Use of Bioluminescence for Detection of Genetically Engineered Microorganisms Released into the Environment

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The persistence and movement of strain JS414 of Xanthomonas campestris pv. campestris, which was genetically engineered to bioluminesce, were monitored during a limited field introduction. Bioluminescence and traditional dilution plate counts were determined. Strain JS414 was applied to cabbage plants and surrounding soil by mist inoculation, by wound inoculation, by scattering infested debris among plants, and by incorporating bacteria into the soil. Bioluminescent X. campestris pv. campestris was detected in plant samples and in the rhizosphere up to 6 weeks after inoculation. Movement to uninoculated plants was detected on one occasion, but movement from the immediate release area was not detected. Strain JS414 was detected in soil samples beneath mist- and wound-inoculated plants only at intentionally infested locations and in aerial samples only on the day of inoculation. Our bioluminescence methods proved to be as sensitive as plating methods for detecting the genetically engineered microorganisms in environmental samples. Our results demonstrate that transgenic incorporation of the luxCDABE operon provides a non-labor-intensive, sensitive detection method for monitoring genetically engineered microorganisms in nature.

Genetically engineered microorganisms (GEMs) may be introduced into the environment to detect (12) or to degrade toxic substances (7), to serve as biological control agents (18), to deliver insecticidal proteins (32), to control frost damage in plants (2, 15, 17), and to enhance nitrogen fixation (3). A major reservation for the release of GEMs has centered around the need to monitor the dispersal and persistence of such organisms in the environment. A recent report by the National Research Council (20) and several other accounts (5, 11, 19) have commented on the paucity of suitable, reliable methods for monitoring GEMs.

Promising new approaches for detecting GEMs have been reviewed by Jain et al. (9). Results obtained with newer technologies suggest that homologous DNA probes can be used to specifically identify certain species of bacteria (31) and to identify the presence of function-specific genes among mixed species of bacteria (23). In other advances workers have used actual tagging of bacteria with reporter genes, such as the lacZ gene (1, 6), or with other selectable traits carried on transposons (21). Another recently developed method relies upon the use of the polymerase chain reaction to amplify small amounts of DNA prior to nucleic acid hybridization (30). However, none of these methods provides real-time or direct detection of bacteria. All rely upon data generated from analyses to reconstruct past events. Also, these methods are generally labor intensive, necessitating multiple extractions and centrifugation steps or serial dilutions and often requiring large amounts of support materials. Moreover, these methods may be subject to background noise caused by the presence of homologous nucleic acid sequences or enzymes present in other microorganisms. The use of bacterial bioluminescence as a reporter gene-encoded phenotype, coupled with broth enrichment culturing or low-light camera imaging, overcomes these disadvantages, since it has negligible background problems and has relatively undemanding time and set-up requirements for assays.

In this report the use of bioluminescence to track a field-released strain of Xanthomonas campestris pv. campestris is described. X. campestris pv. campestris causes black rot, an important worldwide disease of crucifers (33). Previously, the expression of the bioluminescence phenotype in phytopathogenic bacteria has been found not to interfere with the pathogenicity of these organisms (26).

Since work of this nature is novel and exploratory, the scope of this work was necessarily limited to a small-scale field introduction. The experiments were designed to ascertain the general safety of such an experiment and to explore the use of bioluminescence detection. Our results provide a basis for future, more elaborate studies to examine the movement and persistence of GEMs during field introductions.

(Preliminary results of this work were presented at the International Symposium on The Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms at Kiawah Island, S.C., in November 1990.)

MATERIALS AND METHODS

Bacteria and culture conditions. X. campestris pv. campestris JS414 (28, 29), which carries the Lux transposon, Tn4431, and which is strongly and constitutively bioluminescent, was used in all experiments. This strain is similar to its parental strain in pathogenic abilities (29); both can infect the leaves of young plants, where they colonize the vessels of single leaves but are not able to spread systemically within the host. Factors which were deemed to be advantageous in ascertaining the safety of this initial field introduction.

Bacteria were grown on medium 210, a modification of medium 523 (10), in which sucrose is used at a concentration of 5 g/liter. Agar was added to medium 210 broth at a concentration of 15 g/liter when it was needed. The following antibiotics were also added: tetracycline (10 mg/liter; Sigma Chemical Co., St. Louis, Mo.), rifampin (50 mg/liter; Sig-

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ma), cycloheximide (50 mg/liter; Sigma), and benomyl (50 mg/liter; Benlate 50% wettable powder; E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.). Cells were grown at 24 to 27°C.

**Inoculum preparation.** Bacteria were grown to late log phase in 10.0 ml of broth with aeration, chilled on ice, pelleted by centrifugation at 10,000 × g and 4°C, rinsed and resuspended in PM buffer (0.7% K$_2$HPO$_4$, 0.2% KH$_2$PO$_4$, 0.025% MgSO$_4$; pH 7.25) to approximately the original concentration, and diluted 100-fold in PM buffer to give a final concentration of about 10$^7$ CFU/ml. For laboratory studies, the inoculum was used immediately, whereas for the limited field introduction, the suspension was kept on ice until it was used (ca. 1 h).

**Laboratory experiments.** To detect GEMs in soil, inocula were added to 5-g portions of field soil at concentrations of 5 × 10$^1$, 5 × 10$^2$, 5 × 10$^3$, 5, and 5 × 10$^{-1}$ CFU/g of soil. At 2 h after bacterial incorporation, 45 ml of PM buffer was added to each preparation, and the mixture was shaken for 1 h. A 0.1-ml sample of the slurry was added to 3.0 ml of medium 210 broth supplemented with antibiotics, and the preparation was incubated with aeration at 27°C. To assay bioluminescence, a broth sample (0.1 ml) was placed in a cuvette, and total bioluminescence was determined over a period of 10 s by using a Monolight 2000 luminometer (Analytical Luminescence Laboratories, San Diego, Calif.). Bioluminescence measurements were taken at 18- to 24-h intervals.

To detect GEMs in planta, cabbage plants in a growth chamber were inoculated in wounds as described previously (27) with a suspension of strain JS414 in PM buffer. At 2-day intervals, leaves were removed and sampled for bioluminescence with a liquid nitrogen-cooled, charge-coupled device (CCD) camera (Photometrics Ltd., Tucson, Ariz.). Images of bioluminescence were obtained by focusing the CCD camera on the sample and making a 10-min exposure (f/1.2) with the camera cooled to −110°C. Firmware (Photometrics Ltd.) was used to extract positional and quantitative (quanta per minute) data from images. Then the leaf samples were ground in PM buffer in sterile mortars and pestles, and the appropriate dilutions were plated onto medium 210. The numbers of colony-forming units were determined after incubation for 60 h.

**Regulatory conditions for a limited field introduction.** Since strain JS414 is phytopathogenic, permission to conduct a field test with the GEMs was requested from the U.S. Department of Agriculture Animal and Plant Health Inspection Service by submitting a completed Plant Protection and Quarantine form 1001. An environmental assessment was prepared, a finding of no significant impact was issued by the Animal and Plant Health Inspection Service (7a), and plot guidelines were established for a limited environmental introduction of strain JS414 at the Horticulture Crops Unit, E. V. Smith Research Center, Alabama Agricultural Experiment Station, Shorter (Macon County). In accordance with the permit, the test area was fumigated with methyl bromide 10 days prior to transplanting, and 256 cabbage plants at the three- to five-leaf stage were transplanted into a release plot (4 by 4 m) which was surrounded by an unplanted buffer zone (Fig. 1). A guard row of cabbage trap plants was established around the perimeter of the buffer zone.

**Host plants and inoculations.** Two black rot-susceptible cabbage varieties, *Brassica oleracea* var. capitata L. 'Ferry Round Dutch' (Ferry Morris Seed) and *B. oleracea* var. capitata 'Perfect Ball' (Nickerson-Zwaan, Tuitjenhorn, The Netherlands) were planted in Orangeburg sandy loam soil.

Cultivar Ferry Round Dutch plants were planted in the release plot (spaced 10 cm apart), whereas cultivar Perfect Ball plants were planted in the guard rows. The plants in the release plot were subdivided into four plant inoculation treatments (treatments A, B, C, and D) containing 64 plants each.

Treatment A plants were mist inoculated with a suspension of bacteria in PM buffer. A Micro-ULVA sprayer applicator (North American Micron, Reedley, Calif.) was used to deliver the bacteria in droplets having an average diameter of 35 to 45 μm while the other treatments were covered with plastic. The applicator was moved slowly over the plants about 20 cm above their surfaces; we applied about 50 ml of bacteria, until the plants were wet to runoff. Treatment B plants were inoculated by injecting approximately 30 μl of bacteria into the petioles of three different leaves per plant (27). Treatment C plants were inoculated by scattering infested debris among the plants. Infested debris was obtained by inoculating cabbage plants with strain JS414 10 days prior to the field inoculations and verifying the presence of strain JS414 by examining the plants for bioluminescence with the CCD camera (see above) on the evening before the field inoculation. The debris was prepared by shredding leaves and stems of plants displaying bioluminescence. Treatment D plants remained uninoculated.

GEMs were incorporated into the soil at four different locations in the buffer zone (Fig. 1). A preparation of 50 ml of bacteria in PM buffer was mixed with approximately 200 g (wet weight) of soil, and this mixture was placed on the soil surface at the original location.

**GEM sampling.** During 1990 the release plot was sampled for GEMs 1 week before inoculation (April 9), immediately after inoculation (April 16), and at 2-week intervals thereafter until May 28. At 7 weeks after inoculation all plants were tilled under, and the release area was watered to runoff. Soil samples were collected in midsummer (July 17) and the following spring (25 April 1991). Airborne bacteria deposited onto surfaces were detected essentially as described by Lindow et al. (16). Petri dishes
Bioluminescence to Detect Microorganisms

(10 cm) containing medium 210 supplemented with all of the antibiotics described above were arranged in eight radial arrays on the soil surface at 2.8, 3.8, 4.8, 5.8, 7.8, and 9.8 m from the center of the release plot (Fig. 2), were uncovered 10 min prior to the start of mist inoculation, and were allowed to remain uncovered for 40 min. At subsequent sampling dates, petri dishes were placed in the same positions and left uncovered for 30 min.

A total of 20 plants were sampled at each sampling time; for each plant we removed a leaf (5 to 10 g) and a rhizosphere sample (10 to 20 g of soil with associated host plant roots). Four randomly selected plants were taken from each treatment. Similarly, samples were taken from one randomly selected plant in each of the four guard rows. The remaining parts of these plants were discarded to the disposal area. Before inoculation and after the plants were tilled under, only soil samples were collected at random from the surface (top 1 to 2 cm) of the release plot. After inoculation and while the plants were present, 18 soil surface samples (ca. 30 g each) were taken from the buffer zone at 3.8 and 9.8 m from the center of the release plot at positions concurrent with aerial sampling sites (see above); Four of these 18 soil samples were taken at the infested soil sites (Fig. 2). All samples were transported to a laboratory in covered ice chests in the passenger compartment of an automobile.

Detection of bioluminescent bacteria. Field samples were assayed by using the following two methods: (i) colony determinations via serial dilution and plating onto selective media, and (ii) bioluminescence measurements of enrichment cultures in which a luminometer was used. In addition, the bioluminescence of selected leaf samples was determined with the CCD camera prior to either of these determinations. The wet weight of each sample was recorded before it was diluted 10-fold (wt/wt) with sterile distilled water and shaken at 150 rpm at room temperature for 1 h. Leaf samples were prepared as described above (laboratory experiments), while soil and rhizosphere samples were not homogenized.

In the first method, dilutions of bacteria were spiral plated (Spiral Systems Inc., Bethesda, Md.) onto medium 210 supplemented with all of the antibiotics, and the numbers of colony-forming units per gram of sample were determined by using a laser colony counter (Spiral Systems Inc.) and Bacterial Enumeration software (Spiral Systems Inc.). Alternatively, bacteria were manually serially diluted, plated, and counted. In the second method, enrichment cultures were obtained by inoculating 0.1-ml portions of the processed samples into 2.9-ml portions of medium 210 broth supplemented with all of the antibiotics with constant shaking at 27°C. Aliquots (0.1 ml) were assayed at intervals to determine bioluminescence intensity.

Results

Laboratory experiments. (i) Detection of GEMS in soil. The relationship between inoculum level in the artificially infested field soil and the advent and intensity of detectable bioluminescence is shown in Fig. 3. An inoculum of $5 \times 10^3$ CFU/g of soil resulted in immediate bioluminescence, whereas an inoculum of $5 \times 10^4$ CFU/g of soil resulted in detectable bioluminescence after 18 h. Likewise, bioluminescence resulting from an inoculum of $5 \times 10^2$ CFU/g of soil was recorded at 18 h, but the intensity at that time was nearly 10-fold greater than the intensity when the inoculum was $5 \times 10^3$ CFU/g of soil. As few as $5 \times 10^{-1}$ CFU/g of soil in the inoculum were detected by this method. Even though all of the samples eventually bioluminesced at similar intensities, there was a difference in the onset of bioluminescence; a greater initial inoculum resulted in bioluminescence that was detected more quickly.

(ii) Detection of GEMS in plants. Digitized images allowed us to visualize infected areas within leaves without disrupting the leaf tissues. After log transformation of the data, there was a linear relationship between light intensity and bacterial population (Fig. 4). Under these experimental conditions the minimum level that could be detected was $1.5 \times 10^3$ CFU per leaf.

Limited field introduction. (i) Environmental conditions. On the morning of the field introduction, air movement was light, with an average speed of 0.5 miles/h (1.609 km/h) in a southeasterly direction. At the time of release the temperature was 18°C, and the daily maximum was 23°C. The
average minimum and maximum temperatures were 12 and 26°C for 16 to 30 April, 14 and 27°C for 1 to 14 May, and 16 and 27°C for 15 to 28 May, respectively. Rainfall was light over the entire period, and the plants were overhead irrigated as needed. Maximum and minimum air and soil temperatures, precipitation, and anemometer readings were recorded at 24-h intervals.

(ii) Disease development. Disease development was limited, but typical black rot symptoms were observed on wound-inoculated leaves. These leaves absceded, and infections did not proceed systemically. (By design, strain JS414 was derived from a parental strain which was only mildly pathogenic.) Black rot lesions were found on leaf margins of three mist-inoculated plants, whereas no symptoms were observed on uninoculated plants or on plants that were inoculated with infested debris.

(iii) Movement and persistence of strain JS414. On the day of the field introduction, airborne strain JS414 was detected in only 3 of 53 exposed petri dishes (Table 1). These plates were immediately downwind from the mist-inoculated plants. A confluent lawn of bacteria was cultured from one plate, whereas one of the other two plates had 32 colonies and one had 2 colonies. Airborne GEMs were not detected on subsequent sampling dates.

Strain JS414 was recovered from plants and rhizospheres over the entire 6-week sampling period, although by week 6 GEMs were recovered from only one wound-inoculated plant (Tables 1 and 2). GEMs were recovered for up to 4 weeks from the soil-infested locations. At 7 weeks after the plants were tilled under the soil, strain JS414 was not detected in 20 soil samples.

GEMs were consistently recovered from the rhizospheres of wound- and mist-inoculated plants (treatments A and B) but were never recovered from the rhizospheres of debris-inoculated plants (treatment C). In addition, GEMs were never recovered from the soil in the disposal area (data not shown).

Strain JS414 was recovered in only one instance from treatment D plants, and it was not recovered from the trap plants (Table 1). No GEMs were recovered from soil samples in the buffer zone, except from the locations which had been intentionally infested with GEMs. GEMs were never recovered from a random sample of cruciferous and noncruciferous weed leaves in the buffer zone. One year later the fallow soil was resampled after methylbromination, and strain JS414 was not detected in 42 randomly selected soil samples (Tables 1 and 2).

(iv) Sensitivity of bioluminescence detection. Throughout the experiments, the broth enrichment-Lux assay proved to be as sensitive as serial dilution and plating for detecting bacteria (Table 2). The only difference occurred on the day of the field introduction, when the Lux assay successfully detected GEMs in 12 samples which were negative when they were assayed by using the conventional plating method.

To test the ability of digitized images to predict bacterial numbers in environmental samples, leaf samples were taken from the release plot on three different dates, examined with the CCD camera, and subsequently treated to determine the numbers of colony-forming units. In all cases in which bacterial bioluminescence in the leaves was detected with the CCD camera, the values extrapolated from the standard curve were accurate reflections of the bacterial numbers actually recovered from field samples (Fig. 4). Figure 5 shows an example of the image data.

**DISCUSSION**

Bioluminescence is a rare phenotype among rhizosphere and phyllosphere bacteria. To date, only one terrestrial

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**TABLE 1.** Detection of strain JS414 in air, leaf, soil surface, rhizosphere, and weed samples over time by plating and Lux assays

<table>
<thead>
<tr>
<th>Sampling date</th>
<th>No. of positive samples/total no. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Air</td>
</tr>
<tr>
<td>9 April 1990</td>
<td>NA*</td>
</tr>
<tr>
<td>16 April 1990</td>
<td>3/53</td>
</tr>
<tr>
<td>30 April 1990</td>
<td>0/53</td>
</tr>
<tr>
<td>14 May 1990</td>
<td>0/53</td>
</tr>
<tr>
<td>28 May 1990</td>
<td>0/53</td>
</tr>
<tr>
<td>17 July 1990</td>
<td>NA</td>
</tr>
<tr>
<td>25 April 1991</td>
<td>NA</td>
</tr>
</tbody>
</table>

* NA, not applicable.
* The positive samples were samples from the mist-inoculated, wound-inoculated, and debris-inoculated treatments.
* The positive samples were samples from the mist-inoculated, wound-inoculated, and uninoculated treatments.
* The positive samples were samples from the mist-inoculated, debris-inoculated, and uninoculated treatments.
* The positive samples were a sample from the wound-inoculated treatment.

**TABLE 2.** Efficiency of strain JS414 detection as determined by the plating assay and the bioluminescence assay over time in leaf, soil surface, and rhizosphere samples

<table>
<thead>
<tr>
<th>Sampling date</th>
<th>No. of positive samples in plate assay</th>
<th>No. of positive samples in Lux assay</th>
<th>Total no. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 April 1990</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>16 April 1990</td>
<td>7</td>
<td>19</td>
<td>58</td>
</tr>
<tr>
<td>30 April 1990</td>
<td>4</td>
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<td>58</td>
</tr>
<tr>
<td>15 May 1990</td>
<td>8</td>
<td>8</td>
<td>58</td>
</tr>
<tr>
<td>28 May 1990</td>
<td>2</td>
<td>2</td>
<td>58</td>
</tr>
<tr>
<td>17 July 1990</td>
<td>ND*</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>25 April 1991</td>
<td>ND*</td>
<td>0</td>
<td>42</td>
</tr>
</tbody>
</table>

* ND, not determined.
bacterium associated with a soil nematode has been reported to bioluminesce (25). This distinctive phenotype was successfully used to assay for the presence of bioluminescent *X. campestris* pv. *campestris*. Other workers have described the use of bioluminescence to detect bacteria in soil in laboratory studies (8, 22), but this is the first use in a limited field introduction. Bioluminescence detection coupled with broth enrichment culturing proved to be a simple and useful technique for monitoring the movement and persistence of GEMs introduced into the environment. In a typical plating assay to detect bacteria in soil, the sample was first diluted 50-fold and only then were 0.1-ml portions plated out. Moreover, quickly sedimenting particles and any adherent bacteria were probably not plated. In the method which we describe above, the entire 0.1-g sample of soil (not just 1 of 50 parts) and all particles, both sedimented and suspended, can be assayed.

To determine whether bioluminescence data could be used to estimate quantities of bacteria in field samples, known numbers of bacteria were added to soil samples in the laboratory and assayed using the broth enrichment-Lux method. Although precision was lacking, the Lux assay proved to be as sensitive as plate culturing methods in actually detecting the bacteria and provided general infor-
mation about starting numbers of bacteria. This approach is useful because it is easy to perform and allows many more samples to be processed than plating methods allow when given amounts of labor, incubator space, and culture media are used.

The use of a CCD camera provides real-time detection and allows for quantitative measurements of light intensity. Our results show that quantitative measurements are possible without resorting to serial plating and diluting. In prior reports X-ray or photographic film was used for obtaining images of bacterial bioluminescence (26), but these techniques were time consuming and did not result in quantitative data.

Any engineered organism (indeed, any antibiotic-resistant variant, etc.) may differ in subtle ways from the parental strain. Since strain JS414 is constitutively bioluminescent, it is likely to have significantly different energy demands than its parental strain. It is unclear what effect these demands have on the overall competitiveness of the organism. Our data show that under dry, warm weather conditions X. campestris pv. campestris survived up to 6 weeks on the leaves and in the rhizospheres of host plants and to a lesser extent in the soil. No population increase was observed, and by the end of 6 weeks bacteria were detected in association with only one plant. Since sampling was conducted at 2-week intervals, it is possible that a brief population increase could have occurred immediately postinoculation without being detected. Following termination of plant growth, no GEMs were detected in the test area 7 weeks or 1 year later.

GEMs were recovered from the rhizosphere and soil only where they had been inoculated onto plants in an aqueous suspension or where they had been directly added to the soil. In contrast, when the inoculum consisted of debris, the bacteria were recovered only from the phyllosphere. Since the presence of active bacteria in the shredded debris was established by visual observation of bioluminescence shortly before the debris was used, we concluded that the inoculum was viable. Thus, it may be that the bacteria were confined to the plant parts and not readily distributed in the soil.

The lux genes have been expressed in diverse gram-negative bacteria (26), gram-positive bacteria (4, 24), yeasts (13), and plants (14), and thus bioluminescence detection methods should have broad applicability. Larger-scale field introductions and comparative studies of discrete bacterial mutants in susceptible and resistant host plants can now be conducted. Questions such as the role of various phenotypes (e.g., extracellular polysaccharide or pigment production) in environmental persistence and survival of X. campestris pv. campestris can be addressed, as well as the effect of expressing heterologous genes (e.g., lux or other marker genes) and the rate of horizontal gene transfer of the recombinant transposon (Ts4431) into other bacteria. In addition, environmental variables that may affect bacteria, such as soil type, temperature, and water stress, can be explored.

REFERENCES


