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Luminescence-Based Nonextractive Technique for In Situ Detection of *Escherichia coli* in Soil

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Measurement of light output by luminometry was used to estimate quantitatively the cell concentrations of luminescent strains of *Escherichia coli* in liquid culture and inoculated into soil. Strains were constructed in which luciferase production was autoinducible or constitutive. In the former, light output per cell varied considerably during growth but was constant in constitutive strains. In liquid culture, the lower detection limit was in the order of 10^2 cells ml^{-1}. Sensitivity was reduced by approximately 1 order of magnitude for cells inoculated into soil, when 2×10^2 to 6×10^3 cells g of $soil^{-1}$ could be detected. Light output measurements were obtained within 5 min of sampling, and luminometry therefore potentially offers a rapid and sensitive detection technique for genetically engineered microorganisms.

Recent development of molecular-based detection techniques has greatly increased the ability to track microorganisms and introduced genetic material in natural environments. DNA probes enable the detection of specific nucleotide sequences in the presence of high background levels of DNA. By using dot blot hybridization, detection levels in the order of 5×10^4 cells g of $soil^{-1}$ may be achieved under certain circumstances (12), whereas the use of the polymerase chain reaction increases sensitivity by several orders of magnitude (20). The insertion of marker genes such as β-galactosidase (5) allows tracking of genetically engineered microorganisms by cell extraction and subsequent growth on selective media. Although these techniques have enormous implications for the study of microbial ecology, the main impetus for their development has been the potential commercial benefits of genetically engineered microorganisms and the need to monitor such organisms following release into the environment.

None of the techniques described provides in situ detection in the soil. DNA probing requires extraction of cells and removal of humic material prior to DNA extraction. The use of marker genes requires cell extraction, growth, and gene expression, thereby encountering many of the problems associated with traditional dilution plate enumeration techniques. The introduction of fluorescent antibodies to the soil enables specific microorganisms to be detected in situ, but cannot distinguish between living and dead cells. Luminescence-based techniques offer many of the advantages of the above methods and, additionally, the potential for in situ, nonextractive detection of marked cells in soil samples, with recognition of their metabolic state. These techniques involve introduction of genes for luminescence originally cloned from the marine bacterium *Vibrio fischeri*. Seven genes are involved, located on two operons (8, 9). The structural genes, *luxA* and *luxB*, encode luciferase; *luxC*, *luxD*, and *luxE* code for the synthesis and recycling of the aldehyde substrate. *luxI* and *luxR* are involved in regulation of luciferase production (17) via a positive feedback mechanism. The gene product of *luxI* is produced at a low level under noninduced conditions. This level increases with cell growth to a critical concentration, at which it interacts with the gene product of *luxR* to stimulate transcription of *luxICDABE*. Light production is therefore catalyzed by luciferase, which requires a long-chain aldehyde as substrate, oxygen, and a source of reducing equivalents, usually reduced flavin mononucleotide ($FMNH_2$) (6, 10, 11, 21). In *V. fischeri* the in vivo aldehyde substrate is *n*-tetradecyl aldehyde, but *n*-decyl aldehyde must be supplied as exogenous substrate in cells lacking *luxC*, *luxD*, and *luxE*.

Luminescent microorganisms may be detected by five methods: (i) light output measured by luminometry; (ii) DNA probing for, or amplification of, sections of the luciferase gene; (iii) charge-coupled microscopy (14); (iv) X-ray film imaging (19); and (v) enumeration of luminescing colonies (visible by eye) following growth on solid media. Method (i) has the advantage of being nonextractive, whereas methods (iii) and (iv) offer true in situ detection of cells in soil. In addition, techniques for measuring light have high sensitivity and provide a linear response over several orders of magnitude. In this study, we assess the use of luminometry for detection and enumeration of genetically engineered luminescent strains of *Escherichia coli* in liquid culture and following inoculation into soil.

MATERIALS AND METHODS

Bacterial strains and plasmids. Three strains *E. coli* were used: MM294, DH1, and HB101. All three were obtained from the National Collections of Industrial and Marine Bacteria. Luminescent strains were constructed by transforming with plasmids pBTK5, pUCD607, pJE205, and pEMR1. Plasmids pUCD607 and pBTK5 were gifts from C. Kado (18) and G. Stewart (3). Plasmid pJE205 was constructed as described by Engebrecht et al. (8), and pEMR1 was constructed as described in Fig. 1. Details of plasmid constructs are provided in Table 1.

Competent *E. coli* cells, prepared by the method of Mandel and Higa (15), were transformed with plasmid DNA by the method of Cohen et al. (4). Large-scale preparations of plasmid DNA were obtained by CsCl-ethidium bromide density gradient centrifugation (13). Small-scale plasmid preparations were carried out as described by Birnboim and

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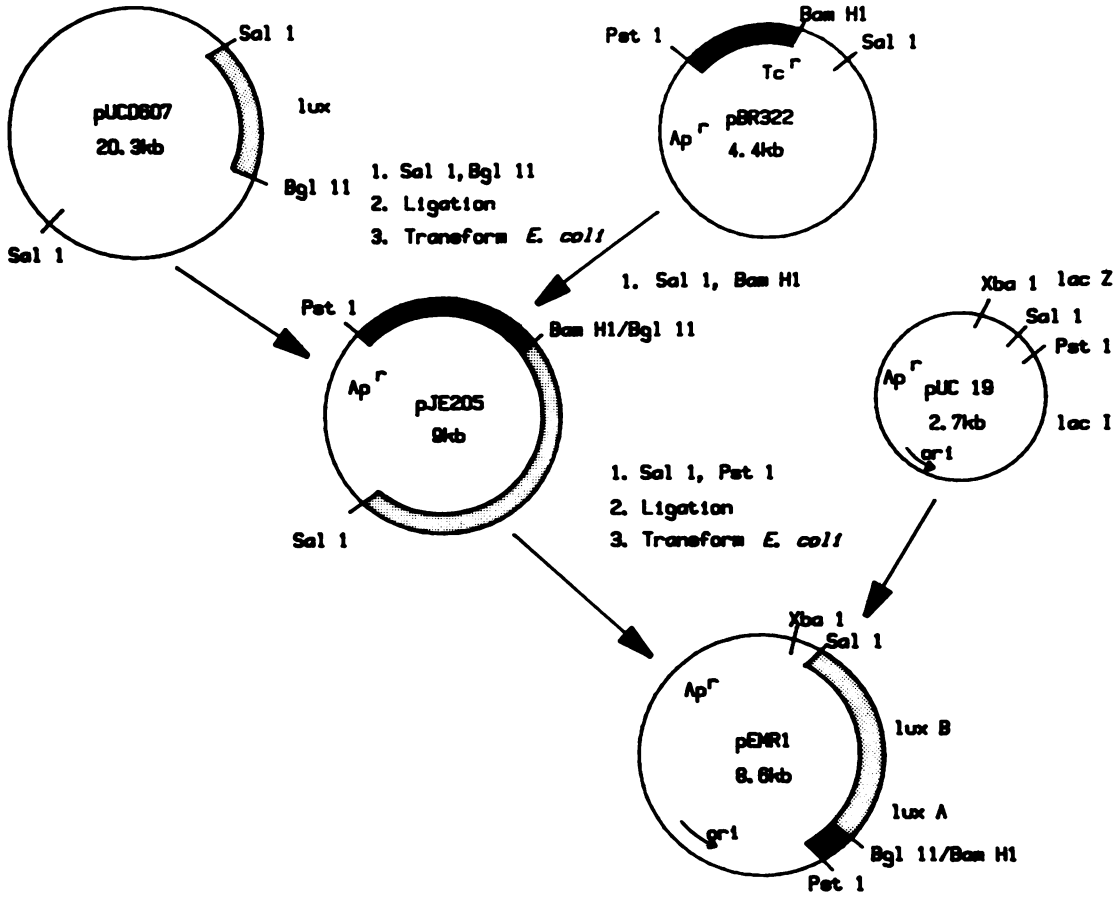

FIG. 1. Construction of plasmids pJE205 and pEMR1. Only the relevant restriction sites are indicated, and maps are not drawn to scale. The 5-kb *SalI-BglII* restriction fragment of pUCD607, which contains *luxA* and *luxB*, was cloned into pBR322 that had been cleaved with *Bam*HI and *Sal*I. The resultant plasmid, pJE205, contains the luciferase genes under the control of the *tet* promoter. The 5.9-kb *SalI-PstI* restriction fragment of pJE205 was inserted into the polylinker cloning site of pUC19 to generate pEMR1. *E. coli* MM294, transformed with either pJE305 or pEMR1, is capable of constitutive light production by adding *n*-decyl aldehyde.

Doly (2). DNA fragments were isolated from agarose gels by the method of McDonnell et al. (16). Restriction endonucleases and T4 DNA ligase were purchased from Amersham, and the conditions used for digestion and ligation of DNA were those recommended by the manufacturer.

Growth and light output in liquid culture. All strains were maintained and grown in L broth or on L broth solidified with 1.5% agar, both containing ampicillin (25 μg/ml) to maintain the plasmid. Batch growth experiments were carried out in triplicate in 250-ml Erlenmeyer flasks containing 100 ml of L broth inoculated with 1 ml of a stationary-phase culture of the appropriate *E. coli* strain. Flasks were incubated at 30°C on a rotary shaker (180 rpm). Samples were removed at regular intervals for measurement of absorbance and light output. The A_{600} of samples (1 ml) was measured and was related to biomass concentration and cell concentration by using standard curves obtained from a culture of *E. coli* MM294 grown in L broth. Light output was measured in triplicate 1-ml samples by using an LKB model 1251 luminometer with output integrated over a 10-s period with continuous mixing and is expressed as relative light units (RLU). Light output was measured in 1-ml samples taken from a 1/10 dilution series, constructed in phosphate-buffered saline, of an exponentially growing culture of *E. coli*. When required, the total cell concentration was estimated by using a Thoma counting chamber and viable concentrations were estimated by the dilution plate method, inoculating Petri dishes containing L agar with samples from a 1/10 dilution plate series and incubating for 24 h at 30°C. Strains containing plasmids bearing *luxC*, *luxD*, and *luxE* produce the aldehyde substrate required for light output. In experiments with strains lacking these genes (i.e., those containing plasmids pJE205 and pEMR1), 1 μl of decyl aldehyde was mixed, by vortexing, with either liquid or soil samples. Luminometer readings were taken within 1 min of sample preparation.

TABLE 1. Plasmids used for transformations of *E. coli* MM294, DH1, and HB101

Plasmid	Genotype	Source or reference
pBTK5	Amp^r *luxRICDABE*	3
pUCD607	Amp^r Km^r Sp^r *luxCDABE*	18
pJE205[a]	Amp^r *luxABE*	This study
pEMR1[a]	Amp^r *luxABE*	This study

[a] Cells containing plasmids pJE205 and pEMR1 are phenotypically dark in the absence of exogenously supplied *n*-decyl aldehyde.

Detection in soil. The soil used was a sandy loam (Boyndie series; pH in distilled water, 4.61; total organic carbon, 2.4%; total nitrogen, 0.1%; cation-exchange capacity, 2.8

TABLE 2. Maximum specific growth rates of host strains and luminescent strains of *E. coli*

Strain	Maximum specific growth rate (h^{-1})[a]	Variance	P^b
MM294	1.00	0.082	
MM294(pBTK5)	0.92	0.013	0.79
MM294(pJE205)	0.96	0.0008	0.52
MM294(pEMR1)	0.89	0.0017	0.86
DH1	0.75	0.053	
DH1(pBTK5)	0.70	0.0009	0.71
DH1(pUCD607)	0.69	0.0017	0.78
HB101	0.63	0.009	
HB101(pUCD607)	0.63	0.0005	0.18

[a] Maximum specific growth rates are the means from three replicate flasks.
[b] Values of P were determined using the Student t test to compare the mean specific growth rate of each host strain with that of the corresponding luminescent strain.

cmol kg^{-1}) sampled from 0 to 20 cm depth from the headland of an arable field near Elgin, North Scotland (Grid reference, NJ224659). Soil was sterilized before use for 1-h intervals at 120°C on three consecutive days. Cells of the appropriate luminescent *E. coli* strain were inoculated into 0.5 g of soil in a cuvette (4 ml), mixed thoroughly, and incubated at 30°C for 0.5 h. The inoculum consisted of 0.5 ml taken from a 1/10 dilution series of an exponentially growing culture of *E. coli*, and light output was measured by luminometry, as described above, after vortexing the slurry.

RESULTS

Batch growth of all strains was characterized by a short or nonexistent lag phase, an exponential phase, a relatively long deceleration phase, and a stationary phase. The maximum specific growth rates of plasmid-bearing strains were not significantly different from those of the host strains (Table 2). In strains containing plasmids bearing the complete *lux* cassette (pBTK5) and a *lux* cassette with a truncated *luxI* gene (pUCD607), growth and bioluminescence followed different patterns. In *E. coli* DH1(pBTK5), light output was autoinducible, decreasing during early exponential growth before increasing sharply and peaking at 9 h during the deceleration phase (Fig. 2a). Similar results were obtained for *E. coli* MM294(pBTK5) (data not shown), but in *E. coli* DH1(pUCD607) (Fig. 2b) and *E. coli* HB101(pUCD 607) (data not shown), light output peaked at 8 h and decreased during the early stationary phase. Plasmid pUCD 607 contains a truncated *lux* cassette under the control of the *tet* promoter (18). Strains harboring pUCD607 should bioluminesce constitutively, but our data indicate partial autoinduction. Luminescence profiles therefore varied with the plasmid rather than the host strain.

Light output per unit biomass increased during growth, as illustrated in Fig. 2c for strains DH1(pBTK5) and DH1(pUCD607). This reflects the full or partial autoinduction, respectively, of luciferase as the biomass concentration increased (7), with differences between plasmids pBTK5 and pUCD607 reflecting differences in plasmid construction. Both plasmids, however, led to variation in the light output per cell with increasing biomass concentration during batch growth, preventing their general use for quantitative detection.

The maximum level of light output was 4.9×10^6 RLU mg of biomass^{-1}, which is equivalent to 0.13 RLU cell^{-1}. The luminometer typically gave constant background levels in the order of 5 RLU, and the standard error for triplicate background readings was typically 3×10^{-3} RLU. In liquid culture, therefore, the lower detection limit during maximum light output was approximately 50 cells ml^{-1}. There is no upper detection limit, but samples containing more than 6×10^6 cells ml^{-1}, equivalent to 8×10^4 RLU, required dilution. The lower detection limit in liquid culture was also determined by measuring light output in 1-ml samples of a 1/10 dilution series of exponentially growing cultures of *E. coli*. Light output was directly proportional to cell concentration down to 10^2 to 10^3 cells ml^{-1} (Fig. 3).

Luminescence profiles for both constitutive strains, MM294(pEMR1) (Fig. 4) and MM294(pJE205) (data not shown), closely followed increases in biomass concentration. A plot of light output against biomass concentration was therefore linear over the full range investigated, and light output per unit biomass showed little variation during growth, with a value of $(3.10 \pm 0.42) \times 10^5$ RLU mg of biomass^{-1}. Extrapolation suggests a lower detection limit of 10^3 cells ml^{-1}. The lower detection limit was also determined by measuring light output by samples of a 1/10 dilution series of exponentially growing cells of *E. coli* MM294 (pEMR1). A linear relationship was obtained, similar to that described above (Fig. 3), but light output by the constitutive strains was lower than that of the autoinducible strains (Table 2). Constitutive strains required an exogenous supply of the luciferase substrate, but this is not thought to have limited light output. Decyl aldehyde is freely permeable to bacterial cells, and increasing the concentrations of and lengths of exposure to the aldehyde did not increase light output but did decrease cell viability. The reduced luminescence of constitutive strains is more likely to have resulted from lower levels of expression of *luxA* and *luxB* genes owing to use of a heterologous promoter.

Light output in soil. Application of luminometry to quantification of cells in soil was assessed by inoculating sterilized soil with a range of concentrations of *E. coli* MM294 (pBTK5), DH1(pUCD607), MM294(pEMR1), and MM294 (pJE205). For each strain, light output was proportional to cell concentration (Fig. 5) although the amount of light output per cell was reduced by approximately 1 order of magnitude compared with cells in liquid culture. Lower detection limits are summarized in Table 3. The most suitable strains are those in which luciferase is produced constitutively. Although these gave lower sensitivity, luminometry was still capable of detecting 2×10^2 to 6×10^3 cells g of soil^{-1}, depending on the strain inoculated. Luminometry therefore provides more sensitive and more rapid enumeration than other nonextractive detection techniques and shows a linear response over the full range of concentrations tested.

DISCUSSION

The introduction into *E. coli* of genes for luminescence provided a means of assessing biomass and cell concentration in liquid culture and in soil. The amount of light output varied with both host and plasmid, but in the most active autoinducible strains a lower detection limit of 50 cells ml^{-1} was achieved for suspended cells. Variation in light output between host cells may reflect differences in regulation of cell metabolism, leading to production of $FMNH_2$, while differences in bioluminescence profiles between plasmids

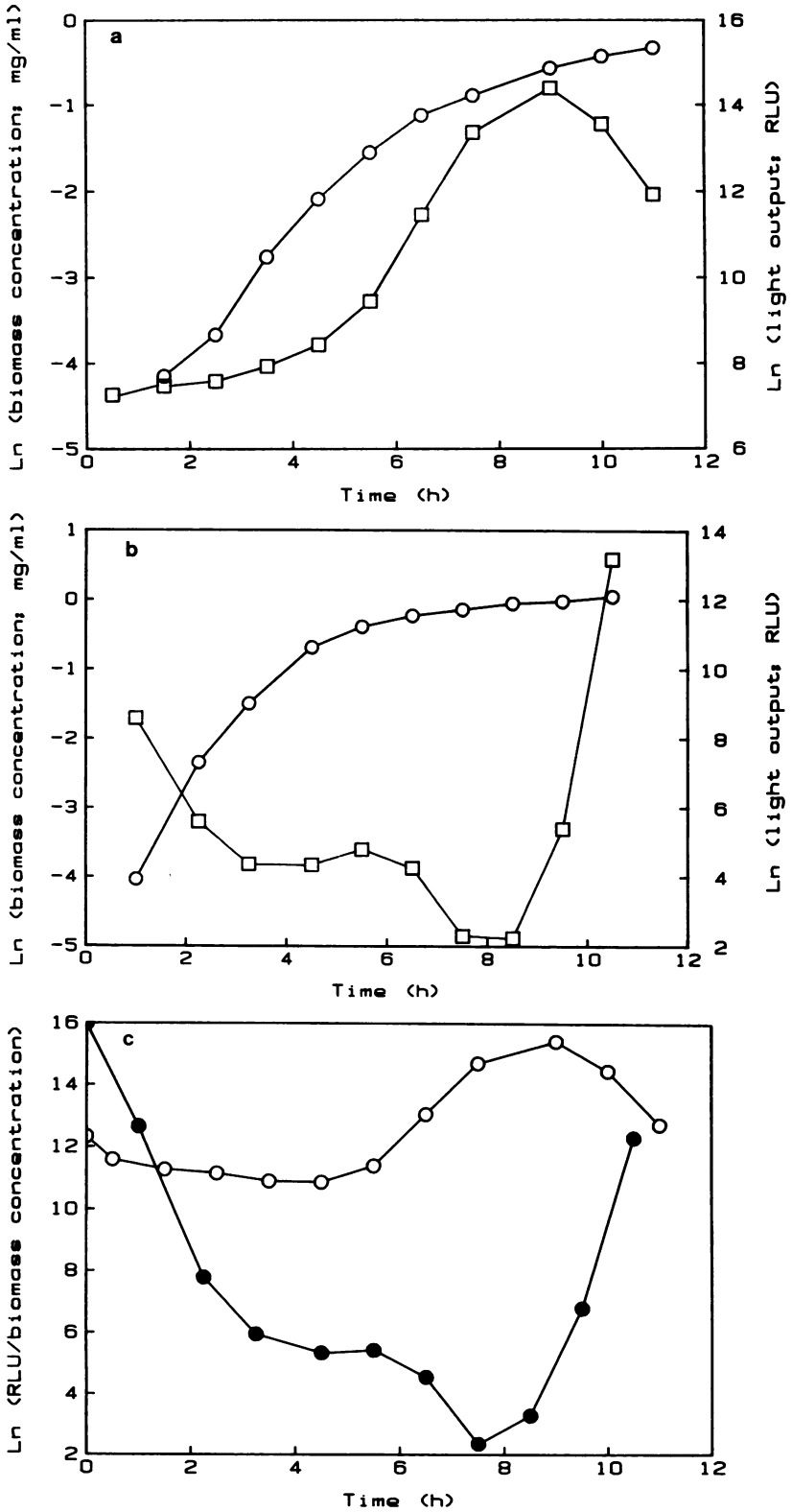

FIG. 2. (a and b) Changes in biomass concentration (○) and light output (□) during growth of *E. coli* DH1(pBTK5) (panel a) and *E. coli* DH1(pUCD607) (panel b) in liquid culture. (c) Changes in light output per unit biomass during growth *E. coli* DH1(pUCD607) (○) and DH1(pBTK5) (●).

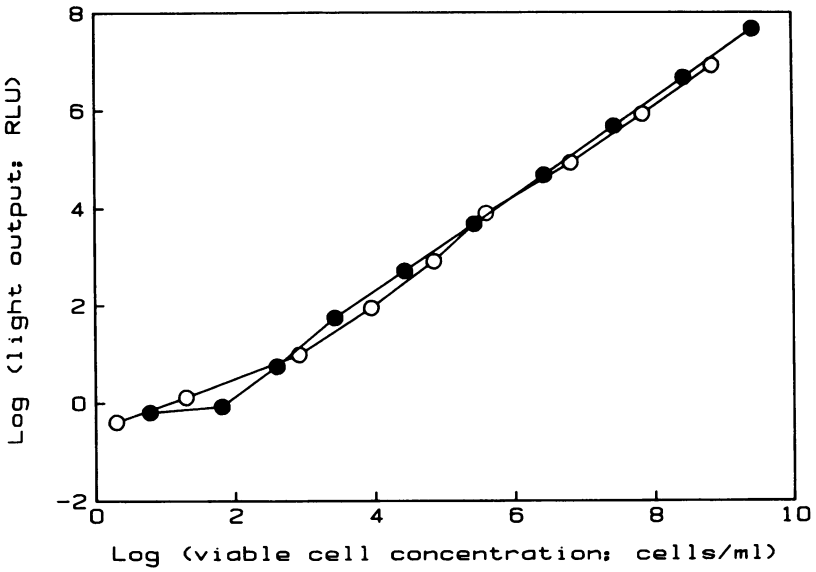

FIG. 3. Light output by exponential-phase cells of *E. coli* DH1(pUCD607) (○) and MM294(pBTK5) (●) diluted in L broth.

pUCD607 and pBTK5 may reflect differences in plasmid copy number. In all strains, light output was proportional to cell concentration over several orders of magnitude. For autoinducible strains, however, light output per cell varied with cell concentration, preventing general use of these strains. For the constitutive strains, light output per cell was independent of cell concentration and could be reliably used to assess cell concentration.

All strains produced less light than *V. fischeri*, the source of the *lux* genes, and a greater understanding of the physiology of luminescence offers the potential for increased light output from *E. coli*. For example, a stronger promoter may increase the expression of the *lux* genes while physiological conditions such as temperature and pH may have been suboptimal for light production. In addition, sensitivity would be increased by a reduction in background light levels measured by the luminometer and a more sensitive photomultiplier tube.

Optimization of conditions for light output is particularly important in soil, where detection levels were up to 1 order of magnitude lower than in liquid culture, owing to quenching of light by soil particles. Light output decreased when cells stopped growing, and luminescence by cells recovering from starvation may be submaximal. Luminometry may therefore provide a measure of microbial activity, while measurement of luminescence potential may be necessary for assessment of biomass under such conditions. Thus, luminometry provides a rapid, nonextractive technique for estimation of cell concentrations within soil, with results obtained within 5 min of sampling. In addition, background luminescence from soil was negligible, and development of the technique offers the potential for distinguishing activity and biomass of the tagged population. Levels of luminescence are likely to vary among genera and strains, but the technique is potentially applicable to any microorganism for which a genetic transformation system has been character-

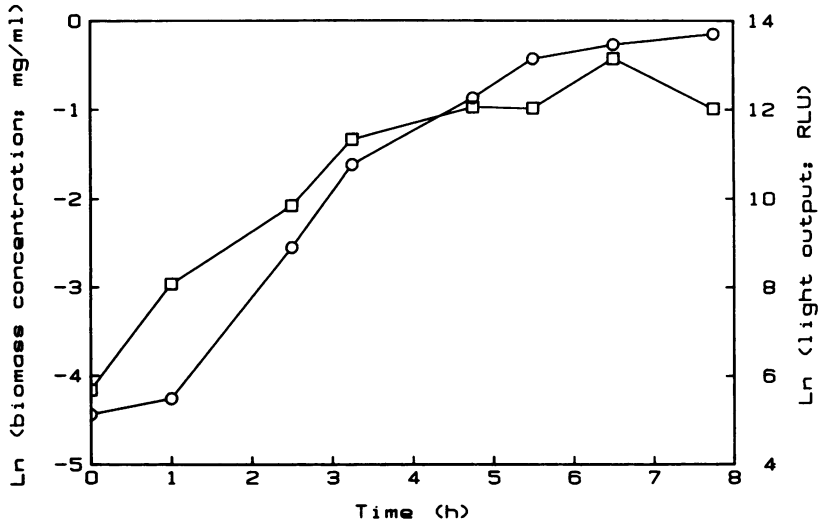

FIG. 4. Changes in biomass concentration (○) and light output (□) during growth of *E. coli* MM294(pEMR1).

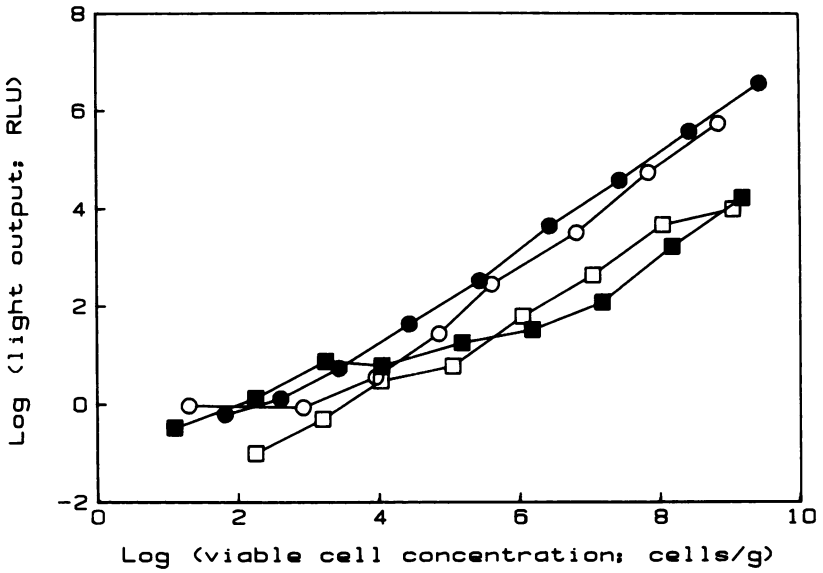

FIG. 5. Light output by exponential-phase cells of *E. coli* DH1(pUCD607) (○), DH1(pBTK5) (●), DH1(pEMR1) (□), and MM294(pJE205) (■) diluted in L broth and inoculated into sterile soil. Standard errors of the mean of triplicate samples with mean luminescence values greater than the lower detection limit (Table 3) were less than 5% of the mean.

ized, and we are currently studying its application to *Erwinia*, *Bacillus*, and *Pseudomonas* spp. In addition, it possesses all the advantages of other molecular-based marker techniques. The luciferase gene has been sequenced (1), allowing construction of primers for the polymerase chain reaction, and the *lux* gene can also be probed for directly. Selective dilution plate counting is possible through visible luminescence of colonies. A further advantage is the potential for true in situ detection of luminescing cells in soil by charge-coupled microscopy (14) and X-ray film imaging (19), which will allow determination of the spatial distribution of introduced organisms.

There is no evidence for a reduction in specific growth rate as a result of luminescence during growth in batch culture, but more detailed studies of growth under carbon and energy limitation are required to determine whether luminescent organisms would be at a disadvantage compared with similar wild-type organisms in natural environments. If this were the case, the problem could be solved by construction of strains with an inducible system for luciferase production. Luminometry therefore provides a sensitive, rapid, nonextractive technique for detection of genetically engineered bacteria in the soil as part of a package of detection techniques based on bioluminescence.

TABLE 3. Lower detection levels for luminescent strains of *E. coli* in liquid culture and inoculated into sterile soil

Strain	Lower detection limit[a] in:	
	Liquid culture (cells/ml)	Soil (cells/g)
DH1(pUCD607)	50	1,000
MM294(pBTK5)	72	199
MM294(pEJ205)	148	806
MM294(pEMR1)	1,211	5,962

[a] Background luminescence was 5 RLU, and the standard error of the mean of triplicate luminometry measurements of a single sample was less than 0.01. The lower detection limit was calculated as the cell concentration giving 6 RLU.

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