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Bioluminescent Most-Probable-Number Method To Enumerate *lux*-Marked *Pseudomonas aeruginosa* UG2Lr in Soil

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Bioluminescence measured with a luminometer and charge-coupled device was an effective marker in most-probable-number assays for *luxAB*-marked *Pseudomonas aeruginosa* UG2Lr in soil. Most-probable-number assays with microtiter plate wells and luminometer tubes gave estimates for UG2Lr that were similar to viable colony counts. Both methods detected five cells per g of soil.

Bioluminescence-based marker technology is successful and promising for tracking genetically engineered microorganisms (GEMs) (6, 9, 16, 26). Recent reviews (12, 13, 19, 21) describe the molecular biology and use of *lux* genes in a variety of microorganisms. Luminescence may be measured by a luminometer (22), scintillation counter (12, 25), or light pipe (6, 10) or may be imaged directly on solid media, soil, food, and plant material by using photographic or X-ray film (6, 17, 21, 28) and charge-coupled devices (CCDs; 7, 9, 15, 16, 21, 27). Individual bacterial cells have been detected by using CCDs without (18) and with a microscope (15, 16, 27).

The detection of bacteria in situ generally requires large numbers of cells, ranging from 1.5×10^4 CFU per leaf (26), 10^4 CFU/cm of root (6), and 7×10^7 cells per g of soil (15) to 6×10^3 cells per g of soil (22). In this study, we investigated a most-probable-number (MPN) method for the detection and enumeration of a bioluminescent *Pseudomonas aeruginosa* UG2Lr strain in soil with a luminometer and CCD. The isolation, growth conditions, and insertion of chromosomal *luxAB* genes into *P. aeruginosa* UG2L and the isolation of a rifampin-resistant UG2Lr strain have been detailed elsewhere (2, 7, 31), with the exception that in this study, cells were grown in tryptic soy broth (TSB; Difco, Detroit, Mich.) rather than minimal salts medium. The sensitivities of the MultiLite luminometer (Biotrace, Plainsboro, N.J.) and charge-coupled Bioview Image Quantifier (BIQ; Biomedical Image Research, Cambridge, United Kingdom) determined for mid-log-phase (12 to 18 h) cells of UG2Lr in TSB culture were 3×10^5 CFU/ml and 6×10^7 CFU/ml, respectively. These sensitivities were reduced by 1 order of magnitude in 1:1 soil-TSB suspensions through soil quenching, as previously observed (10, 22). Five to ten microliters of 10% (vol/vol) ethanolic solution of *n*-decanal (Sigma Chemical Co., St. Louis, Mo.) substrate per ml was added to samples prior to bioluminescence measurements. Amounts greater than 20 μl of *n*-decanal per ml caused a reduction in light emission (data not shown). In addition to instrumentation limitations, light production in *lux* gene-bearing GEMs varies with the bacterial strain (13, 15, 19, 22, 28), gene organization (13, 22, 28), substrate availability, growth phase, and cell metabolic rate (10, 22). In soils in which cells may be inactive or strains are weakly bioluminescent, direct detection of these cells is difficult. Our initial attempt to directly detect 4-day-old UG2Lr inocula in sandy loam soil microcosms with the BIQ revealed that photon images were visible only 6 h after microcosms had received a 2.25-ml TSB-nutrient spike amended with 50 μg of rifampin per ml. Photon images were visible after the original mean ($n = 3$) stationary-phase UG2Lr cells increased from $(9.0 \pm 5.0) \times 10^6$ viable cells per g to $(3.4 \pm 0.15) \times 10^8$ metabolically active cells per g (dry weight) of soil as determined by spread plating (7).

Because of weak light emission and limited photon detection in soil, we developed a simple microplate MPN assay with microtiter plates (Falcon Microtest III; Becton Dickinson, Toronto, Canada) previously used for MPN analyses (8, 11, 23). A bioluminescent MPN technique permits the rapid detection and enumeration of *lux*-bearing cells in soil, and although it requires dilution and nutrient amendment, this method guarantees the high cell densities and metabolic rates required for detection without the use of CCDs or strongly luminescent host cells. The sandy loam soil characteristics, 15.0-g soil microcosm design, inoculation, incubation conditions, and reisolation of introduced microorganisms have been described in detail by Flemming et al. (7).

Standard five- and/or eight-tube replicates were used for MPN assays as described by Alexander (1). Four to ten decimal dilutions were prepared in sterile saline (0.85% [wt/vol]), 1.0-ml samples were inoculated into 10-ml culture tubes and/or luminometer tubes (Mandel Scientific, Guelph, Canada), and 0.1- or 0.2-ml samples were inoculated into microtiter plate wells. With microtiter plates, two or three replicate tests were conducted per plate. Prior to inoculation, 1.0 or 2.0 ml of nonselective TSB or TSB medium containing 50 μg of rifampin per ml was added to sterile tubes or 0.1 or 0.2 ml was added to microtiter plate wells and liquid partially evaporated overnight (11). Concurrently, spread plating was performed as described previously (7), with the exception that some 1-ml samples were spread onto agar plates previously air dried for 2 h in a laminar flow hood. Luminometer and culture tubes and microtiter and petri plates were incubated for 24 h at 30°C without shaking. For bioluminescent scoring of microtiter plates, 15 μl of *n*-decanal solution was spread over lids and photons were collected for 3 to 5 min with the BIQ. On microtiter plates containing 0.2-ml samples, luminescent well images were observed only when a minimum of 7×10^6 UG2Lr cells per ml of suspension was present. The light emissions from tubes were measured with the luminometer after the addition of *n*-decanal. Background luminescence in uninoculated soil and media ranged from 3 to 40 relative light units on the luminometer. After scoring the tubes, MPN estimates were determined from published eight-tube (20) and five-tube (1) tables for decimal dilutions.

Preliminary pure culture studies showed that the mean

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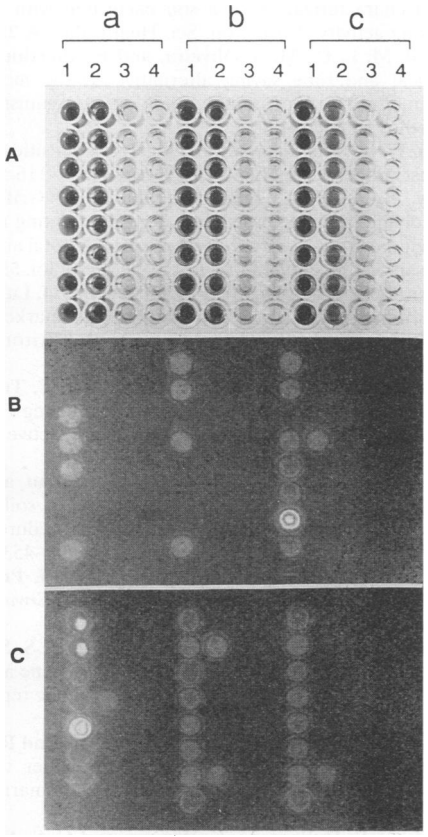

FIG. 1. Bioluminescent eight-well MPN assays on microtiter plates visualized by eye (A) and by photon imaging (B and C). Wells in each dilution series (1 to 4) received 100, 10, 1, and 0.1 mg (dry weight) of soil, respectively, into their TSB-rifampin media. Triplicate microMPN assays (a, b, and c) are shown for soils inoculated with 5 and 22 UG2Lr cells per g (dry weight) of soil (B and C, respectively). Photons were collected for 3 min with the charge-coupled BIQ. The wells emitting light contained *P. aeruginosa* UG2Lr cells and were scored positive.

viable *P. aeruginosa* UG2Lr cell estimates by test tube and microtiter plate five-tube MPN assays were within ±30% (n = 3) of the numbers obtained by plate counts, as determined by inspection of upper and lower 95% confidence limits (data not shown). The MPN results determined with nonselective broth were within ±20% (n = 3) of those obtained by using medium amended with rifampin and cycloheximide at 50 μg/ml each (data not shown).

After counting with a hemocytometer and appropriate dilution of inoculum, 5 to 1,100 mid-log-phase UG2Lr cells per g (dry weight) of soil were added to microcosms to determine the detection limit of bioluminescent microplate MPN (microMPN) assays in this soil. Figure 1 shows microtiter plate (A) and photon images for triplicate eight-tube microMPN assays performed with 0.2 ml of soil suspension and 10-fold soil dilutions from microcosms containing 5 (B) and 22 cells per g (dry weight) of soil (C). From the number of positive wells (4-0-0, 4-0-0, 7-1-0) (Fig. 1B), MPNs were estimated to be 6, 6, and 18 cells per g of soil according to eight-tube tables (20). The viable cell estimates and 95% confidence limits obtained by microMPN and plate counts are summarized in Table 1. Plating and microMPN estimates were similar, and both methods detected as few as five cells per g of soil (Fig. 1B)

TABLE 1. Detection limits of spread plating and eight-well microMPN methods for the estimation of *P. aeruginosa* UG2Lr cells in soil microcosms[a]

Inoculum density (CFU/g of soil)[b]	Mean (n = 3) viable cells/ g (dry wt) of soil		95% confidence limit[c]	
	Plate count ± SD at 95% confidence	Eight-well MPN	Upper	Lower
1.1×10^3	990 ± 210	720	2,200	180
2.2×10^2	250 ± 81	250	950	74
1.1×10^2	52 ± 13	63	220	10
22	13 ± 12	28	93	7
5	11 ± 8	8	48	2

[a] Mid-log-phase cells (18 h) were added to soil 3 h prior to reisolation. Media were amended with rifampin and cycloheximide at 50 μg/ml each.
[b] Determined by spread plating (n = 5) as standard deviation ± 17% of mean inoculum density.
[c] 95% confidence limits for eight-tube MPNs were calculated by multiplying the highest and dividing the lowest estimates by 2.57 (1, 4).

(Table 1). Means were more precise by plating than by MPN methods on the basis of 95% confidence ranges (Table 1), as is commonly noted for MPN techniques (20, 29). Means determined by MPN or viable colony counting were usually from ±10 to ±50% of the actual value (Table 1). Standard eight- and five-tube MPNs gave similar results, with their means differing from the microMPN means by ±4 to 42% (data not shown). With eight-tube tests, however, the confidence limits were slightly smaller since the standard error is reduced with a larger number of replicates per dilution (4). Estimates determined by luminometer tube MPN assays were also similar to those by the microMPN method, differing at most by ±21% of microMPN values (data not shown).

Table 2 presents estimates of *P. aeruginosa* UG2Lr cells in soil microcosms 4 and 11 weeks after inoculation as determined by spread plating and various five-tube MPN assays. Remarkably similar means were obtained by plating and MPN assays (Table 2). Mean estimates from nonselective TSB tubes and wells were equal to or 1.5- to 5-fold higher than those obtained from rifampin-amended broth (data not shown). It was necessary to use rifampin selection for the MPN tubes and wells receiving nonsterile soil diluted less than 1/1,000 because

TABLE 2. Comparison of spread plating and five-tube MPN methods for the estimation of *P. aeruginosa* UG2Lr populations in soil microcosms[a]

Week	Estimation method[b]	10^5 Viable cells/g (dry wt) of soil		
		Mean (n = 3)	95% Confidence limit[c]	
			Upper	Lower
4	Plate counts	15	25	5.7
	MicroMPN	13	79	1.5
	LargeMPN	18	79	3.9
	LumiMPN	23	110	3.9
11	Plate counts	1.2	1.5	0.88
	MicroMPN	2.3	7.9	0.67

[a] Microcosms were inoculated with (5.4 ± 1.8) × 10^8 CFU/g (dry weight) of soil.
[b] Five-tube MPNs were performed with microtiter plates (microMPN), test tubes (largeMPN), and luminometer tubes (lumiMPN).
[c] 95% confidence limits for five-tube MPN values were derived as in Table 1 by using the factor 3.30 (1, 4).

excessive growth of indigenous microorganisms inhibited growth and detectable bioluminescence in some TSB wells containing UG2Lr cells.

Total heterotrophs were also estimated by eight-well microMPN and spread plating using nonselective media. MPN and spread plates were incubated for 1 week at 22°C after the initial 24-h incubation, and wells and tubes were scored positive on the basis of turbidity. The turbidity of microtiter wells was measured with a Bio-Rad 3550 UV microplate reader at 595 nm. Estimates and 95% confidence limits ($n = 3$) for total cultured aerobic heterotrophic microorganisms in the sandy loam soil were $(4.0 \pm 1.2) \times 10^6$ and 2.4×10^6 CFU/g (dry weight) of soil (range, 1.5×10^6 CFU/g to 8.5×10^6 CFU/g) as determined by plate counting and microMPN, respectively. Total heterotroph and UG2Lr populations can, therefore, be determined simultaneously if soil dilutions exceed 10^{-8} and nonselective medium is added to the wells receiving higher soil dilutions.

Although viable colony counts were more precise than MPNs, in cases in which small numbers of replicates were employed (five and eight tubes), MPN estimates were within 1 order of magnitude of colony counts. MicroMPN assays are rapid to perform and require significantly less medium and materials, and data collection from triplicate samples is obtained in minutes. Furthermore, a CCD is not required to achieve sensitive detection of bioluminescent GEMs in soil. The limit of detection in soil by MPN is theoretically better with luminometer tube assays than microMPNs because the sample volumes are 5-fold greater and the luminometer is 10- to 100-fold more sensitive than the BIQ.

MPN analysis is widely used for soil (1, 8, 23), food (32), water (11), and wastewater and in other areas of microbiology (5). Statistical discussions (4, 29) and computer programs (14, 20, 23, 24, 30) have been proposed for estimating MPNs. Research has shown a strong correlation between the bacterial estimates determined by spread plating and MPN (3, 32). Although plating is more precise (20, 29), the precision of MPN techniques is increased by increasing the number of samples per dilution (4, 11, 20) and decreasing the dilution ratios (4, 23). In this study, we used a sandy loam soil common to one of the field research stations at the University of Guelph. It is not known if any problems will be encountered with the MPN-*lux* method using soils with high clay or organic matter contents. A preliminary experiment by researchers using other soils will provide information on the suitability of this MPN method prior to full-scale use of the technique.

MPN can be an effective and rapid method for tracking *lux*-bearing GEMs in soils. The scoring of bioluminescent MPN tubes is rapid, and further identification of the GEM is not required because luminescence is virtually unique in soil (12). Furthermore, CCDs are not a necessity, as this assay was equally effective with luminometer tubes. Microdilution MPN procedures are promising for following the fate of GEMs in natural samples and can be combined with other identification technologies such as PCR and DNA hybridization (8).

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