Field Detection of Phytopathogenic Bacteria by Bioluminescence

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Abstract

Genetic marker systems for tracking microbes released into the environment have greatly increased our ability to assess microbial dispersal and survival and the risks associated with the release of genetically engineered microorganisms. Transgenic incorporation of the *luxCDABE* operon from *Vibrio fischeri* into phytopathogenic bacteria has been used successfully to visualize and determine bacterial colonization in and on plants using a computer-assisted, charge-oupled device (CCD) camera. The dispersal and persistence of bioluminescent *Xanthomonas campestris* pv. *campestris*, causal agent of black rot disease of cabbage, were studied in the field environment in accordance with a USDA/APHIS biotechnology permit. Bioluminescent bacteria in environmental samples were detected with the CCD camera or through bioluminescence measurements of broth enrichment cultures. Colonization of plants with bioluminescent *X. campestris* pv. *campestris* fluctuated with environmental conditions and persistence was coupled with the host's growing season. Dispersal to alternative hosts such as weeds was detected, while movement and persistence in the rhizosphere were limited. Horizontal transfer of the *lux* operon into other leaf- or soil- associated bacteria was not detected.

Introduction

Genetically engineered or modified microorganisms may be introduced into the environment for a variety of reasons, such as to act as biological control agents or bioremediation agents, to enhance nitrogen fixation or to control frost damage on plants (Wilson and Lindow, 1993). A major reservation concerning such releases has centered on the requirement for monitoring the persistence and dispersal of the genetically engineered or modified microbes (GEM). A number of approaches have been used to specifically identify or tag bacteria with reporter genes, such as the *lacZY* gene (Kluepfel and Tonkyn, 1992). However, these methods do not provide direct detection of bacteria, but rely instead on data generated from analyses to reconstruct past events and may be subject to background noise. Bacterial bioluminescence, coupled with broth-enrichment culture and low-light camera imaging enables to overcome these disadvantages and can provide direct, easy and nondestructive detection of recombinant bacteria in the host and their environment.

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Bioluminescence

The emission of light by bioluminescent bacteria is catalyzed by the enzyme luciferase, a heterodimeric enzyme encoded by 2 closely linked genes, *luxA* and *luxB* in the *lux* operon (Meighen, 1991). In the generation of light (590 nm), luciferase oxidizes reduced flavin mononucleotide (FMNH2) and a long-chain fatty aldehyde (RCHO) to yield the corresponding fatty acid and FMH. The other component unique to the system is a fatty acid reductase complex involved in the synthesis and recycling of the aldehyde substrate. The complex contains three proteins, a reductase, transferase and a synthesase encoded by *luxC*, *luxD*, and *luxE*, respectively, found in the *lux* operon (Meighen, 1994). Bacteria expressing all five structural genes can bioluminesce in locations where it would be impossible to add the aldehyde substate (tetradecanal) such as inside plant tissues. The transposon Tn*4431* was introduced into spontaneous rifampicin-resistant derivatives of *Xanthomonas campestris* pathovars in biparental matings with *E. coli* containing a suicide delivery plasmid or via electroporation. The transposon carries the *luxCDABE* genes of the fish bacterium *Vibrio fischeri* as well as a tetracycline resistance gene in a manner which facilitates the transcriptional fusion of the genes into the bacterial chromosomal DNA. If the *lux* genes become inserted into a strong promoter, the mutant bacterial colonies will be bright. Integration into the genome is advantageous because it avoids the variable gene dosage that may occur when the genes are expressed on a plasmid. Strongly and constitutively bioluminescent transposon recipients were selected.

Enumeration of bioluminescent *Xanthomonas campestris* pv. *campestris* in and on leaf samples

Xanthomonas campestris pv. *campestris* (or Xcc), the causal agent of the economically important disease known as black rot of crucifers, penetrates into the host plant through hydathodes or wounds and colonizes the vascular system (Cook *et al.*, 1952). The expression of the bioluminescent phenotype in Xcc was found not to interfere with the pathogenicity or growth characteristics of the strain in different hosts (Dane and Shaw, 1993). Movement and growth of a pathogenic bioluminescent strain (FD91L) were followed in susceptible (cv. Perfect Ball) and resistant (cv. Hancock) cabbage seedlings after wound or mist inoculation with a liquid nitrogen cooled, charge-coupled device (CCD) camera. The CCD camera system allows the visualization and quantification of bioluminescent bacterial population levels without disrupting the ongoing pathogen-host interaction. Images are obtained by focusing the camera on the plants and making a 5- minute exposure with the camera cooled to −110°C. A linear relationship between light intensity and bacterial bioluminescence was detected (Shaw *et al.*, 1992). Wound inoculation techniques were found to bypass the early host response to Xcc infections in the incompatible interaction between pathogen and (resistant) host. Only through hydathode infection did the host fully respond to the pathogen, restricting vascular (endophytic) growth (Williams *et al.*, 1973; Dane and

Shaw, 1993). To determine the relationship between cell density of bioluminescent Xcc in environmental samples and intensity of detectable bioluminescence in enrichment culture, aliquots of inoculum containing 10^7, 10^5, 10^3 or 10 cfu were deposited on leaf disks. Each inoculated leaf disk was added to broth supplemented with antibiotics. The bioluminescence of the broth was sampled over time using a luminometer. An inoculum of 10^7 cfu resulted in detectable bioluminescence at 2 days of incubation in enrichment culture, whereas an inoculum of 10 cfu or less resulted in detectable bioluminescence in 4-5 days of incubation. Similar results were obtained when the inoculum was added to soil collected from the field (Dane and Shaw, 1994). The onset of bioluminescence differed depending on the initial inoculum level.

Field releases and regulatory conditions

Since the recombinant bacteria are phytopathogenic, permission to conduct field releases was requested from the United States Department of Agriculture 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. Plot guidelines and permit conditions were established for a limited environmental introduction of a so-called 'regulated article' at the Horticulture Crops Unit of the E.V. Smith Research Center of the Alabama Agricultural Experiment Station (Shaw *et al.*, 1992).

The first small-scale field experiment was designed to ascertain the general safety of the introduction of the GEM and to explore the use of bioluminescence detection. A weakly pathogenic, brightly bioluminescent strain of Xcc (JS414) was applied to susceptible cabbage plants and surrounding soil by mist inoculation, wound inoculation, by scattering of infested debris among plants or by incorporation into the soil. Airborne bacteria deposited onto surfaces were sampled on sedimentation plates. Field samples were assayed using traditional plating methods and bioluminescence measurements of enrichment cultures. In addition, the bioluminescence of selected leaf samples was determined with the CCD camera. Bioluminescent bacteria were detected in plant samples and in the rhizosphere up to 6 weeks after inoculation. Movement to one uninoculated cabbage plant was detected on one occasion, but movement from the immediate release site was not detected. The bioluminescence detection techniques proved to be as sensitive as traditional plating assays for the detection of bioluminescent bacteria in environmental samples. Since on 2 occasions "dark" mutants were recovered from environmental samples, the high level of bioluminescence in the weakly pathogenic Xcc strain might have been a disadvantage for that organism. The *lux*- or "dark" mutants were found to contain an insertional sequence in the *luxCD* genes.

In subsequent field releases (1991, 1992-1993 and 1996) a pathogenic bioluminescent strain (FD91L) was used to evaluate the differential colonization of host plants (1991) and the effect of alternative biological control agents on the persistence and dispersal of the bioluminescent bacteria (1992, 1996). Annual extensions of the original biotechnology permit with minor modifications were secured from the

USDA/APHIS. Bioluminescent and non-bioluminescent (parental) bacteria were introduced onto plots of susceptible (Perfect Ball) and resistant (Hancock) cabbage plants in the spring of 1991. Movement and dispersal of the bacteria were monitored over time through frequent soil and leaf samplings. Bioluminescent bacteria could be detected on leaf samples with the CCD camera for 2 months as often on leaves of resistant as on susceptible host, especially on areas with lesions caused by weathering, insects or microbes. Resistant hosts can consequently act as important reservoirs of inoculum without detectable symptomatology. Vascular (endophytic) populations were observed in susceptible cabbage only, often before the onset of characteristic V-shaped lesions. Low levels of bioluminescent bacteria, detectable only through broth-enrichment culture, were found thereafter. Bacteria were detectable for a period of 3 months in the phylloplane and for 4 months in the rhizosphere inside plot areas and were not observed to colonize replantings of host and non-host plants (Dane and Shaw, 1994).

In the fall of 1992, bioluminescent bacteria were released onto plots of susceptible cabbage plants which had previously been inoculated with antagonistic xanthomonads to determine the phenomenon of preemptive exclusion. Mist inoculation with *Xanthomonas campestris* pv. *vesicatoria* (Xcv) or a weakly pathogenic strain of Xcc followed by challenge inoculation with the bioluminescent strain one week later, did not restrict the infection of plants with bioluminescent bacteria. Initially, previous inoculation resulted in a pronounced reduction in the number of populations on local lesions and symptomless leaves, but this effect decreased rapidly over time. Hydathodal population development was detectable 10 days after inoculation with bioluminescent bacteria and vascular colonization 9 days later. Epiphytic colonization of bioluminescent bacteria was found to fluctuate with environmental conditions. After a cold spell, only vascular populations were detectable through CCD-camera imaging. Persistence of the recombinant bacteria as a vascular endophyte was associated with the host's growing season (Fig. 1). The mild climatic winter conditions favored not only the survival of the host and persistence of the GEM but also the dispersal to cruciferous and non-cruciferous weeds at the release site as well as in adjacent fields (Dane and Shaw, 1996).

In this year's field release (1996), bioluminescent bacteria were released onto plants treated with plant growth promoting rhizobacteria, antagonistic xanthomonads or fosetyl-Al. Preliminary results showed little effect of treatments on the control of black rot disease of cabbage under field conditions, even though greenhouse experiments had indicated that selected plant growth-promoting rhizobacteria had stimulated the host's defense mechanism and reduced the growth of the bioluminescent pathogen (Dane *et al.,* 1996). The bacteria persisted in the host for less than 2 months after which the high temperature conditions became limiting to cabbage growth confirming results from earlier field releases (Fig. 1).

Summary

Direct evaluation of the spatial distribution of metabolically active bacteria inside the host without disruption of the ongoing host-bacterial interaction has been a major advantage in the use of bioluminescent phytopathogens. Bioluminescence is a rare phenotype among terrestrial bacteria. This distinctive phenotype was successfully used to track the persistence and dispersal of bacteria under varying environmental conditions. Environmental variables such as low temperatures favored the survival of the host and the persistence and dispersal of the bacteria. Survival of the bacteria in the soil after termination of the experiments was consistent with earlier studies conducted with non-recombinant bacteria (Alvarez *et al.*, 1987). The use of *lux* in combination with antibiotic resistance markers allowed rapid and unambiguous selection and identification of the introduced strains. Although we should avoid generalization, our results and those of others with soil-borne root- colonizing bacteria (Kluepfel and Tonkyn, 1992) have shown that there are no inherent dangers in the use of genetically engineered bacteria in the environment. The risk of horizontal gene transfer should be considered. The stability of the *luxCDABE* insert was confirmed through passages *in vitro* and *in planta*, and horizontal gene transfer was not detected in environmental samples. Our results indicate that bioluminescent microbes can be released without perturbation of a given habitat and that future releases of genetically engineered microbes are essential for a better understanding of the microbial ecology of phytopathogenic bacteria and the development of effective disease management strategies.

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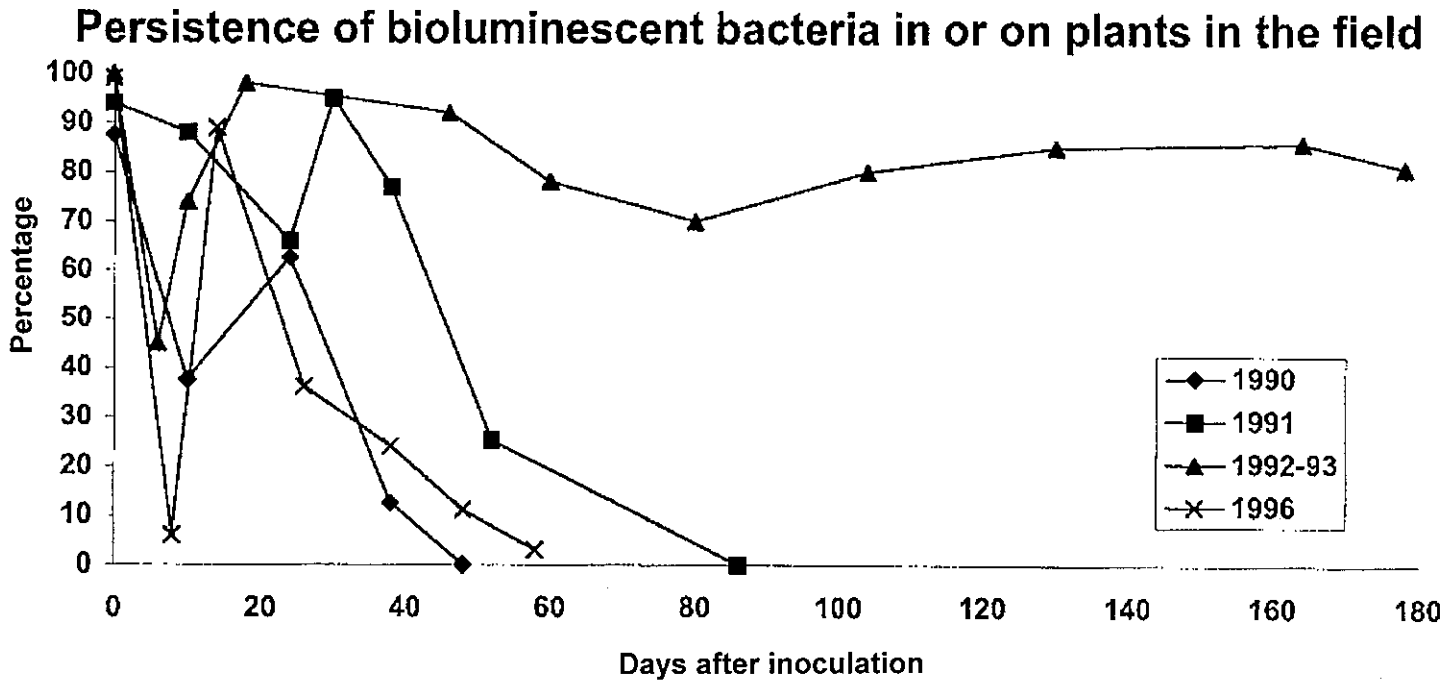

Fig. 1 Percentage of plant samples with bioluminescent *Xanthomonas campestris* pv. *campestris*.

Samples were randomly collected over time from the field release sites.