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Use of Bioluminescence Markers To Detect *Pseudomonas* spp. in the Rhizosphere

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The use of bioluminescence as a sensitive marker for detection of *Pseudomonas* spp. in the rhizosphere was investigated. Continuous expression of the *luxCDABE* genes, required for bioluminescence, was not detectable in the rhizosphere. However, when either a naphthalene-inducible *luxCDABE* construct or a constitutive *luxAB* construct (coding only for the luciferase) was introduced into the *Pseudomonas* cells, light emission could be initiated just prior to measurement by the addition of naphthalene or the substrate for luciferase, *n*-decyl aldehyde, respectively. These *Pseudomonas* cells could successfully be detected in the rhizosphere by using autophotography or optical fiber light measurement techniques. Detection required the presence of 10^3 to 10^4 CFU/cm of root, showing that the bioluminescence technique is at least 1,000-fold more sensitive than β-galactosidase-based systems.

Root colonization is often the limiting step in the use of rhizobacteria as biological control agents. The process of root colonization is very complex. To unravel this process, our knowledge of bacterial behavior in the rhizosphere has to be improved. Since techniques for studying bacteria in the rhizosphere are not adequate, there is an urgent need for the development of new, more efficient techniques.

With this aim we introduced bioluminescence reporter genes as a marker to monitor *Pseudomonas* spp. in the rhizosphere. Previously, bioluminescence has been used successfully for monitoring bacteria in their natural environment (11) and for studying the effect of environmental parameters on gene expression (5). Recently, a bioluminescence reporter plasmid for naphthalene catabolism was developed by insertion of transposon Tn*4431*, carrying the promoterless *luxCDABE* genes of *Vibrio fischerii* (11), into a naphthalene catabolic plasmid in *Pseudomonas fluorescens* 5R (7). The resulting strain, 5RL, produces light upon exposure to naphthalene or to the inducing metabolite, salicylate. Strain 5RL has successfully been employed as a biosensor for naphthalene in soil by using fiber optics for the detection of light production (7).

The present study was undertaken to determine whether bioluminescence could be used to monitor bacteria directly in the rhizosphere.

Use of a naphthalene-inducible bioluminescence reporter system in the rhizosphere. Surface-sterilized, germinated soy beans were inoculated by dipping them into a bacterial suspension (10^7 or 10^8 CFU/ml) of *P. fluorescens* 5RL (Table 1). To approximately 120 ml of a sterilized sand-vermiculite (1:1) mixture, 10 ml of plant nutrient solution (12), containing 10^7 CFU/ml, was added. This inoculated sand-vermiculite mixture was put into plastic bags (16 by 6 cm), and the inoculated soybeans were planted. After 4 to 14 days of growth, the roots were taken out of the bags. Most of the adhering sand-vermiculite particles were removed by gentle shaking of the root system, and the plants were placed on water-soaked filter paper and subsequently sealed in plastic. Bioluminescence on soybean roots was detected either by autophotography or by using a flexible light pipe (2, 7). For autophotographs, the soybean roots were sealed in plastic bags and subsequently exposed in the dark to a Kodak X-Omat RP XRP5 film for 3 to 5 h. The flexible light pipe equipped with a collimating beam probe was placed 1 to 2 mm above the root to collect the light emitted by the bacteria on the root. The light pipe was connected to an Oriel (Stratford, Conn.) digital display model 7070 with a photomultiplier tube model 77340 (2, 7). Measurements were performed in a dark room. The degree of colonization of the roots by the applied bacteria was determined after shaking 1-cm root pieces vigorously for 30 s in the presence of glass beads (100 to 200 μm in diameter) followed by dilution plating on King's B medium (6) supplemented with the appropriate antibiotics. In the sand-vermiculite mixture, *P. fluorescens* 5RL was able to colonize soybean roots at up to 10^5 CFU/cm of root (Fig. 1). Addition of naphthalene to the roots induced the bioluminescence genes in these bacterial cells. Autophotographs prepared after this induction showed the presence of the bioluminescent bacteria on the root system (Fig. 1). When the light pipe was used to measure the light emission on different spots on the root, signals from 0.1 to 20 nA were recorded (Fig. 1). This result showed that bioluminescent bacteria on the root can be detected both by using autophotography as well as by using the liquid light pipe.

Use of constitutively expressed *luxCDABE* reporter systems in the rhizosphere. In order to test whether bacteria with constitutive light production could be monitored on the root system, bacteria carrying constitutively expressed *luxCDABE* constructs were used. For plasmid-encoded constitutive bioluminescence, plasmid pLW1 was constructed. A random *Bgl*II digest of the chromosomal DNA of *Pseudomonas putida* M114 (Table 1) was screened for strong promoter activity in an expression vector (for details, see reference 13). After this screening, a 4.6-kb fragment that

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TABLE 1. Bacterial strains and plasmids

Strain (plasmid)	Characteristics of plasmid or strain	Reference
E. coli		
DH1(pUCD615)	*luxCDABE* promoter probe	10
HB101(pUCD623)	Suicide plasmid containing the *luxCDABE* transposon promoter probe Tn*4431*	11
1164(pLW1)	pUCD615, containing a constitutive promoter in front of *luxCDABE*	This study
WA803(pDB30)	Suicide plasmid containing Tn*5-lux*, a Tn*5* derivative in which the *luxAB* genes are expressed from the *neo* promoter	1
Pseudomonas spp.		
5RL	*P. fluorescens* 5R containing the naphthalene-inducible bioluminescence plasmid pUTK21	7
M114	*Pseudomonas* sp.	9
LT2-139	*P. putida* AD8-27 in which Tn*4431* is present behind a constitutive promoter	This study
PB2204	*P. putida*	2
WCS374	*P. fluorescens*	4

showed very strong, constitutive promoter activity in several bacterial backgrounds (*Escherichia coli*, *Rhizobium* spp., and *Pseudomonas* spp. [13]) was obtained. This fragment was cloned in front of the *luxCDABE* genes in pUCD615 (Table 1). The resulting constitutive bioluminescence plasmid, pLW1, was introduced in the root-colonizing *P. fluorescens* WCS374, causing a bright bioluminescent phenotype; 1 ml of a culture in LB (Luria-Bertani [8]) medium with an optical density at 620 nm of 0.25 emitted light signals reaching 24 nA when measured with the light pipe. Since loss of this plasmid from the WCS374 population in the rhizosphere was dramatic (80 to 90% of the cells had lost the plasmid after six days in the rhizosphere), we also used chromosomally encoded bioluminescence genes. Chromosomally encoded bioluminescence was obtained by using transposon Tn*4431*, which contains the promoterless *luxCDABE* cassette (11). *E. coli* HB101(pUCD623), carrying Tn*4431* on a suicide plasmid (11), was allowed to conjugate with the root-colonizing *P. putida* AD8-27. After this mating, tetracycline-resistant transposants were selected and bright bioluminescent phenotypes were elected from the selection plates and further characterized. Strain LT2-139 is such a Tn*4431* derivative with a bright constitutive bioluminescent phenotype. One milliliter of a culture in LB medium (optical density at 620 nm = 0.25) results in 340-nA signals as measured with the light pipe, which is at least 10-fold brighter than that of strain WCS374(pLW1) (see above). The bioluminescent ability of strain LT2-139 was observed under various growth conditions (e.g., on minimal salt and rich media as well as on iron- and phosphate-poor media). Furthermore, the insertion of the transposon into the chromosome of strain LT2-139 affected neither the growth nor the root-colonizing ability of the strain (data not shown). Soybean plants were inoculated with either LT2-139 or WCS374(pLW1). Detection of bioluminescence by using autophotographs with seedlings (up to 5 days old) or larger root systems (plants 9 to 14 days old [Fig. 2]) was unsuccessful. However, after addition of nutrients in the form of LB medium, the root system inoculated by either of these strains showed intense black root patterns on the autophotographs (data not shown). Addition of *n*-decyl aldehyde, the substrate for luciferase, resulted in some black spots on the autoradiogram of the roots, but only when the plants had been inoculated with strain LT2-139. Using the light pipe, light signals in the nanoampere range were detected in the presence of *n*-decyl aldehyde on those root parts on which at least 10^5 CFU/cm of root were present (data not shown). The lack of light detection on plants inoculated with WCS374(pLW1) will in part be due to disappearance of the plasmid pLW1 from the population of WCS374 cells, since only 10 to 20% of the cells recovered from the roots contained the bioluminescence plasmid.

When LT2-139 cells from the rhizosphere were resuspended in LB medium and the bioluminescence of this suspension was monitored in time, the bioluminescence appeared to increase gradually. In contrast, addition of

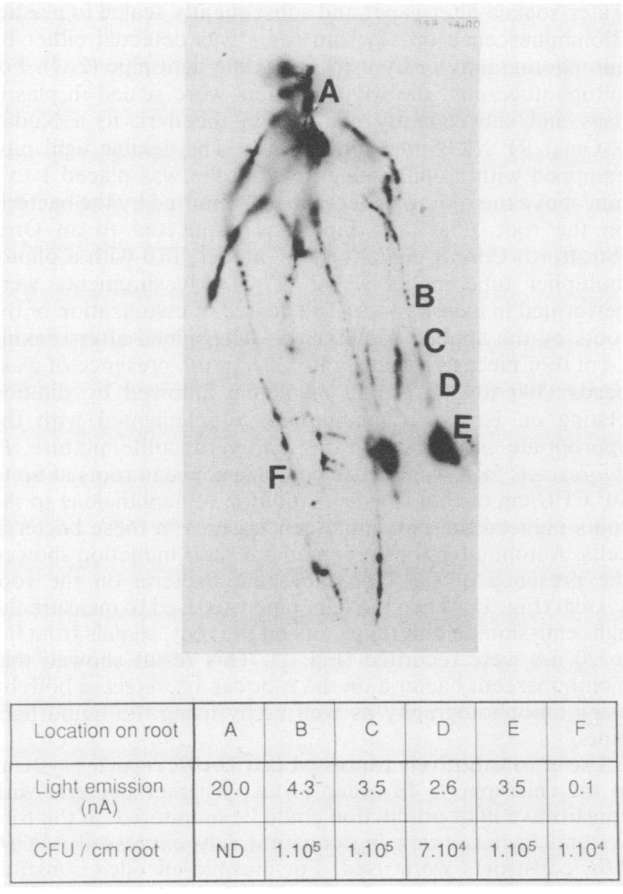

FIG. 1. Naphthalene-inducible bioluminescence in *P. fluorescens* 5RL detected by autophotography. The letters are placed next to locations on the roots where light pipe measurements were performed and where 1-cm root pieces were taken to determine the number of CFU present, as described in the text.

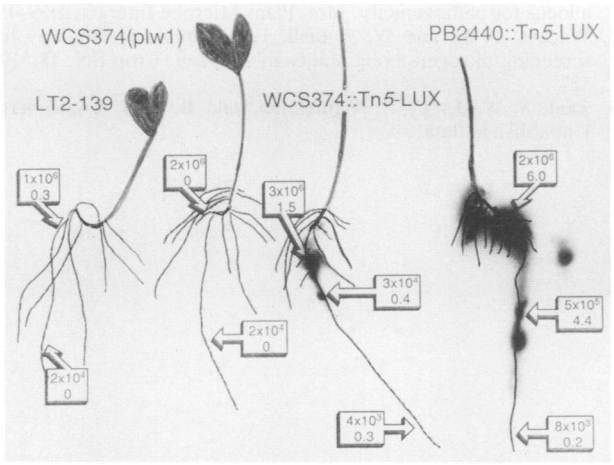

FIG. 2. Autophotograph of roots inoculated with cells containing a *luxCDABE* construct [LT2-139 and WCS374(pLW1)] and a *luxAB* construct (WCS374::Tn*5*-*lux* and PB2440::Tn*5*-*lux*). Arrows point to spots on the roots on which light pipe measurements were performed (numbers are given at the bottom of the squares in nanoamperes) and on which the number of CFU was determined by sampling 1-cm root pieces as described in the text (numbers are given at the top of the squares in CFU per centimeter of root.

n-decyl aldehyde to the suspension resulted in a sudden increase which faded again after 15 to 20 min. These findings suggest that the low bioluminescence activity of cells in the rhizosphere is reduced primarily because of a lack of the energy-demanding aldehyde substrate for the bioluminescence reaction. Most likely the cells in the rhizosphere are not able to synthesize enough of the aldehyde substrate for a bright bioluminescence reaction. Therefore, we examined cells which do not have the energy demand of the synthesis of the long-chain aldehyde. This was done by constructing cells which produce the luciferase (encoded by the *luxAB* genes) but not the enzymes involved in the synthesis of the aldehyde substrate (*luxCDE*).

Use of a *luxAB* reporter system in the rhizosphere. A Tn*5* derivative which contains the *luxAB* genes of *Vibrio harveyi* under the control of the constitutively expressed *neo* promoter (1) was introduced into both *P. fluorescens* WCS374 and *P. putida* PB2440 (Table 1). After addition of *n*-decyl aldehyde, the resulting strains produced bright bioluminescence on plates. Logarithmic cultures of both these strains showed similar bioluminescence characteristics; after addition of aldehyde to 1 ml of a culture (optical density at 620 nm = 0.25), signals ranging from 250 to 300 nA were recorded with the light pipe. Either of these strains could be detected on soybean roots upon addition of *n*-decyl aldehyde on autophotographs as well as by using the light pipe (Fig. 2). The signals obtained with the light pipe are positively correlated with the number of bacteria present on the root; on root parts containing approximately 10^5 to 10^6 CFU/cm of root, the light pipe registered signals of 1 to 10 nA, while signals of 0.1 to 1.0 nA were detected on root parts on which 10^3 to 10^4 CFU/cm of root were present.

The results described here suggest that the constitutive expression of bioluminescence (*luxCDABE* behind constitutive promoters) does not occur at high levels in the rhizosphere. This is most likely because of the high energy demand on these cells to synthesize the aldehyde substrate continuously. Therefore, these cells cannot be detected by the techniques used in this study without the addition of nutrients. However, the addition of nutrient solutions influences the distribution and numbers of cells in the rhizosphere and would therefore probably bias the results. Sensitive detection of cells without addition of nutrients can be achieved by using (i) cells in which the *luxCDABE* genes are induced just prior to detection as shown by the use of the naphthalene-inducible bioluminescent strain 5RL or (ii) cells in which only *luxAB* genes are expressed (WCS374::Tn*5*-*lux* or PB2440::Tn*5*-*lux*), resulting in cells that can only produce light upon the addition of the substrate for luciferase. These cells can be detected on roots by optical light measurement techniques and by autophotography. The latter clearly shows the distribution of the cells along the root system. Furthermore, autophotography demonstrates which sites are colonized preferentially by the applied bacteria, such as the root tips (Fig. 1) and the sites at which lateral roots emerge (Fig. 2). Such observations are extremely valuable for studies involving the dynamics of bacterial adhesion to, and colonization of, plant roots. The minimum number of cells required for detection is 10^3 to 10^4 CFU/cm of root, which makes bioluminescence at least 1,000-fold more sensitive than β-galactosidase-based systems (3).

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REFERENCES

1. **Boivin, R., F. P. Chalifour, and P. Dion.** 1988. Construction of a Tn*5* derivative encoding bioluminescence and its introduction in *Pseudomonas*, *Agrobacterium*, and *Rhizobium*. Mol. Gen. Genet. **213**:50–55.
2. **Burlage, R. S., G. S. Sayler, and F. Larimer.** 1990. Monitoring of naphthalene catabolism by bioluminescence with *nah-lux* transcriptional fusions. J. Bacteriol. **172**:4749–4757.
3. **De Weger, L. A., L. C. Dekkers, and B. J. J. Lugtenberg.** Use of reporter bacteria for studying the availability of phosphate in the rhizosphere. *In* C. Keel, B. Knoller, and G. Défago (ed.), Plant growth-promoting rhizobacteria. Proceedings of the International Workshop on Plant Growth-Promoting Rhizobacteria, October 1990, Interlaken, Switzerland. International Organization for Biological and Integrated Control of Noxious Animals and Plants, in press.
4. **Geels, F. G., and B. Schippers.** 1983. Selection of antagonistic fluorescent *Pseudomonas* spp. and their persistence following treatment of seed potatoes. Phytopathol. Z. **108**:193–206.
5. **Gutterson, N., W. Howie, and T. Suslow.** 1990. Enhancing efficiencies of biocontrol agents by use of biotechnology, p. 749–765. *In* R. Baker and P. Dunn (ed.), New direction in biological control: alternatives for suppressing agricultural pests and diseases. Alan R. Liss, Inc., New York.
6. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. **44**:301–307.
7. **King, J. M. H., P. M. Digrazia, B. Applegate, R. Burlage, J. Sanseverino, P. Dunbar, F. Larimer, and G. S. Sayler.** 1990. Rapid, sensitive bioluminescent reporter technology for naphthalene exposure and biodegradation. Science **249**:778–781.
8. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **O'Gara, F., P. Treacy, M. O'Sullivan, and P. Higgins.** 1986. Biological control of phytopathogens by *Pseudomonas* spp: genetic aspects of siderophore production and root colonization, p. 331–339. *In* T. Swinburne (ed.), Iron siderophores and plant diseases. Plenum Publishing Corp., New York.

10. **Rogowsky, P. M., T. J. Close, J. A. Chimera, J. J. Shaw, and C. I. Kado.** 1987. Regulation of the *vir* genes of *Agrobacterium tumefaciens* plasmid pTiC58. J. Bacteriol. **169**:5101–5112.
11. **Shaw, J. J., L. G. Settles, and C. I. Kado.** 1987. Transposon Tn*4431* mutagenesis of *Xanthomonas campestris* pv. *campestris*: characterization of a nonpathogenic mutant and cloning of a locus for pathogenicity. Mol. Plant-Microbe Interact. **1**:39–45.
12. **Wacket, T. J., and W. J. Brill.** 1976. Simple rapid assay for screening nitrogen fixing ability in soybean. Crop Sci. **15**:519–523.
13. **Zaat, S. A. J., C. A. Wijffelman, and B. J. J. Lugtenberg.** Unpublished data.