to distinguish *P. putida* colonies with different plasmids by the color of luminescence. However, differentiating microscopic populations (microcolonies or individual cells) by this phenotype still remains a challenge for future research with specialized instrumentation.

It is preferable to insert the marker system into the chromosome of the organism, where its stability may be higher, with the further advantage that chromosomal genes are intrinsically less likely to be rapidly disseminated in the environment by genetic transfer than plasmid-borne genes. On the basis of these three fusions and mini-Tn*5* derivatives (10, 18), we have also developed a number of delivery vectors that may allow the stable insertion of eukaryotic luciferase genes into bacterial chromosomes (38). Since bioluminescence has been demonstrated to be an unequivocal phenotype for tracking microorganisms in the environment (9, 32), the use of these tools could be very reliable when monitoring two strains in the presence of indigenous microorganisms is desired. We are currently using these fusions to monitor simultaneous gene transfer of two plasmids in soil microorganisms.

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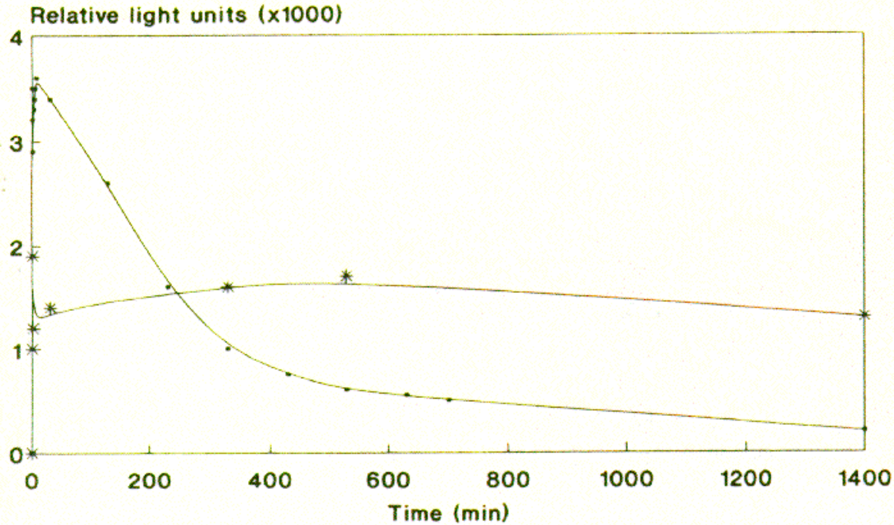

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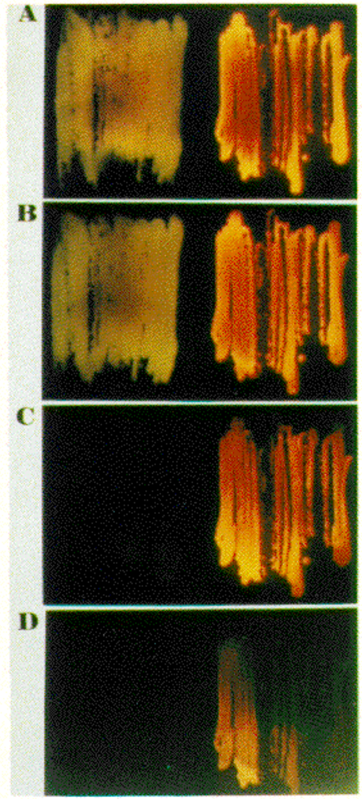

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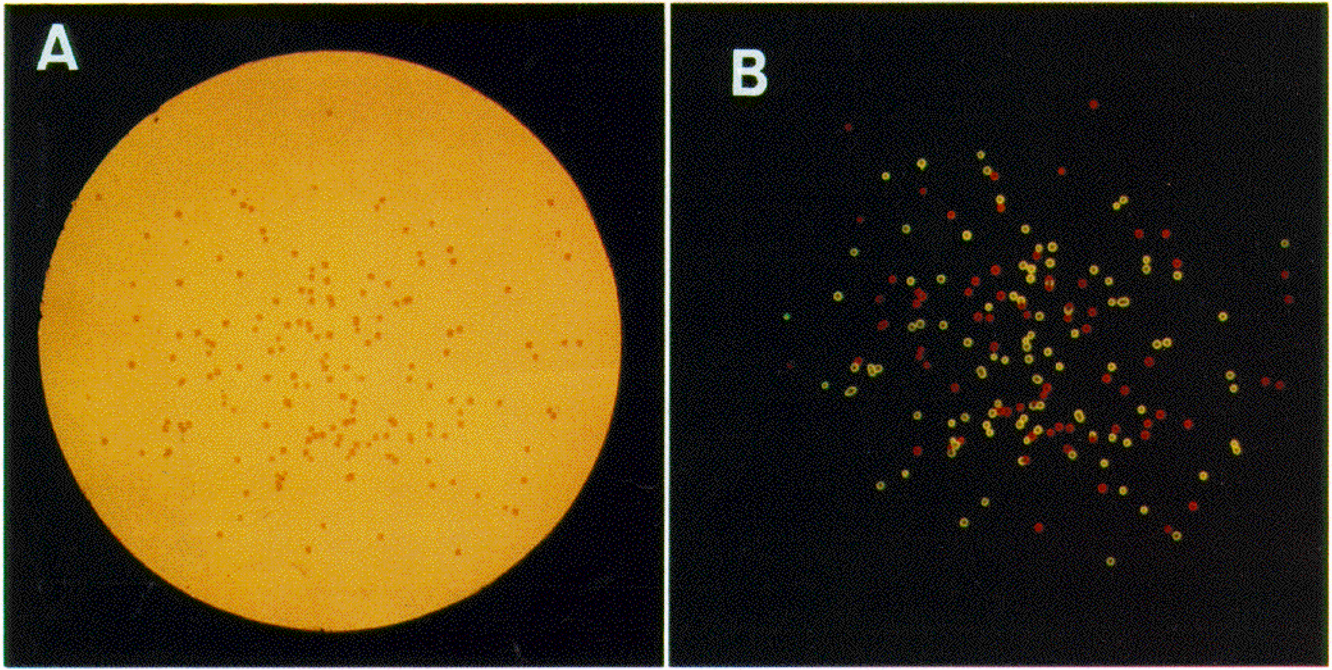
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