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# Simultaneous detection of *gfp*-marked *Moraxella* sp. G21r and *lux*-marked *Ralstonia eutrophas* H850Lr using most-probable-number method

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**Abstract**

The green fluorescent protein encoded by *gfp* gene and the luminescent protein encoded by *luxAB* genes were used as markers to detect *p*-nitrophenol (PNP)-degrading *Moraxella* sp. G21r and polychlorinated biphenyl (PCB)-degrading *Ralstonia eutrophas* H850Lr cells, respectively, in mixed liquid cultures and in soil samples using a most-probable-number (MPN) assay. Population estimates for both *gfp*-marked G21r and *lux*-marked H850Lr by using MPN assays were similar to viable colony counts. The MPN assay with microtiter plates permitted the simultaneous detection of fluorescent and luminescent bacteria in soil samples faster than conventional plate counting. 

*Keywords:* *gfp*-marked *Moraxella* sp. G21r; *lux*-marked *Ralstonia eutrophas* H850Lr; MPN assay

## 1. Introduction

Genetically engineered microorganisms (GEMs) may have the potential for various applications in crop production, biodegradation of toxic compounds, and biological control (Amy et al., 1985; Milewski, 1985; Morgan and Watkinson, 1989; Drahos, 1991). Although recombinant DNA techniques have allowed the development of altered bacterial strains, there may be possible environmental risks associated with using GEMs. For this reason, suitable detection techniques are needed to detect and enumerate GEMs in the environment.

The detection of bacteria in situ has been conducted using spread plating that requires viable, culturable and detectable numbers of cells per gram of soil (Rattray et al., 1990; Mahro et al., 1992). In this study, we investigated marker systems including the green fluorescent protein (GFP) (Errampalli et al., 1999a) and bioluminescence (*luxAB*) with a most-probable-number (MPN) method which can be simultaneously used in enumerating GEMs in soil and aquatic samples. In order to develop a dual detection technique for fluorescent and luminescent bacteria simultaneously in soil environments, we used *Moraxella* sp. G21r and *Ralstonia eutrophas*

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H850Lr as marked strains because both could be used for bioremediation. The G21r strain can degrade *p*-nitrophenol (PNP) and H850Lr can degrade PCBs (Tresse et al., 1998; Van Dyke et al., 1996). This paper describes a technique that is simpler and faster than the conventional plate counting for *gfp*-marked G21r and *lux*-marked H850Lr in mixed liquid cultures and soil samples.

## 2. Materials and methods

### *2.1. Bacterial strains, growth conditions, and chemicals*

The bacterial strains used in this study were *Moraxella* sp. G21r and *R. eutrophas* H850Lr containing *gfp* genes and *luxAB* genes, respectively. G21r and H850Lr were maintained on tryptic soy agar (TSA) medium supplemented with kanamycin (50 μg/ml) and rifampicin (50 μg/ml). Cultures of G21r and H850Lr were grown for 36 h at 30°C at 200 rpm in 30 ml of tryptic soy broth (TSB). For long-term storage, cultures were maintained at − 70°C in 50% (v/v) glycerol.

Kanamycin, rifampicin, and cycloheximide were purchased from Sigma (St. Louis, MO, USA). TSA and TSB were from Difco (Detroit, MI, USA). Microtiter plates and *n*-decanal were purchased from Fisher Scientific (Toronto, Ont., Canada).

### *2.2. Preparation of pure cultures and mixed cultures in broth and soil*

To prepare equal cell numbers of G21r and H850Lr grown in broth before MPN assays, cultures of G21r and H850Lr were diluted in sterile 0.85% (w/v) NaCl to an O.D. of 1.0 at 600 nm, and each culture was diluted again to give a final cell number from $1\times10^{1}$ to $1\times10^{6}$ $ml^{-1}$. To identify the correlation between O.D. and culturable cell numbers, plate counting was carried out at the same time. Each pure culture at various cell concentrations, from $10^{6}$ to 1 $ml^{-1}$, was mixed with the same volume of another culture at different ratios to make combinations of G21r to H850Lr or H850Lr to G21r from $1{:}10^{6}$ to 1:1, respectively.

The mixed culture of G21r and H850Lr for the soil experiment was prepared with only the 1:1 ratio prior to being inoculated into soil samples. A sandy loam soil (75% sand, 18% silt, 7% clay) was collected from field plots at the University of Guelph Cambridge Research Station, Canada. The soil characteristics were described previously (Flemming et al., 1994). The cells (about $1\times10^{7}$ CFU/g) were mixed into 50 g of moist soil contained in a 100 ml beaker. The soil samples were incubated at 24°C in the dark for 30 days. Periodically, 1 g of soil sample was removed and diluted in sterile 0.85% (w/v) NaCl to a final volume of 10 ml, and these samples were used for MPN assays.

### *2.3. MPN assays*

Two hundreds μl of mixed culture samples of various combinations were placed in the first well of the microtiter plates which consist of 12 wells containing 180 μl of TSB medium supplemented with kanamycin and rifampicin at concentrations of 50 μg/ml, respectively. Serial 10-fold dilutions were performed from the second well to the 11th well with transfers of 20 μl to the next wells. The 12th well containing only TSB medium supplemented with 50 μg/ml kanamycin and 50 μg/ml rifampicin was used as the control. All MPN assays were carried out in triplicate and based on per g dry weight soil sample.

### *2.4. Detection of fluorescence and luminescence*

Microtiter plates containing mixed cultures of serial decimal dilutions were incubated for 2 days at 30°C in the dark. After incubation, the fluorescence intensities of GFP and luminescence by LuxAB activity in microtiter plates were detected using a FL500 fluorescent plate reader (Labsystems, Helsinki, Finland) and a luminescent detector (BIQ, Cambridge, UK), respectively. For fluorescent detection, positive and negative numbers were determined based on the control which was the 12th well. In the luminescent detection, 20 μl of 50% (v/v) ethanolic solution of *n*-decanal (Sigma) substrate per ml was added to the lid of microtiter plates, and incubated for 30 s prior to bioluminescence measurement. Positive numbers were determined by photon images based on light emission of LuxAB in the

presence of the *n*-decanal vapour (Flemming et al., 1994).

### 2.5. Enumeration of G21r and H850Lr

MPN estimates were determined from previously published four-tube tables for decimal dilutions (Vincent, 1970). For example, from the number of total positive wells (4-4-3-0-0-0-0-0-0-0 ($n = 11$, total positives), 4-4-4-4-4-4-2-0-0-0 ($n = 26$, total positives), 4-4-4-4-4-4-4-4-1-0 ($n = 33$ positives total)), MPNs were estimated to be $1.0 \times 10^2$, $5.8 \times 10^5$, and $3.1 \times 10^7$ cells per g of dry soil (or ml of liquid culture) according to four-tube tables (Vincent, 1970).

### 2.6. Data analysis

Following an analysis of variance, significant differences between plate counts and microMPNs were identified using a two-tailed Student's *t*-test at the 0.01 level of significance (Bailey, 1995).

## 3. Results and discussion

### 3.1. Simultaneous detection of G21r and H850Lr in mixed liquid cultures

In a previous study, we constructed a *gfp*-marked *Moraxella* sp. G21 strain (Tresse et al., 1998; Errampalli et al., 1999b). Subsequently, a spontaneous rifampicin-resistant strain, designated G21r, was isolated from G21. H850Lr is resistant to both rifampicin and kanamycin (Van Dyke et al., 1996). We examined the simultaneous detection of both marked strains in mixed broth cultures. MPN cell estimates of G21r and H850Lr in the 1:1 cell ratio mixture were similar to the cell numbers obtained by plate counts in which G21r was initially present at $4.5 \pm 1.3 \times 10^8$ CFU/ml and H850Lr at $5.7 \pm 0.2 \times 10^8$ CFU/ml (Table 1). In addition, the plate count of the two strains mixed in 1:1 ratio was $5.3 \pm 1.0 \times 10^8$ CFU/ml. These results verify that we used equivalent cell numbers for G21r and H850Lr. As previously reported (Flemming et al., 1994), cell estimates by MPN assays were useful as an alternate method to viable plate counts.

Table 1
Simultaneous detection of *Moraxella* G21r and *Ralstonia* H850Lr in mixed liquid cultures[a] after 2 days incubation at 30°C

| Inoculum ratios[b] (G21r:H850Lr) | Population estimates[c]/ml (Four-well MPN[d]) | |
|---|---|---|
| | G21r | H850Lr |
| 1:1 | $(4.0 \pm 1.6) \times 10^8$ | $(1.3 \pm 0.5) \times 10^9$ |
| $10^{-1}$:1 | $(1.5 \pm 0.4) \times 10^7$ | $(1.3 \pm 0.5) \times 10^9$ |
| $10^{-2}$:1 | $(7.2 \pm 2.4) \times 10^5$ | $(5.0 \pm 1.6) \times 10^8$ |
| $10^{-3}$:1 | $(3.5 \pm 2.0) \times 10^4$ | $(1.3 \pm 0.5) \times 10^9$ |
| $10^{-4}$:1 | $(2.6 \pm 0.8) \times 10^3$ | $(1.3 \pm 0.5) \times 10^9$ |
| $10^{-5}$:1 | $(7.2 \pm 2.4) \times 10^1$ | $(5.0 \pm 1.6) \times 10^8$ |
| $10^{-6}$:1 | $(1.7 \pm 0.0) \times 10^1$ | $(1.3 \pm 0.5) \times 10^9$ |
| 1:$10^{-1}$ | $(4.0 \pm 1.6) \times 10^8$ | $(1.9 \pm 1.0) \times 10^8$ |
| 1:$10^{-2}$ | $(5.0 \pm 1.6) \times 10^8$ | $(6.3 \pm 3.5) \times 10^6$ |
| 1:$10^{-3}$ | $(5.0 \pm 1.6) \times 10^8$ | $(2.2 \pm 0.8) \times 10^5$ |
| 1:$10^{-4}$ | $(5.0 \pm 1.6) \times 10^8$ | $(1.0 \pm 0.7) \times 10^4$ |
| 1:$10^{-5}$ | $(4.0 \pm 1.6) \times 10^8$ | $(1.2 \pm 0.4) \times 10^2$ |
| 1:$10^{-6}$ | $(4.0 \pm 1.6) \times 10^8$ | $(1.7 \pm 0.0) \times 10^1$ |

[a] Media were amended with rifampicin and kanamycin at 50 μg/ml each.
[b] G21r initially used was $4.5 \pm 1.3 \times 10^8$ ($n = 3$). H850Lr was $5.7 \pm 0.2 \times 10^8$ ($n = 3$). CFU/ml and the mixture of two strains was $5.3 \pm 1.0 \times 10^8$ ($n = 3$) CFU/ml. Various mixing ratios of two strains were prepared after serial decimal dilutions of one against the other.
[c] Data are means of three replicates ± S.D.
[d] Four-well MPNs were performed with microtiter plates.

To further examine the feasibility of simultaneous detection, we estimated the numbers of both strains mixed in various ratios. To detect G21r and H850Lr simultaneously in liquid cultures at cell ratios, the same MPN plates were used for fluorescent and luminescent detections. As shown in Table 1, the initial inoculum density of G21r decreased. G21r cell numbers also decreased while there was no significant difference in H850Lr cell numbers after 2 days. H850Lr cell numbers also exhibited a similar pattern in that cell numbers decreased with the smaller inoculum size without significantly affecting G21r numbers. These results indicated that G21r and H850Lr in mixed liquid cultures can be simultaneously and unambiguously detected without interference from each other. In 10-fold dilutions, two strains showed equal population estimates of 17 cells (Table 1). In another study, *Pseudomonas aeruginosa* UG2Lr cells marked with *luxAB* genes were detected at numbers as low as five cells per g of soil using a MPN assay (Flemming et al., 1994) using

the eight-well MPN. However, in the present study, the four-well MPN was used.

### 3.2. Comparison of spread plating and MPN assays in soil microcosms

To further examine the relationship between spread plating and MPN assays in estimating cell numbers per g dry soil, two to seven 10-fold dilutions of G21r and H850Lr cultures were individually inoculated into soil. One g of soil containing each strain was diluted with 10 ml of saline, and 200 μl of soil dilutant was transferred into microtiter wells containing TSB supplemented with kanamycin, rifampicin, and cycloheximide at 50 μg/ml each. After 2-day incubation at 30°C in the dark, cells were enumerated by fluorescent and luminescent detection and the four-well MPN table (Vincent, 1970). For both G21r and H850Lr, similar estimates were obtained from plate counting and MPN assays. For example, using the $10^{-5}$ dilution, the two strains were estimated to be $1.7 \times 10^3$ cells per g dry soil by the MPN assay while the plate count estimates were higher but in the same range at $6.3 \times 10^3$ and $4.9 \times 10^3$ cells per g dry soil for G21r and H850Lr, respectively (Table 2). These results suggest that MPN assays with microtiter plates instead of plate counting could be used with soil samples as well as in liquid cultures. However, the MPN values were lower.

### 3.3. Simultaneous detection of G21r and H850Lr in sterile and non-sterile soil microcosms

Equal numbers of G21r and H850Lr cells were inoculated into sterile and non-sterile soil and their populations estimated over a 30-day period. Periodically, 1 g soil samples were removed for microMPN assays for total cell counting and fluorescent and luminescent detection. As shown in Fig. 1A, G21r and H850Lr in sterile soil exhibited higher numbers of CFU/g dry soil than those in non-sterile soil. This may be due to other microorganisms in the non-sterile soil competing with G21r and H850Lr, whereas no competing indigenous microorganisms were present in the autoclaved soil, except G21r and H850Lr. This also indicates that although other cells are present in soil microcosms, G21r and H850Lr could be detected by MPN assays. This suggests that GEMs containing Tn5-delivered *gfp* or *luxAB* markers can be used effectively to detect survival in soil microcosms. Since we performed the MPN assay after incubating cells for 2 days in microtiter plate prior to fluorescence and luminescence measurements, we in fact enumerated the culturable G21r and H850Lr cells in soil sample over the 30-day period. This explains the almost identical population estimates of G21r and H850Lr shown in Fig. 1B and 1C, respectively.

To determine whether other microbial cells possessed fluorescence or luminescence, microMPN

Table 2
Comparison of plate counts and population estimates for *Moraxella* G21r and *Ralstonia* H850Lr in soil microcosms[a]

| Dilution ($\log_{10}$)[c] | Cell estimates[b] (cells/g dry soil) | | | |
|---|---|---|---|---|
| | G21r[d] | | H850Lr | |
| | Plate counts | MicroMPN[d] | Plate counts | MicroMPN[d] |
| 0 | $(6.3\pm1.6)\times10^{8x}$ | $(3.6\pm2.1)\times10^{8x}$ | $(4.9\pm0.6)\times10^{8x}$ | $(7.3\pm2.4)\times10^{8x}$ |
| −2 | $(6.3\pm1.6)\times10^{6x}$ | $(6.3\pm3.5)\times10^{6x}$ | $(4.9\pm0.6)\times10^{6x}$ | $(3.0\pm1.3)\times10^{6x}$ |
| −4 | $(6.3\pm1.6)\times10^{4x}$ | $(1.3\pm0.6)\times10^{5x}$ | $(4.9\pm0.6)\times10^{4x}$ | $(1.9\pm1.0)\times10^{4x}$ |
| −5 | $(6.3\pm1.6)\times10^{3x}$ | $(2.2\pm0.8)\times10^{3x}$ | $(4.9\pm0.6)\times10^{3y}$ | $(2.2\pm0.8)\times10^{3x}$ |
| −6 | $(6.3\pm1.6)\times10^{2x}$ | $(7.7\pm4.0)\times10^{2x}$ | $(4.9\pm0.6)\times10^{2y}$ | $(1.7\pm0.0)\times10^{2x}$ |
| −7 | $(6.3\pm1.6)\times10^{1x}$ | $(2.6\pm0.8)\times10^{1x}$ | $(4.9\pm0.6)\times10^{1y}$ | $(2.2\pm0.8)\times10^{1x}$ |

[a] Initial cell numbers of G21r and H850Lr were $(6.3\pm1.6)\times10^8$ and $(4.9\pm0.6)\times10^8$ CFU per g of soil, respectively. Media were amended with rifampicin, kanamycin, and cycloheximide at 50 μg/ml each.

[b] Data are means of three replicates±S.D. Superscript *x* and *y*: values followed by the same letter on a given dilution number are not significantly different ($P = 0.01$) between plate counts and MicroMPN on each strain as determined by two-tailed Student's *t*-test.

[c] Serial decimal dilutions were performed to seven times with sterile saline (0.85% NaCl) before G21r or H850Lr was inoculated in soil.

[d] Four-well MPNs were performed with microtiter plates (microMPN).

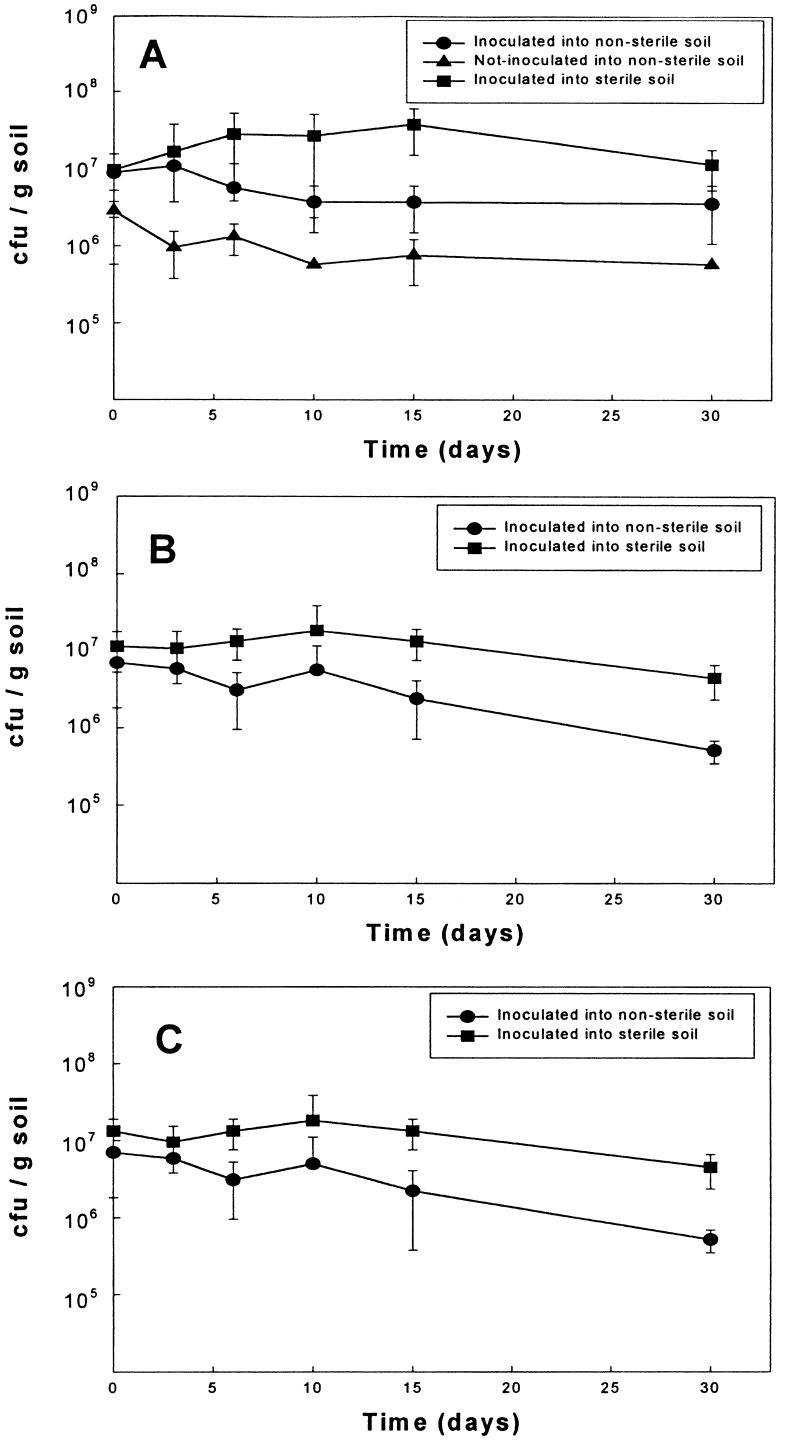


Fig. 1. Comparisons of population estimates by (A) total cell counting, (B) fluorescent detection of *Moraxella* G21r and (C) luminescent detection of *Ralstonia* H850Lr (C) for 30 days in sterile and non-sterile soil. Data are means of three replicates±S.D.

assays were carried out with autoclaved and non-sterile soils in which neither G21r nor H850Lr were inoculated. Although other cells were present in non-sterile soil (Fig. 1A), no fluorescence or luminescence was ever detected (data not shown). These results indicate that the detection methods were able to estimate the survival of these strains simultaneously for a month using MPN assays. Although the conventional plate counting was more precise than MPN assays with microtiter plates, it requires more time. Here, we demonstrated that MPN assays are simple and rapid to simultaneously detect a *gfp*-marked strain by fluorescence and a *lux*-marked strain by luminescence in a mixed soil sample. Compared to the conventional plate counting, this MPN assay with microtiter plates permitted a faster dual detection of soil samples for enumeration of different bacterial species.

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