Microb Releases (1993) 1:203–207

Microbial Releases

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Detection of genetically engineered bioluminescent pseudomonads in potato rhizosphere at different temperatures

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Abstract. Six strains of pseudomonads were genetically engineered to bioluminesce. Following potato seed-piece inoculation, enumeration, and distribution of bioluminescent (Lux^+), pseudomonads were evaluated in the rhizosphere 1 month after seeding. Plants were grown in nonsterile field soil at 12, 20, and 28° C in growth chambers. When colonies were seen on plates, decyl-aldehyde was added and Lux^+ pseudomonads started to emit light. The enumeration of Lux^+ pseudomonads was possible using dilution plating on a general medium (TSA·B), since the Lux^+ pseudomonads represented most of the bacterial population recovered from the rhizosphere, and sensitivity of counts was slightly enhanced by using a selective medium (PAF·Kan·B). Rhizosphere colonization by Lux^+ pseudomonads decreased from 6.4 and 6.3 log [(cfu/g root dry weight) + 1] at 12° C to 5.7 and 5.6 at 20° C and to 5.4 and 5.5 at 28° C on TSA·B and PAF·Kan·B medium, respectively. The distribution of Lux^+ pseudomonads along the root was observed by embedding roots into agar. At 12° C, entire root systems were colonized, but only the upper part of the root system was colonized at 20 and 28° C. Proportion of roots colonized by Lux^+ pseudomonads decreased from 7.1 to 1.6 to 0.004% with increasing temperature of 12, 20, and 28° C. These results demonstrate that the *luxAB* genes are a useful bacterial marker to study root colonization in nonsterile soil.

Key words: Bioluminescence – Genetic marker – Rhizosphere colonization – *Pseudomonas putita* – *P. fluorescens*

Introduction

Root-colonization studies are limited by the lack of adequate bacterial markers, especially when nonsterile soil is used (Kloepper and Beauchamp 1992). Colony morphology, intrinsic antibiotic profiles, spontaneous antibiotic resistance, immunofluorescence, and DNA hybridization can be used to recover introduced strains. Under some conditions, colony morphology, antibiotic resistance, and immunofluorescence reactions may be altered after rhizobacteria are introduced in soil. Impurities can affect the quality of immunofluorescence reactions and DNA hybridizations, in turn affecting the accuracy of the enumeration.

The use of novel phenotypes may allow better recovery of introduced rhizobacteria (Drahos et al. 1986; Kluepfel et al. 1991). Bioluminescence, which is the production of light by a biological system, can be used to mark bacteria (Meighen 1991). Several genes involved in bioluminescence have been cloned and used to transform various gram-negative and gram-positive bacteria (Meighen 1991). Light production is catalyzed by the luciferase enzyme which oxidizes $FMNH_2$ and a long-chain fatty acid. A Tn*5-LuxAB* has been constructed which carries luciferase genes from *Vibrio harveyi* (Boivin et al. 1988). The *luxCDE* genes, encoding the production of a long fatty acid, are absent on this Tn*5* derivative, thereby decreasing the metabolic load for engineered bacteria. When *luxAB*-engineered (Lux^+) bacteria are exposed to an aldehyde, this compound can cross the membrane freely, allowing the bacteria to bioluminesce in the dark (Legocki et al. 1986).

Most luminous bacterial species are from marine environments; an exception is the terrestrial *Xenorhabdus luminescens* (Meighen 1991). *X. luminescens* normally lives in symbiosis with insect-pathogenic nematodes (Meighen 1991), but it is absent in soil bacterial population (Colepicolo et al. 1989). Therefore, bioluminescence is a useful marker for terrestrial bacteria, allowing the visualization of Lux^+ bacteria on roots and in N_2-fixing nodules without interference from indigenous bacteria (de Weger 1991; Fravel et al. 1990; O'Kane et al. 1988).

The main objective of this study was to evaluate the use of the *luxAB* genes as a marker for root-colonizing bacteria when plants were grown in nonsterile field soil. The effect of temperature on the population dynamics

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of engineered Lux$^+$ pseudomonads was followed at 12, 20, and 28° C in the potato (*Solanum tuberosum* L.) rhizosphere using dilution plating. The numbers of Lux$^+$ pseudomonads recovered on selective and nonselective media were compared, and their distribution on roots was evaluated by embedding roots in agar.

Materials and methods

Bacterial strains

Pseudomonas putida strains 61.9A, 86.139, GR7.4, and GR20.5, and *P. fluorescens* strains 17.148 and GR20.3 were supplied by Esso Ag Biologicals (Saskatoon, Canada). Rhizobacteria were maintained for long-term storage at −80° C in *Pseudomonas* broth (PB) (tryptone 10 g, peptone 10 g, K_2HPO_4 1.5 g, $MgSO_4$ 1.5 g, and glycerol 10 g per liter) plus 15% glycerol. Except where noted, strains were routinely grown on *Pseudomonas* Agar F (PAF; Difco Laboratories, Detroit, Mich.). All strains but 86.139 were previously selected as plant-growth-promoting rhizobacteria (PGPR) on rapeseed or soybean (Kloepper et al. 1988; Polonenko et al. 1987). *Escherichia coli* strain WA803 (pDLB30) carrying the Tn*5-luxAB* genes of *V. harveyi* was used as the donor, and *Pseudomonas* sp. strain RB100C as a reference transconjugant pseudomonad (Boivin et al. 1988). These two strains were obtained from Dr. P. Dion (Département de Phytologie, Faculté des Sciences de l'Agriculture et de l'Alimentation, Université Laval, Ste-Foy, Quebec).

For all these bacteria, resistance to carbenicillin and ampicillin (50 µg/ml each) was determined on PAF medium. Pseudomonad strains had intrinsic resistance to these antibiotics, whereas *E. coli* strain WA803 (pDLB30) was sensitive.

Marking bacteria with luxAB

The donor *E. coli* strain WA803 (pDLB30) was maintained on nutrient agar (Difco Laboratories, Detroit, Mich.) supplemented with 2 g/l yeast extract (NAYE), 25 µg/ml chloramphenicol, and 12 µg/ml kanamycin. *E. coli* was grown on nutrient broth plus yeast extract (NBYE) and antibiotics. The recipient pseudomonad strains were grown in NBYE. After 16 h growth at room temperature, 0.1 ml of both bacterial suspensions were filtered through 0.20-µm sterile nitrocellulose filters. The bacteria on the filter were washed twice with 2 ml of 0.1 M $MgSO_4$, and the filters harboring bacteria were placed on NAYE for 24 h. Cells from the filters were suspended in 10-ml and 100-µl samples were then placed on PAF plus 50 µg/ml kanamycin (PAF·Kan) for the first conjugation assay. Transconjugants growing on PAF·Kan were selected for fluorescent pigments under UV ligth (254 nm) and their luminescence in the dark. Bioluminescence was observed after the addition of 100 µl of *N*-decyl-aldehyde (Sigma, St. Louis, Mo.) to the inside of the petri dish lid. Intrinsic resistance to carbenicillin (PAF·Kan plus 50 µg/ml carbenicillin at pH 7.2) and ampicillin (PAF·Kan plus 50 µg/ml ampicillin at pH 5.5) in recipient cells was used to eliminate the *E. coli*; however, several transfer cycles were required to purify pseudomonads designated 89.139L and GR20.3L, respectively.

In a second trial, spontaneous mutants resistant to rifampicin and nalidixic acid (50 µg/ml each) were selected before conjugation. After conjugation, transconjugants were selected on PAF·Kan plus rifampicin, and streaked on PAF·Kan plus nalidixic acid. The colonies were free of *E. coli* after two purification cycles. The transconjugants were designated 17.148L, 61.9AL, GR7.4L, and GR20.5L. All mutants were selected to have a generation time similar to the wild type in PB.

Growth chamber experiment

The marked strains were grown for 24 h in PB at 27° C. Bacteria were washed twice in M buffer [0.2 mM K_2HPO_4-KH_2PO_4, 140 mM NaCl, 1 mM $MgSO_4$, 0.15 mM $MnCl_2$, 0.5 mM $CaCl_2$; pH 7.2 (Dazzo and Brill 1978)], and the optical density was adjusted to 1.0 (A_{660}). The potato cultivar 'Superior' (Elite class 1) was provided by C. Parent (Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec, Centre de production de pomme de terre de semence, Pointe-aux-Outardes, Québec). Seed pieces were cut from the tuber with a 3-cm-diameter melon-ball scoop and contained only one eye. After suberization at room temperature for 12–24 h, seed pieces were soaked in 1 ml of bacterial suspensions of strains 17.148L, 61.9AL, 89.139L, GR7.4L, GR20.3L, and GR20.5L per tuber for 20 min. For controls, seed pieces were dipped in M buffer without bacterial suspension. One seed piece was planted per 10-cm-diameter pot containing 500 g of nonsterile field soil (loamy sand, pH 6.0) adjusted to 75% of field capacity. The soil moisture was kept at about 75% field capacity with water provided from below. The pots were placed in growth chambers at 12, 20 and 28° C with a 16-h photoperiod. The experimental design was a split-plot design, with temperatures as the main-plot factor and bacterial treatments as the subplot factor. Eight replicates were used. One month after seeding, root systems were removed carefully from the soil and vigorously shaken to dislodge loosely adhering soil. Roots were placed in plastic bags on ice until they were processed.

Rhizosphere populations

For even replicates (i.e., four of eight), the root systems were placed in 100 ml M buffer, agitated at 200 rpm for 30 min, and dilutions were prepared. Roots were then dried for 48 h at 65° C and weighed. Dilution plating was performed on Tryptic Soy Agar (Difco Laboratories, Detroit, Mich.) plus 100 µg/ml benomyl [Benlate 50WP, DuPont, Canada (TSA·B)] and PAF·Kan supplemented with benomyl (PAF·Kan·B). The cultivatable, aerobic bacterial population on roots was evaluated on TSA·B and PAF·Kan·B and is referred to as the total bacterial population. The Lux$^+$ pseudomonad populations were counted in the dark on TSA·B and PAF·Kan·B after exposure to decyl-aldehyde vapor. The bacterial counts were performed on plates with 30–300 CFU, and counts of less than 30 CFU were considered as missing values in the analyses.

Distribution of Lux$^+$ pseudomonads on roots

For odd replicates, each root system was dissected into its numerous fibrous roots with sterilized forceps and scalpel. These roots were embedded in molten PAF·Kan·B to identify the number of roots colonized by the marked strains. Bacteria were allowed to grow for 3 days for the bacterial Lux$^+$ counts or for 5 days for embedded roots. Plates were exposed to decyl-aldehyde vapor, and luminescent root length was measured.

Data analysis

All data were tested for homogeneity of variances (Little and Hills 1978) using Bartlett's test (Steel and Torrie 1980) prior to the analysis of variance. Homogeneity of variances was obtained by using the $\log_{10}$ [(cfu/g root dry weight) + 1] transformations. The addition of 1 was required to avoid log(0) in the analysis (Gomez and Gomez 1984). The analyses of variance were performed using the General Linear Models (GLM) procedure of SAS software (SAS Institute, Cary, N. C.). The GLM procedure was used since there were unequal numbers of observations for Lux$^+$ pseudomonads counts due to treatments with less than 30 Lux$^+$ CFU.

Results

Marking bacteria with luxAB

Conjugation of pseudomonads with WA803(pDLB30) resulted in the production of luminescent mutants. These marked strains emitted light after the addition of decyl-aldehyde as observed visually in a darkroom. For the reference transconjugant strain RB100C and the other pseudomonads, from 1 to 15 colonies were detected per plate derived from the bacterial suspension without dilution, indicating that the frequency of transconjugant per recipient cell was equivalent to 1×10^{-6}. The introduced *luxAB* genes in pseudomonads appeared stable since after more than 20 passages on PAF medium, none of the mutants had lost its ability to emit ligth.

Growth chamber experiment

Rhizosphere populations. No light-emitting bacteria were detected on TSA·B and PAF·Kan·B media prior to addition of decyl-aldehyde. The only bioluminescent bacteria were pseudomonads. When there were too many colonies per plate, only diffuse light was detected from bioluminescent colonies.

Total bacterial population was higher on TSA·B than on PAF·Kan·B, but bioluminescent bacterial populations were similar on both media. Increased temperature did not significantly ($P<0.05$) affect total bacterial populations on TSA·B and PAF·Kan·B (Figs. 1, 2). On average, the log [(cfu/g root dry weight) + 1] of the total bacterial populations were between 7.4 and 7.7 on TSA·B and between 6.7 and 7.0 on PAF·B at tested temperatures. In contrast, bioluminescent strains were significantly affected by increasing temperatures (strain and temperature $P<0.001$: TSA·B; Strain*Temperature $P<0.001$: PAF·Kan·B) (Figs. 1, 2). The magnitude of decrease in Lux^+ populations with increased temperatures was different among strains. On TSA·B and PAF·Kan·B, respectively, mean populations of Lux^+ rhizobacteria decreased from 6.4 and 6.3 log [(cfu/g root dry weight) + 1] at 12° C to 5.7 and 5.6 at 20° C and to 5.4 and 5.5 at 28° C. The higher log [(cfu/g root dry weight) + 1] reflected the lower root weights at 12° C.

Distribution of Lux^+ pseudomonads on roots. Embedding the roots into molten agar allowed the growth of bioluminescent bacteria, and when plates were exposed to decyl-aldehyde it was possible to visually identify colonized roots (Fig. 3). At 12° C, most roots were entirely colonized by the Lux^+ pseudomonads. At 20 and 28° C, the first 5–8 cm of the roots proximal to the seed-piece were frequently colonized, whereas root tips were less frequently colonized. The percent of roots colonized by the Lux^+ pseudomonads was significantly affected by the temperatures ($P<0.001$). On average, the number of roots colonized by Lux^+ pseudomonads decreased linearly ($P<0.01$) from 84% to 57% and 29% at 12, 20, and 28° C, respectively.

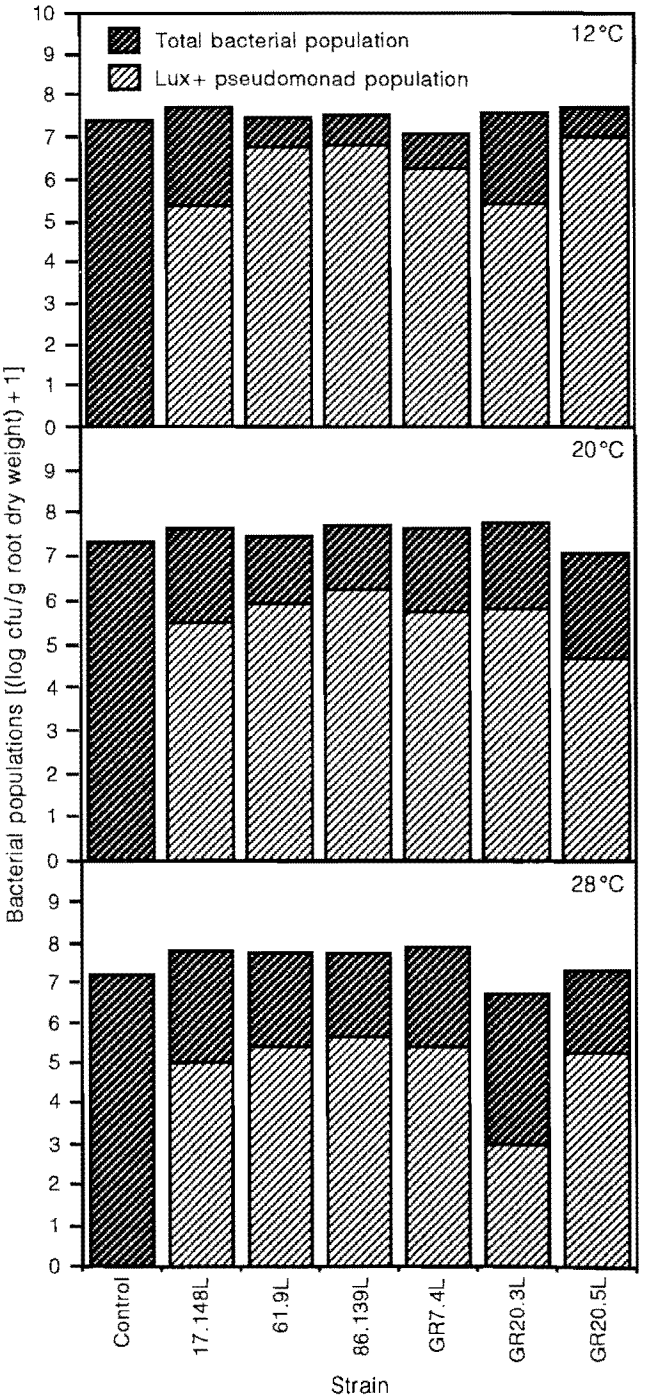

Fig. 1. Total bacterial and Lux^+ pseudomonad populations on TSA·B at 12, 20, and 28° C

Discussion

These results suggest a new approach for studying root colonization in nonsterile soil. This bioluminescent approach is limited to microorganisms without an intrinsic ability to emit light, and thus should be useful for almost all soil bacteria. Because of its simplicity, this technique is suitable for specific recovery of an introduced strain without being time-consuming and requiring specialized equipment. In a dark room, bioluminescence

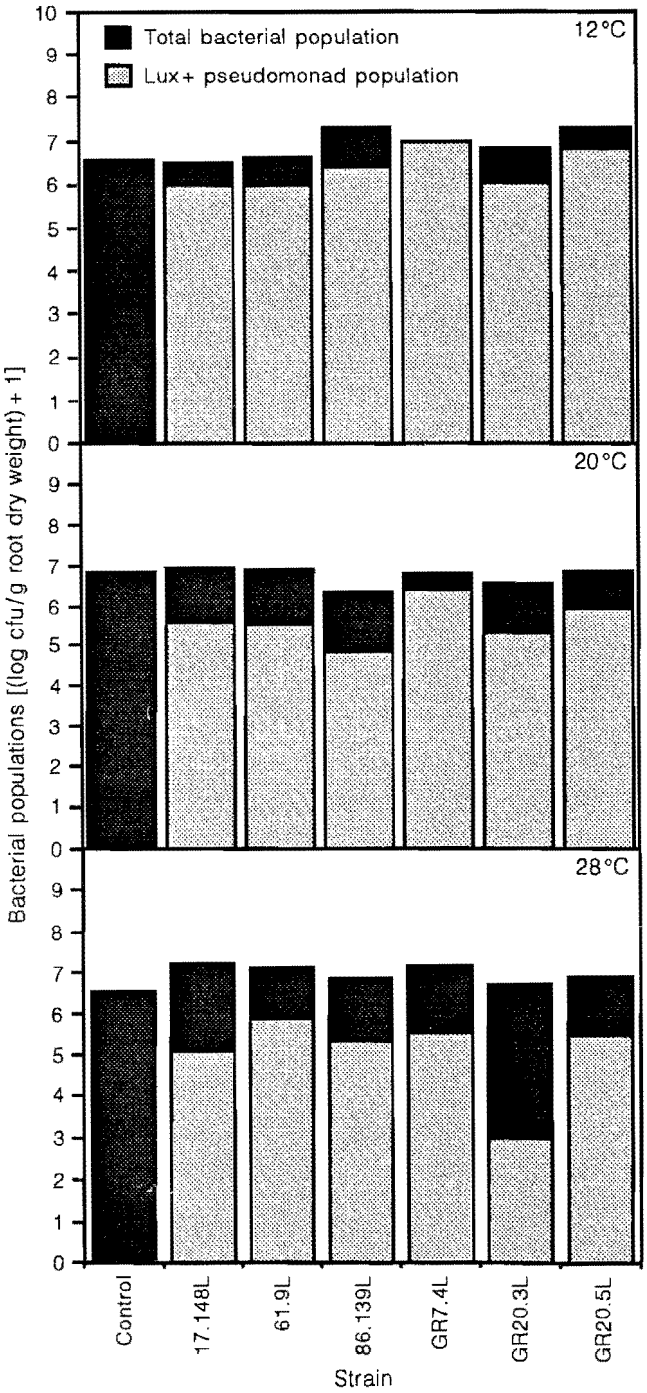

Fig. 2. Total bacterial and Lux$^+$ pseudomonad populations on PAF·Kan·B at 12, 20, and 28°C

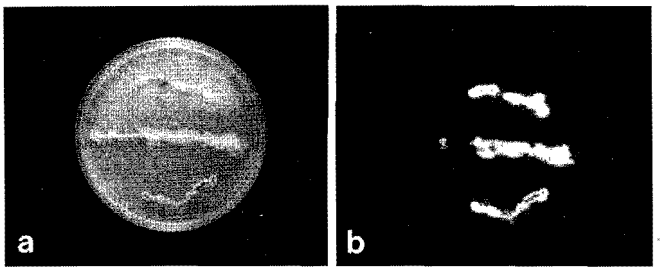

Fig. 3a, b. Pictures (35 mm) of strain GR7.4L growth from potato root embedded in PAF·Kan·B medium. View illuminated by incident light (**a**) and by bacterial bioluminescence (**b**)

can be observed from actively growing colonies after the addition of an aldehyde. Quantitative information can be obtained by counting bioluminescent colonies, whereas qualitative information can be obtained by embedding roots in molten agar followed by visualization of the bioluminescent outgrowth. Photographs of luminescent bacteria can be taken using 35-mm film.

Root-colonization studies require the selective recovery of introduced bacteria from the indigenous rhizosphere population. The most frequently used marking system employs antibiotic-resistant mutants (Bahme and Schroth 1987; Juhnke et al. 1987; Kloepper et al. 1980; Kluepfel et al. 1991). Similarly, in studies with E6(pUCD607) (Fravel et al. 1990) and Mud*lac*-engineered strains (Seong et al. 1991), only mutants were used. In fact, with nonsterile soil, it would have been difficult, if not impossible, to selectively recover wild-type rhizobacteria with tested media. This is particularly true for strains 86.139L and GR20.3L which are not marked with antibiotic resistance. Fravel et al. (1990) were not able to selectively recover wild-type *Enterobacter cloacae* strain E6 among other microflora, because too many bacteria were able to grow on Luria agar amended with ampicillin. However, the bioluminescent derivative E6(pUCD607) was recovered on Luria agar amended with ampicillin and kanamycin.

In contrast to the use of antibiotic-marked strains, Lux$^+$ pseudomonads can be identified and counted on a general medium at appropriate dilutions. However, the bacterial background may influence the detection of the Lux$^+$ bacteria, since luminescence is dependent on the physiological stage of the bacteria (Shaw et al. 1987). TSA·B full strength was used for total bacterial counts to allow good growth of Lux$^+$ bacteria and better bioluminescent counts. The addition of kanamycin and benomyl to the PAF medium decreased the bacterial and fungal background and improved the recovery of the Lux$^+$ pseudomonads. Under these optimal conditions counts were easily performed visually in a dark room and engineered pseudomonads were at a comparable physiological stage and under more uniform growth conditions resulting in similar light production.

The Tn*5-luxAB* was a stable marker in pseudomonads during the course of this study. Transfer of *luxAB* genes to other rhizosphere inhabitants was not observed. Only colonies with a typical pseudomonad phenotype were observed to bioluminesce after decyl-aldehyde addition. Similarly, in a field release of a Tn*5* *P. fluorescens* derivative, Bakker et al. (1991) were not able to detect Tn*5* transfer to other pseudomonads.

Rhizosphere colonization is affected by temperature (Davies and Whitbread 1989; Loper et al. 1985; Roult et al. 1963; Seong et al. 1991). Temperature affects plant growth and development, which in turn can alter the quantity and quality of the root exudates (Curl and Truelove 1986). This can influence the colonization of the root system. Moreover, temperature can influence

growth of the introduced strain and indigenous microorganisms (Stanier 1986), and the competition among rhizosphere microorganisms, creating complex soil-plant-microorganism interactions. At all the tested temperatures, the Lux^+ pseudomonads colonized the root system. The Lux^+ pseudomonad population constituted 7 and 24% of the total bacterial population at 12° C, which decreased to 2 and 8% at 20° C, and to 0.4 and 3% at 28° C on TSA·B and PAF·Kan·B, respectively. High temperatures have been previously shown to decrease pseudomonad survival in the potato rhizosphere (Loper et al. 1985). In contrast, Seong et al. (1991) reported that a strain with optimal growth at 19° C more effectively colonized the maize rhizosphere at 18° C than a strain having an optimal growth temperature of 28° C. None of the Lux^+ pseudomonads tested in this study had a higher rhizosphere population at 28° C than at 12° C. This could be explained by the arctic origin of the wild-type rhizobacteria (Lifshitz et al. 1986).

Tn*5-luxAB* derivatives offer an alternative to spontaneous antibiotic-resistant mutants as a selective marker in microbial ecology studies. In addition, bioluminescent genes are relatively harmless and can be used for assessing risks involved in field releases of genetically engineered bacteria as proposed by Shaw et al. (1992).

Acknowledgements. We thank H. Bisonnette for technical assistance. We are grateful to all those who provided us with bacterial cultures. This work was supported by grants from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche and from the Ministère de l'Enseignement Supérieur et de la Recherche du Québec, "les actions structurantes". C. J. B. gratefully acknowledges the Natural Sciences and Engineering Research Council of Canada for a postgraduate scholarship.

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