APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1995, p. 2950–2957 Vol. 61, No. 8
0099-2240/95/$04.00+0

Characterization of Rhizosphere Colonization by Luminescent *Enterobacter cloacae* at the Population and Single-Cell Levels

E. A. S. RATTRAY,[1]* J. I. PROSSER,[2] L. A. GLOVER,[2] AND K. KILLHAM[1]

Department of Plant and Soil Science, Meston Building, University of Aberdeen, Aberdeen AB9 2UE,[1] and Department of Molecular and Cell Biology, Marischal College, University of Aberdeen, Aberdeen AB9 1AS,[2] United Kingdom

Received 25 April 1994/Accepted 3 June 1995

A bioluminescence marker system was used to characterize colonization of the rhizosphere by a bacterial inoculum, both in terms of population activity and at the single-cell level. Plasmid pQF70/44, which contains *luxAB* genes under the control of a strong constitutive phage promoter, was introduced into the rhizobacterium and model biocontrol agent *Enterobacter cloacae*. Light output from the *lux*-modified strain was detected by luminometry of samples from growing cultures of *E. cloacae* and from inoculated soil and wheat root samples. The minimum detection limits for fully active cells under optimum conditions were 90 and 445 cells g^{-1} for liquid culture and soil, respectively. The metabolic activities of the *lux*-marked population of *E. cloacae*, characterized by luminometry, contrasted in rhizosphere and nonrhizosphere soil. Cells in the rhizosphere were active, and there was a linear relationship between light output and cell concentration. The activity of cells in nonrhizosphere soil could not be detected unless the soil was supplied with substrate. Novel use of a charge-coupled device is reported for the spatial characterization of rhizosphere colonization by *E. cloacae* (pQF70/44) at the single-cell and population levels. Used macroscopically, the charge-coupled device identified differences in colonization due to competition from indigenous soil organisms. The *lux*-marked bacterium was able to colonize all depths of roots in the absence of competition but was restricted to the spermosphere in the presence of competition (nonsterile soil). The combined use of luminometry, dilution plate counts, and charge-coupled device imaging (macroscopic and microscopic) demonstrates the potential of luminescence-based detection as a powerful system for assessment of rhizosphere colonization by *lux*-marked microbial inocula.

The increasing interest in the use of microbial inocula has led to the development of molecular marker systems to enable the assessment of microbial survival and dispersal, which is necessary to quantify the benefits and risks associated with their environmental release. Such marker systems include *lacZY* (12), *xylE* (43), antibiotic or metal resistance genes (3), and luminescence-based techniques (4, 14, 32). All of these marker systems involve the introduction of the appropriate genes, either plasmid or chromosomally borne, to enable the detection of extracted, viable cells containing the marker gene. Luminescence-based detection provides additional advantages, through the nonextractive detection of light by luminometry from samples containing *lux*-modified cells (28) and single-cell detection through charge-coupled device (CCD)-enhanced microscopy (33). The production of light depends on the metabolic activity of the *lux*-modified cells. Therefore, luminometry enables the activity of a marked population to be assessed in terms of both actual and potential activity (23, 28, 27). When cells are growing exponentially, the light output has been shown to be directly proportional to the microbial biomass (28). However, stationary-phase or starved cells require a period of activation for light output to be proportional to biomass (potential luminometry) (23). Luminometry will also, therefore, provide information on the potential for activation of cells after prolonged incubation in the soil.

* Corresponding author. Mailing address: Department of Plant and Soil Science, Meston Building, University of Aberdeen, Aberdeen AB9 2UE, United Kingdom. Phone: 224-273725. Fax: 224-272268.

The light output from certain *lux*-modified cells is sufficient to enable single-cell detection. The method developed by Silcock et al. (33) required immobilization of cells on agar and incubation for a short period of time to allow for activation of dormant cells. Combined luminometric and CCD technology, therefore, enables detection of active cells, with the latter also providing information on the location of marked inocula in the environment.

The potential uses of microbial inocula in the environment include legume inoculation with rhizobia (25), biological control (40), plant growth regulation (19), and detoxification (5). In many instances, such inocula must colonize the rhizosphere of the host plant to fulfill their role. The rhizosphere is seen, therefore, as a target niche for the introduction of microbial inocula, and their use, coupled with the need for greater understanding of the ecology of inocula in the rhizosphere, requires adequate detection methods to enable nonextractive, in situ monitoring of rhizosphere colonization. The *lux* genes have been cloned into a variety of bacteria (9, 15, 28, 32), including the potential biocontrol agents *Pseudomonas fluorescens* (1) and *Enterobacter cloacae* (13). Since the rhizosphere will often be the target environment for the possible use of these potential biocontrol agents, it is important to understand their rhizosphere colonization. The rhizosphere ecology of fluorescent pseudomonads has been studied extensively (e.g., see references 2, 6, 10, 36, 37, and 39), although that of *E. cloacae* has received very little attention. *E. cloacae* has been shown to antagonize *Pythium* spp. (18), *Fusarium* spp. (34), and *Rhizopus* spp. (42).

The rhizosphere is a zone of enhanced biological activity in

the soil, with microbial population densities that are greater than those in the bulk soil. Although the rhizosphere is generally a nutrient-limited environment, colonization is generally associated with greater substrate activation than that which occurs in bulk soil (41). Because light output from *lux*-modified inocula is dependent on metabolic activity, the rhizosphere represents a zone in which luminescence-based detection may have considerable potential. While other workers (e.g., see references 4, 11, and 31) have used some aspects of bioluminescence to study rhizosphere ecology, other aspects need to be considered to exploit the full potential of this powerful technique. The aim of these studies was to investigate the use of luminescence-based detection in the rhizosphere at the population and the single cell levels by (i) comparing the activities of *lux*-modified *E. cloacae* cells in the rhizosphere of wheat and in the bulk soil, by using luminometry; (ii) developing the use of a CCD for in situ detection of rhizosphere colonization of wheat by *E. cloacae* and comparing CCD imaging with data obtained by dilution plate counts; and (iii) developing a microculture technique for the detection of single *lux*-modified cells in the wheat rhizosphere.

MATERIALS AND METHODS

Bacterial strains and culturing conditions. Plasmid pQF70/44 contains *luxAB* genes from *Vibrio harveyi* under the control of the phage-specific promoter from *Pseudomonas syringae* pv. phaseolicola and a gene for carbenicillin resistance (38). *E. cloacae* was grown in L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose) at 25°C. Luria-Bertani (LB) agar was prepared by the addition of 1.5% (wt/vol) bacteriological agar to L broth. Carbenicillin was used at a concentration of 300 μg ml^{-1} (Sigma Chemical Co.).

DNA manipulations. Competent cells of *E. cloacae* were prepared in the manner described by Mandel and Higa (21) and transformed with plasmid DNA (pQF70/44) according to the method described by Cohen et al. (7). Luminescent recombinants were detected on agar plates containing carbenicillin (incubated for 24 h at 25°C) by using a CCD after the addition of 2 μl of the luciferase substrate *n*-decyl aldehyde to the lid of the petri dish.

Visualization of luminescent colonies with the CCD. Luminescent, viable colonies on agar plates were visualized by using a nitrogen-cooled, slowly scanning CCD camera (385 by 578 pixels, each 22 μm square) (model 1 nitrogen-cooled camera; Wright Instruments Ltd., Enfield, United Kingdom) and an associated computer-imaging system (Dell system 310). For bright-field visualization, the exposure time was 0.01 s. The dark-field exposure time was 10 to 120 s, after the application of 2 μl of *n*-decyl aldehyde to the lid of the petri dish. The CCD was enclosed in a light-tight box with a removable door to reduce entry of extraneous light during dark-field exposures.

Measurement of growth and luminescence during batch growth. 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 recombinant strain. The flasks were incubated at 25°C on a rotary shaker (180 rpm). Samples were removed at regular intervals, and the biomass concentrations were determined by relating the A_{550} to a standard curve obtained from a culture of the appropriate strain with a known biomass concentration. Light output was measured, after the addition of 1 μl of *n*-decyl aldehyde, in triplicate 1-ml samples by using an LKB model 1251 luminometer, with output integrated over a 10-s period with continuous mixing, and was expressed in relative light units (RLU). For strains encoding *luxAB*, 1 μl of *n*-decyl aldehyde was mixed with 1 ml of culture, and the luminometer readings were taken 2 min after preparation.

Preparation of microbial inocula. Microbial inocula were prepared from cultures grown to late-log phase in L broth containing the appropriate antibiotics. The cells were centrifuged (1,400 × *g*; 30 min; 16°C) and washed twice in an equal volume of sterile Ringer's solution (1/4 strength) (Oxoid). After centrifugation, the bacterial pellet was resuspended in an equal volume of Ringer's solution, and a dilution series was prepared in Ringer's solution for determination of viable cell concentrations by plating on LB agar containing the appropriate antibiotics and incubation at 25°C for 24 h.

Minimum detection levels in liquid culture and soil. Inocula were prepared as described above, and a dilution series was constructed in L broth. Triplicate 1-ml samples were added directly to luminometer cuvettes, and triplicate samples from each dilution were added to 0.5 g of Craibstone soil (Countesswell series; pH [distilled water] 6.46; organic carbon, 2.47%; organic nitrogen, 0.27%; cation exchange capacity, 7.40 cmol kg^{-1}) in a luminometer cuvette, to obtain a range of cell concentrations in a soil slurry (15, 29). The soil slurry was vigorously mixed, and luminescence was measured following addition of *n*-decyl aldehyde. The soil slurry was continuously mixed during measurement of light output in the luminometer. Viable cells in the soil slurry were determined by dilution plates using LB agar containing carbenicillin to relate light output to viable cell concentration.

Light output from soil samples inoculated with luminescent *E. cloacae*. Soil samples (10 g; −33 kPa; 100 kg m^{-3}) were inoculated with a range of cell concentrations (10^4 to 10^8 cells g^{-1} of soil) of *E. cloacae*(pQF70/44) and incubated at 15°C. The bacterial inoculum was prepared as described previously and inoculated into soil by adding 0.5 ml of cell solution (in 10-μl aliquots) and equilibrating the soil to the appropriate matric potential. After 7 days, light output and viable cell counts were determined. Light output was measured in 1-ml samples from a 1/10 dilution (prepared in 1/4-strength Ringer's solution) of inoculated soil, after the addition of 1 μl of *n*-decyl aldehyde, as described above. Actual luminescence was obtained by performing the luminescence assay immediately after sampling. Potential luminescence was determined by measuring light output after incubating the soil suspension (1/10 dilution) at 25°C for 2 h in a 1% solution of double-strength 523 medium (1% sucrose, 0.8% casein hydrolysate, 0.4% yeast extract, 0.39% $K_2HPO_4 \cdot 3H_2O$, 0.03% $MgSO_4 \cdot 7H_2O$) (24). Viable cell counts on LB agar containing carbenicillin and cycloheximide (50 μg ml^{-1}) were determined.

Light output from seedling samples inoculated with luminescent *E. cloacae* by using luminometry. Microbial inocula were prepared as described above and inoculated onto sterile seeds. Sterile wheat seeds were prepared by soaking in 2% (vol/vol) hypochlorite for 15 min, washing in sterile water for 30 min, and leaving for 16 h in a petri dish containing water-moistened sterile filter paper. The sterile seeds were inoculated with luminescent bacteria by coating with a mixture of cells and carboxymethyl cellulose (2:1), to give concentrations of 10^8 and 10^7 cells $seed^{-1}$. The seeds were sown in replicate pots containing nonsterile Craibstone soil (−33 kPa; 100 kg m^{-3}). The pots were incubated for 7 days at 15°C under a light bank (86 μmol m^{-2} s^{-1}). After 7 days, the seedlings were removed from the pots, and the roots were transferred to 10 ml of Ringer's solution (1/4 strength). Actual and potential luminescence and corresponding viable cell counts were determined from the root samples as described previously.

Preparation and inoculation of the wheat-filter paper microcosms. Microbial inocula were prepared as described for inoculation onto sterilized wheat seeds. The sterile seeds were inoculated with luminescent bacteria by coating with a mixture of cells (from a dilution series) and carboxymethyl cellulose (2:1), to give a concentration of 10^8 cells $seed^{-1}$. Inoculated seeds were placed on filter paper (Whatman no. 42) contained within a petri dish. The filter paper had previously been adjusted to a matric potential of −33 kPa according to moisture release characteristics for filter paper (Whatman no. 42) (16). The petri dishes were incubated at 15°C under a light bank (86 μmol m^{-2} s^{-1}) for 7 days.

Preparation and inoculation of wheat-soil sheet microcosms. Microbial inocula of *E. cloacae* (pQF70/44) were prepared and used as seed coating as described. The coated seeds were placed in sheet microcosms containing repacked Craibstone soil at a bulk density of 100 kg m^{-3} and a matric potential of −33 or −1,500 kPa (adjusted according to the moisture release characteristic of Craibstone soil). The sheet microcosms consisted of two sheets of Perspex (25 by 25 by 0.3 cm) held apart by 1-cm strips of Perspex and containing Craibstone soil and a single coated wheat seed. Soil (sieved to less than 3 mm and packed to 100 kg m^{-3}) was adjusted to a matric potential of −33 or −1,500 kPa by adding water according to the moisture release characteristic. The inoculated seed was placed 1 cm from the top of the microcosm and grown in a plant growth cabinet under a light bank (86 μmol m^{-2} s^{-1}) with a 16-h photoperiod. The microcosms were maintained at a constant matric potential (−33 or −1,500 kPa) by daily gravimetric adjustment of the moisture content. The required volume of water was added to the surface of the soil, after the removal of the top sheet of Perspex. The thin layer of soil (1 cm) allowed rapid equilibration of the matric potential. The loss of water through evaporation and evapotranspiration was negligible in terms of the overall moisture content of the soil. Replicate microcosms for each matric potential were harvested, and root colonization was determined by macroscopic CCD imaging and dilution plate counting (26).

Macroscopic detection of rhizosphere colonization by luminescent *E. cloacae* by using the CCD. Wheat seedlings from filter paper and soil sheet microcosms were sampled at 7 days. The seedlings grown on filter paper were imaged by using the CCD directly in the petri dish, after the application of 2 μl of *n*-decyl aldehyde to the lid. Seedlings grown in soil sheet microcosms were placed either on moist filter paper or in an agar overlay, after loosely adhering soil was removed by washing briefly in Ringer's solution. Agar overlays were obtained by placing the plants on solidified LB agar (in petri dishes) and covering with molten agar (42°C). Seedlings in agar overlays were incubated at 25°C for 2 h prior to imaging. Samples were imaged for 0.01 s in the light to obtain a bright-field image. Dark-field images were obtained (ranging from 1 s to 30 min) after storage for 15 min in the dark within the light-tight box (with 2 μl of *n*-decyl aldehyde added to the lid of the petri dish) to eliminate any autofluorescence from the shoot and leaves.

Viable cell enumeration of rhizosphere samples. After 7 and 14 days at 15°C, the wheat seedlings were carefully removed from the soil, and the roots were harvested for viable cell enumeration. The roots were shaken to remove loosely associated soil, sectioned at intervals of 5 cm from the seed, placed in 1/4-strength Ringer's solution, and hand shaken for 1 min. The soil suspension represented the ectorhizosphere fraction. The roots were then rinsed in sterile

1/4-strength Ringer's solution to remove any further soil. The endorhizosphere fraction (which includes the rhizoplane) was obtained by sonicating the roots in an ultrasonication bath (Decon FS minor sonic bath; Decon Ultrasonics Ltd., East Sussex, United Kingdom) for 30 min. The shoot was sampled by cutting into 2-cm pieces and by suspension in 1/4-strength Ringer's solution and sonication as described above. Dilution plate counts on LB agar (containing carbenicillin and cycloheximide) were performed in triplicate on each fraction for viable cell enumeration, and luminescent colonies were counted by using the CCD. Standard errors of the means were calculated, and analysis of variance was performed to determine differences between matric stress values for each fraction.

Single-cell detection in the rhizosphere by using the CCD. Root samples were obtained from seedlings grown in the sheet microcosms containing Craibstone soil that had been inoculated with *lux*-modified *E. cloacae*. A section of root (1 cm) was placed in an Eppendorf tube containing 200 μl of Ringer's solution (1/4 strength), and the tube was shaken to remove loose soil. The root was removed and placed on a microculture slide (30), and the slide was incubated for 2 h at 30°C. Microculture slides were observed by using a transmitted-light microscope equipped with Apochromat ×12.5 and ×25 objectives (Jenamed Variant; Carl Reiss, Jena, Germany) and attached to the CCD via a C mount. An aliquot (1 μl) of *n*-decyl aldehyde was injected into the agar adjacent to the cells to be imaged prior to dark-field exposures. Bright-field exposures had an exposure time of 0.01 s, while dark-field images were obtained over a 30-min period.

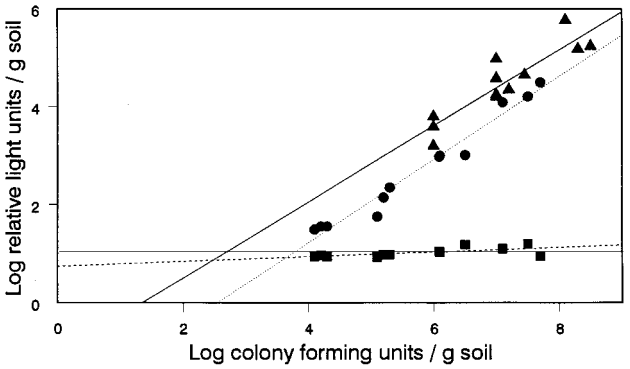


FIG. 1. Light output in relation to CFU of *E. cloacae*(pQF70/44) in nonsterile soil in the presence (●) ($r^2 = 0.97$) and absence (■) ($r^2 = 0.34$) of added substrates and in rhizosphere samples in the absence of added substrates (▲) ($r^2 = 0.88$). ——, limit of detection of the luminometer.

RESULTS

Characterization of *lux*-modified bacteria. *E. cloacae* exhibited the usual growth curve characteristic of batch growth and the luminescence profile during growth similar to those of other *lux*-marked bacteria (15, 28) (figure not shown). The maximum specific growth rate of the plasmid-bearing strain (0.89 ± 0.07) was not significantly ($P \leq 0.05$ [calculated by Student's *t* test]) different from that of the parent strain (0.81 ± 0.08). Light output for the *lux*-modified strain closely followed increases in biomass concentration.

The lower detection limits were determined for exponentially growing cells suspended in liquid culture and inoculated into soil. Background luminescence from the luminometer was approximately 9 RLU, with standard error typically 0.01 for triplicate readings of a single sample. This background level was subtracted from all samples prior to plotting. There was a linear relationship between viable cell concentrations and light output for both liquid culture ($r^2 = 0.997$) and nonsterile soil ($r^2 = 0.982$) as reported for *Escherichia coli* (28) and *Erwinia carotovora* (15). Lower detection limits were calculated by extrapolation of lines calculated by linear regression log-log plots of light output against cell concentration to a value of 1 RLU above the background level. In liquid culture, the lower detection limit for *E. cloacae* (pQF70/44) was 90 ± 10 cells ml^{-1}, while the lower detection limit in nonsterile soil was 445 ± 50 cells ml^{-1}.

Actual and potential luminescence following prolonged incubation in soil and the rhizosphere. To determine whether *E. cloacae* remained active in bulk soil and the rhizosphere after a 7-day incubation, luminescence assays were performed to assess both actual and potential activities. Figure 1 represents scatter plots for both actual and potential luminescence of *E. cloacae* 7 days after inoculation of nonsterile soil and from rhizosphere samples with a range of cell concentrations. Background RLU were not subtracted from these data, but the background levels are indicated in the figure. The data demonstrate that actual activity from starved cells in soil for 7 days was barely above background luminescence, whereas potential luminescence (i.e., incubation with substrate) in nonsterile soil, which involves activation of the cells, resulted in a linear relationship between cell numbers and relative light output. There was no change in the viable cell concentration after incubation with substrate for 2 h. The light output per cell (after activation) for cells inoculated into nonsterile soil was $(5.60 \pm 0.98) \times 10^{-4}$ RLU $cell^{-1}$. There was an approximately linear relationship between actual activity (measured by light output) and cell numbers for 7-day-old rhizosphere samples containing *lux*-marked cells, even in the absence of added substrate (Fig. 1). The actual luminescence from cells in the rhizosphere of 7-day-old seedlings was much greater (>1 order of magnitude) than the levels observed for potential luminescence of starved cells in soil, with light output per cell being $(2.74 \pm 0.82) \times 10^{-3}$ RLU $cell^{-1}$, resulting in a minimum detection level of 574 ± 116 cells g^{-1} (Fig. 1). When substrates were added to rhizosphere samples, growth of the inoculated cells occurred within the 2-h incubation.

Determination of rhizosphere colonization by *E. cloacae* by using CCD-enhanced imaging and viable cell enumeration. CCD-enhanced images of root samples from wheat-filter paper microcosms inoculated with *lux*-modified *E. cloacae* were obtained after 7 days (Fig. 2a and b). Dark-field images of roots containing *E. cloacae* on filter paper showed high levels of luminescence (Fig. 2b). Under sterile conditions on filter paper, *E. cloacae* appeared to colonize the entire root system (Fig. 2a and b). In nonsterile soil at −33 kPa (Fig. 3a), luminescence was observed only in the spermosphere and the upper roots (Fig. 3b). Lack of complete colonization when the indigenous population is present suggests that *E. cloacae* may be susceptible to competition. The pattern of colonization, in nonsterile soil, was confirmed by dilution plate counting data (Fig. 4a and b), although the data suggest greater root colonization than visualization through CCD imaging. The dilution plate counting data showed that *E. cloacae* predominantly colonized the spermosphere and the 0- to 5-cm fraction of the roots (Fig. 4a). Cells were detected down to the 10- to 15-cm and 5- to 10-cm fraction only at −33 and −1,500 kPa, respectively, with the highest numbers being detected in the endorhizosphere (Fig. 4a). Cells either were not present in the lower root fractions or were below the level of detection. Variability of CFU increased with increasing distance from the seed (i.e., as the number of cells decreased), such that significant ($P \leq 0.05$) differences were found only when cells were absent from below 10 cm, at the high matric stress (−1,500 kPa). As matric stress was increased to −1,500 kPa, the numbers of *lux*-marked *E. cloacae* cells that could be detected at the lower depths of the roots decreased, with cells remaining predominantly around the seed (Fig. 4b and 5c). Matric stress was found to limit both vertical dispersion and the metabolic activity of an inoculum in the rhizosphere. CCD-enhanced imaging of wheat seedlings maintained at −1,500 kPa for 7 days showed no luminescence when imaged on moist filter paper (Fig. 5b), contrasting with the result obtained at −33

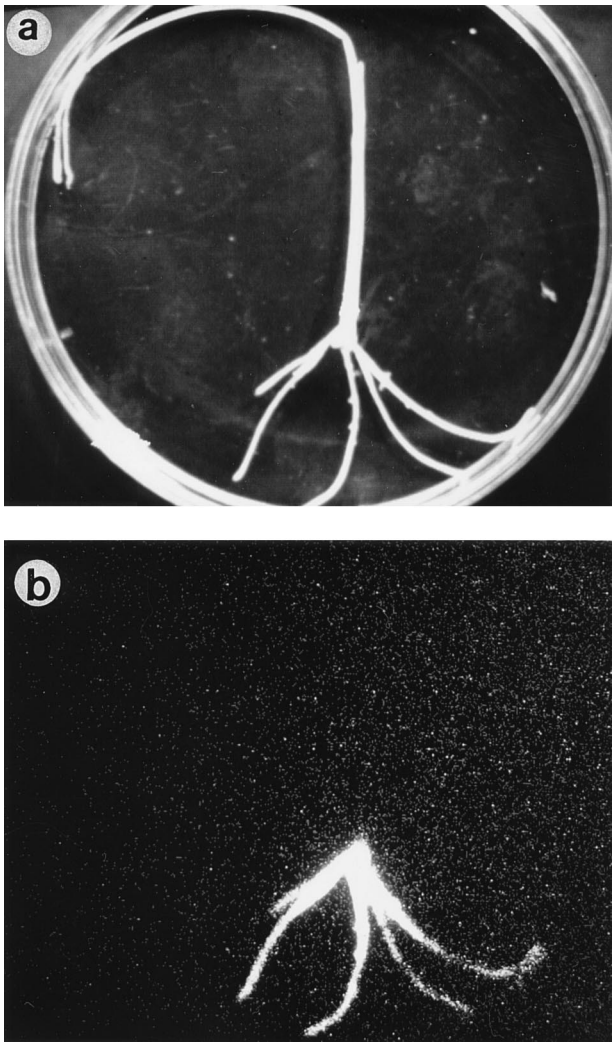


FIG. 2. In situ visualization of *E. cloacae*(pQF70/44) colonizing wheat roots under sterile conditions grown on filter paper in petri dishes at 7 days by using a CCD. Bright-field exposure (0.01 s) (a) and dark-field exposure (10 s) (b) after the application of 2 μl of *n*-decyl aldehyde to the lid of the petri dish are shown.

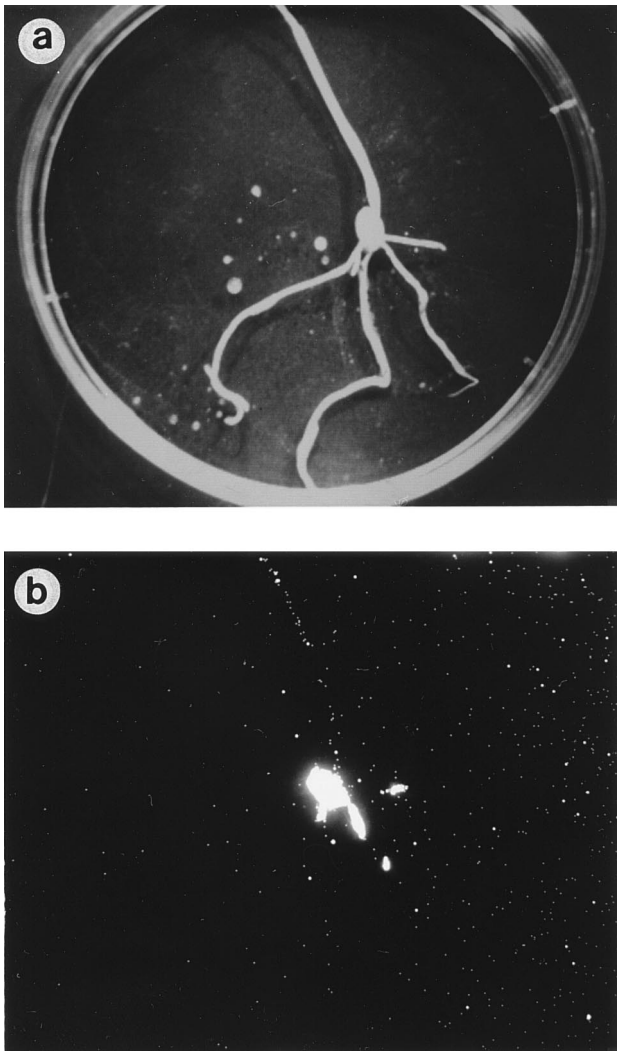


FIG. 3. In situ visualization of *E. cloacae*(pQF70/44) colonizing wheat roots grown in nonsterile soil at −33 kPa in sheet microcosms at 7 days. Bright-field exposure (0.01 s) (a) and dark-field exposure (15 min) (b), after the application of 2 μl of *n*-decyl aldehyde to the lid of the petri dish, are shown.

kPa (Fig. 3b). However, when the seedling was embedded in agar, luminescence was observed around the seed after a 2-h incubation at 25°C (Fig. 5c).

Single-cell detection in the rhizosphere by using the CCD. Single cells of *lux*-modified *E. cloacae* were imaged in the rhizosphere of wheat roots by using a CCD camera attached to a microscope (Fig. 6). Larger areas of intense luminescence were visualized on the root surface, indicating the presence of microcolonies. Dark-field exposures of 30 min were sufficient to detect single cells in rhizosphere samples. Comparison of the dark- and bright-field exposures (Fig. 6a and b) allowed identification of individual *E. cloacae* cells within the wheat rhizosphere (Fig. 6a).

DISCUSSION

The introduction of plasmid pQF70/44 into *E. cloacae* resulted in high levels of light production, enabling its rhizosphere colonization to be characterized through luminescence technology. Such high levels of light resulted in minimum detection levels of 90 cells ml^{-1} in liquid culture and 574 cells g^{-1} in rhizosphere soil. The development of highly luminescent strains has led to single-cell detection in soil (33) and the phylloplane (38) by using CCD-enhanced microscopy. High levels of light output were probably due to the multicopy nature of the plasmid, with approximately 13 copies being reported for *E. coli* and *P. syringae*, combined with a strong heterologous promoter (38). The presence of the strong phage promoter has been responsible for the increase in sensitivity compared with that for the tetracycline promoter from pBR322 that has been used previously in other *lux*-modified bacteria, such as *P. fluorescens*(pFAC510) (1).

Measurement of light output through luminometry has enabled the assessment of inoculum activity in soil (27), since luminometric activity is related to metabolic activity (23, 24). In all luminescent strains, light output per unit of biomass is constant during exponential growth but falls during stationary phase because of a decline in actual metabolic activity (1, 24, 28). Figure 1 demonstrates the low level of metabolic activity (actual activity) of cells in soil measured by luminometry and illustrates the activation of marked cells with substrate (poten-

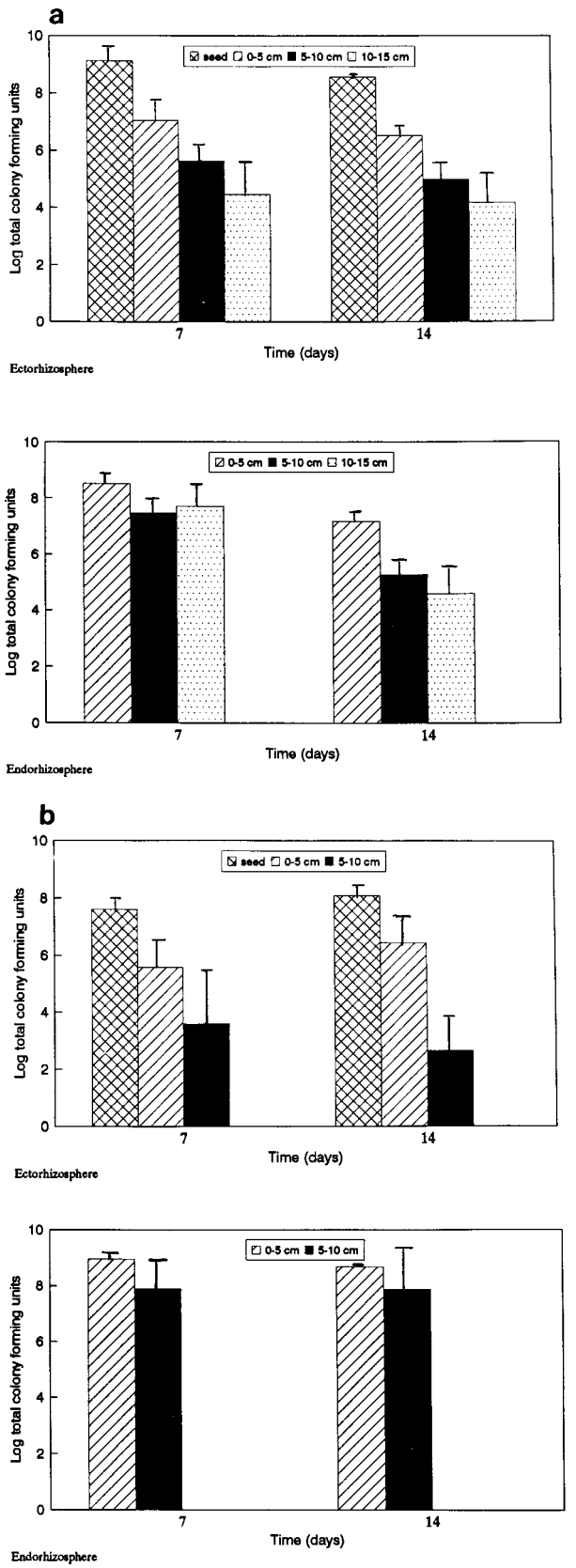
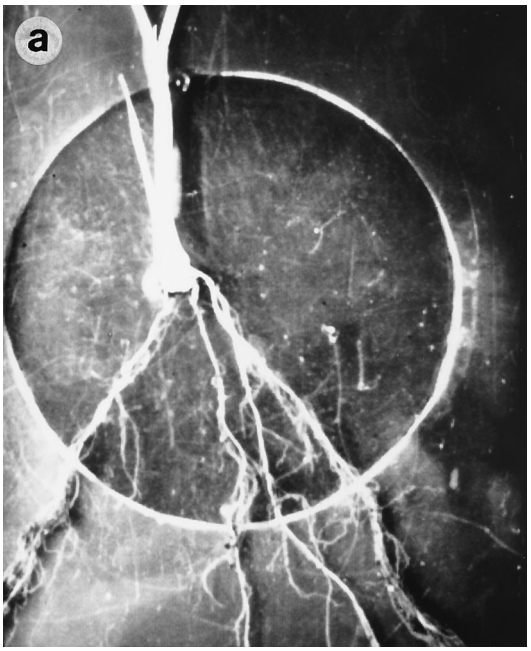


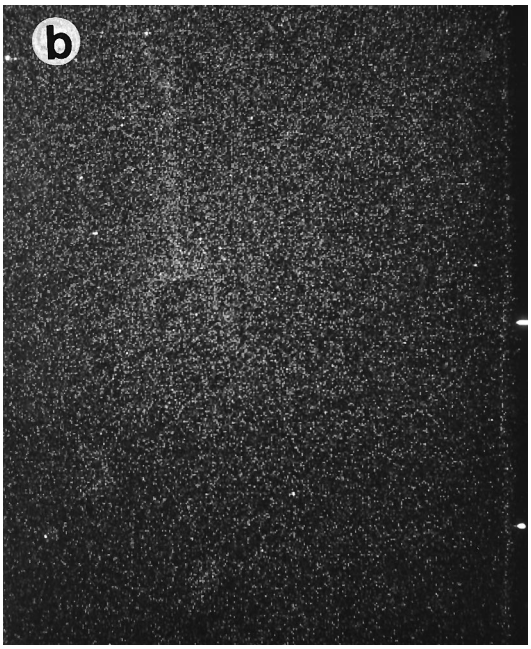
FIG. 4. Total CFU with respect to time and depth in the rhizosphere at −33 kPa (a) and −1,500 kPa (b) for *E. cloacae*(pQF70/44). Bars indicate standard errors of the means.

tial luminescence), resulting in a direct relationship between luminescence and cell concentration (Fig. 1). Potential luminescence, in terms of RLU cell^{-1}, depends on the period of prior starvation, as shown through previous work by Meikle et al. (23), although this does not affect the relationship between luminescence and cell concentration. The potential luminescence assay has previously been shown to provide data which correlate with other measures of microbial activity (e.g., dehydrogenase activity) (24). The rhizosphere provides the microbial population with substrate, such that actual luminescence is directly related to cell concentration (Fig. 1).

The presence of active cells in the rhizosphere was confirmed by using CCD-enhanced imaging. Under sterile conditions and in nonsterile soil, areas of luminescent activity were observed at a matric potential of −33 kPa (Fig. 2 and 3). The luminescence observed in Fig. 2 and 3 reflects actual activity in the rhizosphere resulting from utilization of substrate produced in situ, since no exogenous substrate for metabolism was provided. When cells were inoculated onto seeds and placed into soil at −1,500 kPa, CCD-enhanced imaging was unable to detect any luminescence (Fig. 5). This suggests a lack of activity of the *lux*-marked cells at −1,500 kPa, even in the rhizosphere. A matric potential of −1,500 kPa has been reported to be the threshold for activity for many bacterial species in soil (17). Under these circumstances (at −1,500 kPa), substrate activation was required to detect the presence of luminescent cells. In the present study, agar overlays were employed to activate the cells in the rhizosphere, enabling visualization of the cells around the seed (Fig. 5c). This type of assay is analogous to assaying potential luminescence (23), whereby a substrate is added to activate cells to obtain maximum luminescence. Luminescence detected after substrate activation indicates potential activity and may provide an estimation of the total marked population. This was demonstrated by the luminescence observed around the seed at −1,500 kPa, which corresponded to dilution plate counting data. Discrepancies were noted, however, between CCD imaging and viable cell counts. Viable cells were detected only at lower depths (e.g., 5 to 10 cm; −1,500 kPa) by dilution plate counts and not through CCD imaging, even after substrate activation. These discrepancies may have occurred through lack of sensitivity of the CCD or through contamination of roots at lower depths when they were destructively sampled. Furthermore, the competition from indigenous microorganisms colonizing the rhizosphere may prevent in situ CCD imaging because of reduced light output (33).

Colonization by *E. cloacae* appears to have been restricted by interactions with indigenous soil bacteria. In the absence of indigenous populations, *E. cloacae* was able to colonize the entire root system. The inability of *E. cloacae* to colonize in the presence of indigenous bacteria may be due to competition or differences in organic compounds present, since it has been hypothesized that microbes in the rhizosphere may modify the material released from the roots (30). These results are in agreement with those described by Fravel et al. (13), who showed that *E. cloacae* was located predominantly in the spermosphere. Our results suggest that *E. cloacae* colonizes the endorhizosphere in greater numbers than the ectorhizosphere, since at −33 kPa 10-fold more cells were detected in the former fraction. This contrasts with colonization profiles reported for another abundant rhizobacterium, *P. fluorescens* (29). This may be due to physiological differences between *P. fluorescens* and *E. cloacae*. It has been suggested that *Enterobacter* species produce considerable extracellular polysaccharides, which can cause an increase in adsorption to surfaces (e.g., the rhizoplane) (20). In addition, cells which are ad-





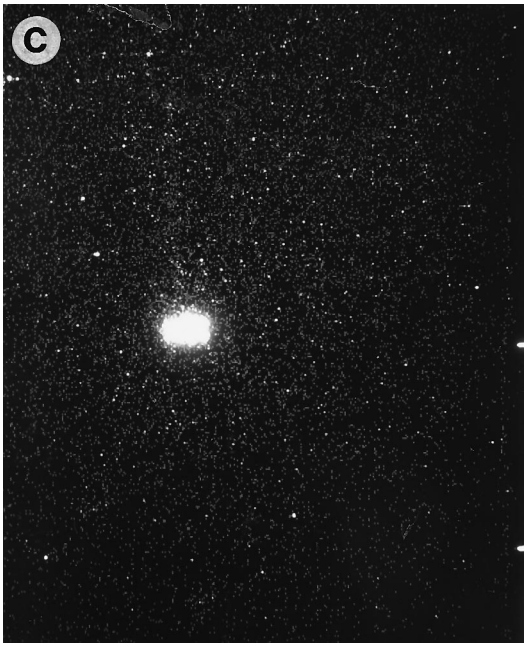


FIG. 5. In situ visualization of *E. cloacae*(pQF70/44) colonizing wheat roots grown in nonsterile soil at −1,500 kPa in sheet microcosms at 7 days. (a) Bright-field exposure (0.01 s); (b) dark-field exposure (15 min) of seedling on moist filter paper; and (c) dark-field exposure (30 min) of seedling in an agar overlay, after the application of 2 μl of *n*-decyl aldehyde to the lid of the petri dish.

sorbed to surfaces are not readily available for translocation, thus preventing effective vertical dispersion within the root system. Although the CCD enabled qualitative, spatial visualization of an inoculum in the rhizosphere, it was unable to distinguish readily between luminescence resulting from cells in the endo- and in the ectorhizospheres. However, the CCD did provide rapid information on the vertical dispersion and activity (both actual and potential) of an inoculum within the rhizosphere.

Single cells and microcolonies of *lux*-marked *E. cloacae* were imaged by using CCD-enhanced microscopy in the rhizosphere of wheat seedlings (Fig. 6). The CCD has previously been used successfully to image single bacterial cells in soil (33) and mammalian cells in culture (8). Silcock et al. (33) noted that the presence of soil quenched some of the light produced by bacterial cells, whereas the imaging of mammalian cells did not suffer from background quenching (8). Light produced by cells in the rhizosphere did appear to be quenched, not only because of the presence of soil but also because of lysates exuded from the roots. Single cells could not be identified on the rhizoplane; this may be due to cells being predominantly present as microcolonies but may also result from technical difficulties in focusing on the root surface. Viable cell counts certainly suggest that *E. cloacae* was distributed between the ectorhizosphere and the endorhizosphere (including the rhizoplane) (Fig. 4).

Bioluminescence markers have been used to detect bacteria in a variety of environments, for example, colonization of plant surfaces by inocula (e.g., see references 4, 11, 13, and 31), to detect disease progression by phytopathogenic bacteria (22, 32), and to detect contaminating bacteria in food (35). Fravel et al. (13) demonstrated the advantage of bioluminescence for estimating the extent of spatial distribution of *E. cloacae* on lettuce roots by autophotography. The method developed by these workers was relatively insensitive and time-consuming (requiring overnight incubation plus long exposure times) (13). Shaw et al. (31) increased the sensitivity by using a CCD camera to test the ability of digitized images to predict bacterial numbers on leaf surfaces following the introduction of *Xanthomonas campestris* marked with *lux* into field plots. Although Shaw et al. (31) found that their Lux assay could not give precise values of numbers of their marked bacterium, the assay proved to be as sensitive as plate counts. Beauchamp et al. (4) used a combination of luminometry, dilution plate counting, and CCD imaging to assess root colonization by fluorescent pseudomonads. To obtain quantitative detection of their *lux*-marked strain, Beauchamp et al. (4) relied on the

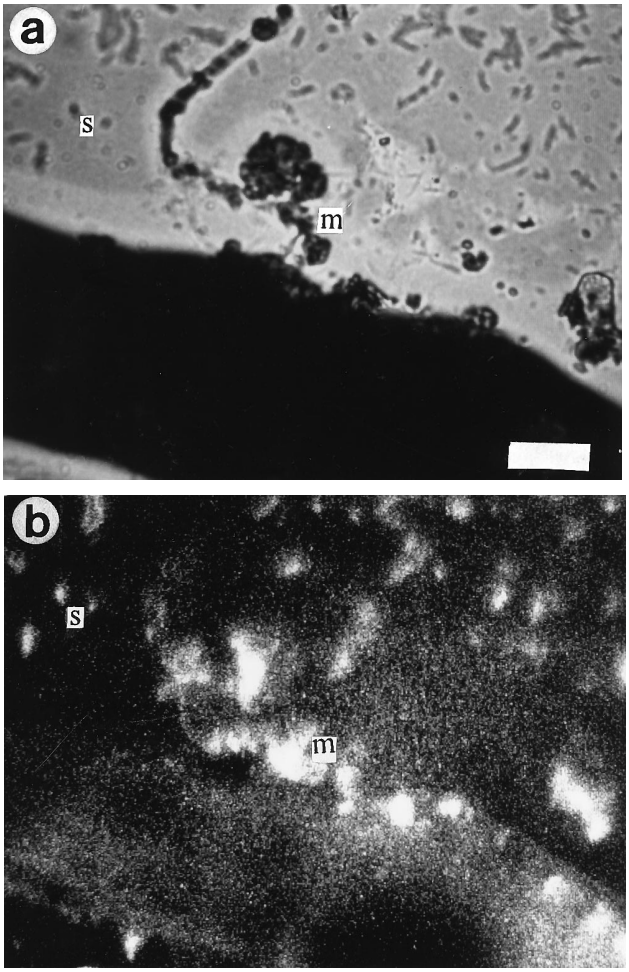


FIG. 6. CCD image-enhanced exposures of *E. cloacae*(pQF70/44) in the wheat rhizosphere imaged in microculture in a bright-field (0.02-s) exposure (a) and a dark-field (30-min) exposure (b). Single cells (s) and microcolonies (m) are denoted. Scale bar, 10 μm (data from Prosser [26; printed with permission from the Society of General Microbiology]).

growth of the marked bacteria in a broth enrichment technique. The results reported in our paper demonstrated the use of multicopy *lux*-containing plasmid, combined with a strong heterologous promoter (HcM) to acquire additional information regarding the in situ activities of marked inocula and the spatial locations of single cells in the wheat rhizosphere by using a CCD. The low endogenous background of bioluminescence in the soil (33) makes *lux* an ideal marker for environmental studies. Quantification of bioluminescence on the basis of light output per cell may enable assessment of the efficacy and activity of biocontrol agents and elucidation in the mechanisms involved in biocontrol of root pathogens.

In conclusion, a marker system should, ideally, be sensitive, specific, and rapid and should allow for screening of large numbers of samples. The *lux* marker system has many of these features. In common with other molecular marker systems, *lux* enables the detection of viable cells, providing a visual marker and a selective marker when it is combined with antibiotic resistance (29). The main advantages of bioluminescence techniques, however, are the ability to assess metabolic activity, both actual and potential activity, and to obtain spatial information on plant-microbe interactions in situ.

ACKNOWLEDGMENTS

This study was carried out under a contract from the Department of the Environment (London, United Kingdom) coordinated by J. Lynch (HRI, Littlehampton, United Kingdom).

REFERENCES

1. **Amin-Hanjani, S. A., A. Meikle, L. A. Glover, J. I. Prosser, and K. Killham.** 1993. Plasmid and chromosomally encoded luminescence marker system for the detection of *Pseudomonas fluorescens* in the soil. Mol. Ecol. **2:**47–54.
2. **Bahme, J. B., and M. N. Schroth.** 1987. Spatial-temporal colonization patterns of a rhizobacterium on underground organs of potato. Phytopathology **77:**1093–1100.
3. **Bale, M. J., J. C. Fry, and M. J. Day.** 1987. Plasmid transfer between strains of *Pseudomonas aeruginosa* on membrane filters attached to river stones. J. Gen. Microbiol. **133:**3099–3107.
4. **Beauchamp, C. J., J. W. Kloepper, and P. A. Lemke.** 1993. Luminometeric analyses of plant root colonisation by bioluminescent pseudomonads. Can. J. Microbiol. **39:**434–441.
5. **Chakrabarty, A. M.** 1985. Genetically manipulated microorganisms and their products in the oil service industries. Trends Biotechnol. **3:**32–38.
6. **Chao, W. L., E. B. Nelson, G. E. Harmen, and H. C. Hoch.** 1986. Colonization of the rhizosphere by biological control agents applied to seeds. Phytopathology **76:**60–65.
7. **Cohen, S. N., A. C. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria; genetic transformation of *Escherichia coli* R-factor. Proc. Natl. Acad. Sci. USA **69:**210–214.
8. **Conner, J. A.** 1986. Digital imaging of free Ca charges and of spatial gradients in growing processes in single mammalian central nervous system cells. Proc. Natl. Acad. Sci. USA **83:**6179–6183.
9. **Cook, N., D. J. Silcock, R. N. Waterhouse, J. I. Prosser, L. A. Clover, and K. Killham.** 1993. Construction and detection of bioluminescent strains of *Bacillus subtilis*. J. Appl. Bacteriol. **75:**350–359.
10. **Davies, K. G., and R. Whitbread.** 1989. Factors affecting the colonisation of a root system by fluorescent pseudomonads: the effect of water, temperature and soil microflora. Plant Soil **116:**247–256.
11. **De Weger, L. A., P. Dunbar, W. F. Mahafee, B. J. J. Lugtenberg, and G. S. Sayler.** 1991. Use of bioluminescence markers to detect *Pseudomonas* spp. in the rhizosphere. Appl. Environ. Microbiol. **57:**3641–3644.
12. **Drahos, D. J., B. C. Hamming, and S. Macpherson.** 1986. Tracking recombinant organisms in the environment: β-galactosidase as a selectable nonantibiotic marker for fluorescent pseudomonads. Bio/Technology **4:**439–443.
13. **Fravel, D. R., R. D. Lumsden, and D. P. Roberts.** 1990. *In situ* visualisation of the biocontrol rhizobacterium *Enterobacter cloacae* with bioluminescence. Plant Soil **125:**233–238.
14. **Grant, F. A., L. A. Glover, K. Killham, and J. I. Prosser.** 1991. Luminescence based viable cell enumeration of *Erwinia carotovora* in soil. Soil Biol. Biochem. **23:**1021–1024.
15. **Grant, F. A., J. I. Prosser, K. Killham, and L. A. Glover.** 1992. Luminescence based detection of *Erwinia carotovora* subsp. *carotovora* in soil. Soil Biol. Biochem. **24:**961–967.
16. **Greacen, E. L., G. R. Walker, and P. G. Cook.** 1989. Procedure for filter paper method of measuring soil water suction. Divisions of soils divisional report no. 108. Commonwealth Scientific and Industrial Research Organisation, Adelaide, Australia.
17. **Griffin, D. M.** 1972. The influence of water, p. 71–112. *In* D. M. Griffin (ed.), Ecology of soil fungi. Chapman and Hall, London.
18. **Hadar, Y., G. E. Harmen, A. G. Taylor, and J. M. Horton.** 1983. Effects of pregermination of pea and cucumber seeds and of seed treatment with *Enterobacter cloacae* on rots caused by *Pythium* spp. Phytopathology **73:** 1322–1325.
19. **Kloepper, J. W., D. J. Hume, F. M. Scher, C. Singleton, B. Tipping, M. Laliberte, K. Frauley, T. Kutchaw, C. Simonson, R. Lifshitz, I. Zaleska, and L. Lee.** 1988. Plant growth-promoting rhizobacteria on canola (rapeseed). Plant Dis. **72:**42–46.
20. **Lynch, J. M., and E. Bragg.** 1985. Microorganisms and soil aggregate stability. Adv. Soil Sci. **2:**133–171.
21. **Mandel, M., and A. Higa.** 1970. Calcium dependent bacteriophage DNA infection. J. Mol. Biol. **53:**159–162.
22. **McLennan, K., L. A. Glover, K. Killham, and J. I. Prosser.** 1992. Luminescence-based detection of *Erwinia carotovora* associated with rotting potato tubers. Lett. Appl. Microbiol. **15:**121–124.
23. **Meikle, A., L. A. Glover, K. Killham, and J. I. Prosser.** 1994. Potential luminescence as an indicator of activation of genetically modified *Pseudomonas fluorescens* in liquid culture and soil. Soil Biol. Biochem. **26:**747–755.
24. **Meikle, A., K. Killham, J. I. Prosser, and L. A. Glover.** 1992. Luminometric measurement of population activity of genetically modified *Pseudomonas fluorescens* in soil. FEMS Microbiol. Lett. **99:**217–220.
25. **Paau, A. S.** 1989. Improvement of rhizobium inoculants. Appl. Environ. Microbiol. **55:**862–865.
26. **Prosser, J. I.** 1994. Molecular marker systems for the detection of genetically modified microorganisms in the environment. Microbiology **140:**5–17.

27. **Rattray, E. A. S., J. I. Prosser, L. A. Glover, and K. Killham.** 1992. Matric potential in relation to survival and activity of a genetically modified inoculum in soil. Soil Biol. Biochem. **5:**421–425.
28. **Rattray, E. A. S., J. I. Prosser, K. Killham, and L. A. Glover.** 1990. Luminescence-based non-extractive detection technique for in situ detection of *Escherichia coli* in soil. Appl. Environ. Microbiol. **56:**3368–3374.
29. **Rattray, E. A. S., J. A. Tyrrell, J. I. Prosser, L. A. Glover, and K. Killham.** 1993. Effect of soil bulk density and temperature on wheat rhizosphere colonisation by *lux*-marked *Pseudomonas fluorescens*. Eur. J. Soil Biol. **29:** 73–82.
30. **Rovira, A. D.** 1969. Plant root exudates and their influence upon soil microorganism. *In* G. F. Baker and W. C. Synder (ed.), Ecology of soil-borne plant pathogens prelude to biological control. John Murray, London.
31. **Shaw, J. J., F. Dane, D. Geiger, and J. W. Kloepper.** 1992. Use of bioluminescence for detection of genetically engineered microorganisms released into the environment. Appl. Environ. Microbiol. **58:**267–273.
32. **Shaw, J. J., and C. I. Kado.** 1986. Development of a *Vibrio* bioluminescence gene-set to monitor phytopathogenic bacteria during the ongoing disease process in a non-disruptive manner. Bio/Technology **4:**560–564.
33. **Silcock, D. J., R. N. Waterhouse, L. A. Glover, J. I. Prosser, and K. Killham.** 1992. Detection of a single genetically modified bacterial cell in soil by using charge coupled device-enhanced microscopy. Appl. Environ. Microbiol. **58:** 2444–2448.
34. **Sneh, B., M. Dapler, Y. Elad, and R. Baker.** 1984. Chlamydospore germination of *Fusarium oxysporum* f. sp. *cucumerinum* as affected by fluorescent and lytic bacteria from a *Fusarium*-suppressive soil. Phytopathology **74:**1115–1124.
35. **Stewart, G. S. A. B., and P. Williams.** 1992. *Lux* genes and the applications of bacterial bioluminescence. J. Gen. Microbiol. **138:**1289–1300.
36. **van Elsas, J. D., J. T. Trevors, D. Jain, A. C. Wolters, C. E. Heijnen, and L. S. van Overbeek.** 1992. Survival of, and root colonisation by, alginate-encapsulated *Pseudomonas fluorescens* cells following introduction into soils. Biol. Fertil. Soils **14:**14–22.
37. **van Elsas, J. D., J. T. Trevors, and L. S. Overbeek.** 1991. Influence of soil properties on the vertical movement of genetically-marked *Pseudomonas fluorescens* through large soil microcosms. Biol. Fertil. Soils **10:**249–255.
38. **Waterhouse, R. N., D. J. Silcock, H. L. White, H. Buhariwalla, and L. A. Glover.** 1993. The cloning and characterisation of phage promoters, directing high expression of luciferase in *Pseudomonas syringae* pv. *phaseolicola* allowing single cell and microcolony detection. Mol. Ecol. **2:**285–294.
39. **Weller, D. M.** 1984. Distribution of a take-all suppressive strain of *Pseudomonas fluorescens* on seminal roots of winter wheat. Appl. Environ. Microbiol. **48:**897–899.
40. **Weller, D. M.** 1988. Biological control of soil-borne pathogens in the rhizosphere bacteria. Annu. Rev. Phytopathol. **26:**379–407.
41. **Whipps, J. M.** 1990. Carbon economy, p. 59–98. *In* J. M. Lynch (ed.), The rhizosphere. Wiley, Chichester.
42. **Wilson, C. L., J. D. Franklin, and L. Pusey.** 1987. Biological control of *Rhizopus* rot of peach with *Enterobacteria cloacae*. Phytopathology **77:**303–305.
43. **Winstanley, C., A. W. Morgan, R. W. Pickup, J. G. Jones, and J. R. Saunders.** 1989. Differential regulation of lambda P_L and P_R promoters by a c1 repressor in a broad-host-range thermoregulated plasmid marker system. Appl. Environ. Microbiol. **55:**771–777.