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Construction of a Tn*5* derivative encoding bioluminescence and its introduction in *Pseudomonas, Agrobacterium* and *Rhizobium*

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Summary. A simple method based upon the use of a Tn*5* derivative, Tn*5*-Lux, has been devised for the introduction and stable expression of the character of bioluminescence in a variety of gram-negative bacteria. In Tn*5*-Lux, the *lux*AB genes of *Vibrio harveyi* encoding luciferase are inserted on a *Sal*I – *Bgl*II fragment between the kanamycin resistance (Km^r) gene and the right insertion sequence. The transposon derivative was placed on a transposition suicide vehicle by in situ recombination with the Tn*5* suicide vector pGS9, to yield pDB30. Mating between *Escherichia coli* WA803 (pDB30) and a strain from our laboratory, *Pseudomonas* sp. RB100C, gave a Km^r transfer frequency of 10^{-6} per recipient, a value 10 times lower than that obtained with the original suicide vehicle pGS9. Tn*5*-Lux was also introduced by insertion mutagenesis in other strains of gram-negative soil bacteria. The bioluminescence marker was expressed in the presence of n-decanal, and was monitored as chemiluminescence in a liquid scintillation counter. The recorded light intensities were fairly comparable among the strains, and ranged between 0.2 to 1.8×10^6 cpm for a cell density of 10^3 colony forming units/ml. Nodules initiated by bioluminescent strains of *Rhizobium leguminosarum* on two different hosts were compared for intensity of the bioluminescence they produced.

Key words: Bioluminescence – Tn*5* derivative – *Pseudomonas* sp. – *Agrobacterium* sp. – *Rhizobium* sp.

Introduction

The bioluminescence character has proved to be a useful marker for the localization and enumeration of plant-associated bacteria (Legocki et al. 1986, 1987; Shaw and Kado 1986, 1987). Bioluminescence can be detected in a non-disruptive manner either visually, photographically or with very sensitive electronic equipment (Shaw et al. 1987). The *lux*AB genes of *Vibrio harveyi,* first cloned by Belas et al. (1982) and almost simultaneously by Cohn et al. (1983), are among the available bioluminescence genes and code for two distinct subunits of a bacterial luciferase.

*Lux*AB genes have already been successfully expressed in *Bradyrhizobium japonicum,* after their introduction by recombination in the bacterial chromosome (Legocki et al. 1986, 1987). The other reports of introduction of *lux* genes in plant-associated bacteria have made use of the *lux*-ABCDE operon of *V. fischeri* that includes genes for aldehyde production by the fatty acid reductase pathway. This operon has been cloned in a broad-host-range plasmid, and then transferred to representatives of the genera *Agrobacterium, Rhizobium, Erwinia, Pseudomonas* and *Xanthomonas* (Shaw and Kado 1986). Promoterless transposons containing the *lux* genes, such as the mini-Mu *lux* of Engebrecht et al. (1985) or Tn*4431* of Shaw and Kado (1987), serve to monitor gene expression by introduction downstream of indigenous promoters. In several of these previous studies, kanamycin resistance (Km^r) was used as the positive selection marker for co-transfer of *lux* genes, with the latter placed upstream of the Km^r gene (Engebrecht et al. 1985; Legocki et al. 1986; Shaw and Kado 1986).

Although several Tn*5* derivatives have already been constructed in vitro, including some that contain mobilization genes, replication origins and genes determining antibiotic resistance or catabolic properties (De Vos et al. 1986; Krishnapillai et al. 1986; Walker and Pemberton 1987), to our knowledge no Tn*5* derivative encoding bioluminescence has yet been obtained. In this report, we describe cloning of the *lux*AB genes in Tn*5* downstream of the Km^r gene and promoter. The transposon derivative, named Tn*5*-Lux, has been used for the stable maintenance and constitutive expression of the character of bioluminescence in strains of *Pseudomonas, Agrobacterium,* and *Rhizobium* spp.

Materials and methods

Plasmids and bacterial strains. Plasmids used in this study are listed in Table 1. Plasmid pFIT001, which was the source of *lux* genes used in this study, is a derivative of pBR322 where the two contiguous sites for *Hin*dIII and *Eco*RI have been inactivated (Legocki et al. 1986). In pFIT001, the *lux*AB genes of the *V. harveyi* DNA insert are expressed constitutively from the "anti-tet" promoter of the tetracycline resistance (Tc^r) gene. The locations of *Sal*I and *Bgl*II sites on pFIT001 were mapped by restriction analysis. The relevant characteristics of the *Escherichia coli* strains used in this study are as follows; HB101 is streptomycin resistant (Sm^r) and auxotrophic for proline, leucine, thymine and thiamine, whereas WA803 is Sm^s and auxotrophic for methionine and thiamine (Selvaraj and Iyer 1983). Other bacteria used in this work are listed in Table 2.

Media and antibiotics. Ammonium sulfate-mannitol agar (AMA) was the minimal medium used to maintain strains of *Pseudomonas* and *Agrobacterium,* and consisted of SM-N

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Table 1. Plasmids used in this study

Plasmid	Size (kb)	Relevant phenotype[a]	Source or derivation
pBR322	4.4	Ap^r, Tc^r	Bolivar et al. (1977)
pDB20	3.0	Ap^r	Deletion derivative of pBR322, this work
pDB25	8.7	Ap^r, Km^r, Bl^r	Tn*5* insertion on pDB20, this work
pFIT001	8.2	Ap^r, Lux^+	Legocki et al. (1986)
pDB27	9.4	Ap^r, Km^r, Lux^+	Replacement of a *SalI* fragment of pFIT001, this work
pDB28	9.6	Ap^r, Km^r, Bl^s, Lux^+	Replacement of a *BglII* fragment of pDB25, this work
pGS9	30.5	Cm^r, Km^r, Bl^r, Tra^+	Selvaraj and Iyer (1983)
pDB30	31.4	Cm^r, Km^r, Bl^s, Lux^+, Tra^+	In situ replacement on pGS9 of Tn*5* by Tn*5*-Lux

[a] Ap, ampicillin; Tc, tetracycline; Km, kanamycin; Bl, bleomycin; Cm, chloramphenicol; Lux, bioluminescence; Tra, transfer ability

Table 2. Strains of *Pseudomonas, Agrobacterium* and *Rhizobium* spp. used in this study

Strain	Relevant phenotype		Source or derivation[b]
	Kanamycin resistance	Bioluminescence (relative light units)[a]	
Pseudomonas sp.			
RB100C	–		Boivin et al. (1987)
RB100C L	+	11.7±1.7	Mating with RB100C, selection on AMA[c] containing kanamycin (Km)
Pseudomonas putida			
CH183	–		Rossignol and Dion (1985)
CH183 L	+	14.2±1.3	Mating with CH183, selection on AMA[c] containing Km
Agrobacterium tumefaciens			
C58	–		J. Tempé, CNRS, Orsay, France
C58 L	+	6.4±0.54	Mating with C58, selection on YAA[c] containing Km
Agrobacterium rhizogenes			
A4	–		G. Bécard, Université Laval, Canada
A4 L	+	2.7±0.24	Mating with A4, selection on YAA[c] containing Km
ATCC 15834	–		G. Bécard, Université Laval, Canada
15834 L	+	9.0±0.81	Mating with ATCC 15834, selection on YAA[c] containing Km
Agrobacterium radiobacter			
K84	–		A. Kerr, Waite Agricultural Research Institute, Australia
K84 L	+	4.2±0.58	Mating with K84, selection on YAA[c] containing Km
Rhizobium meliloti			
A3	–		L.M. Bordeleau, Agriculture Canada
A3RIF	–		Spontaneous Rif^r mutant of A3 (C. Beaulieu)
A3RIF L	+	6.7±0.44	Mating with A3RIF, selection on YMA[c] containing rifampicin (Rif) and Km
3Doa20a	–		L.M. Bordeleau, Agriculture Canada
3Doa20a L	+	5.0±0.89	Mating with 3Doa20a, selection on YAA[c] containing Km
Rhizobium leguminosarum biovar *viceae*			
175B2	–		J. Burton, Nitragin, USA
175B2 L	+	5.5±0.41	Mating with 175B2, selection on YAA[c] containing Km
175F9	–		J. Burton, Nitragin, USA
175F9 L	+	9.3±0.58	Mating with 175F9, selection on YAA[c] containing Km
175F19	–		R.S. Smith, Nitragin, USA
175F19 L	+	4.6±0.72	Mating with 175F19, selection on YAA[c] containing Km

[a] Relative light units expressed as cpm obtained for a cell density of 10^3 colony forming units/ml bioluminescence medium and then divided by 10^5. Values are means of 5 replicates ±standard error

[b] *Escherichia coli* WA803 (pDB30) was used as the donor strain for the matings

[c] AMA, ammonium sulfate-mannitol agar; YAA, yeast extract-adonitol agar; YMA, yeast extract-mannitol agar

salts (Klapwijk et al. 1977), 0.5 g/l ammonium sulfate, 5 g/l mannitol and 15 g/l agar (Difco). Yeast extract-nutrient broth (YNB) and yeast extract-nutrient agar (YNA) were the liquid and solid complex media used for *E. coli* and contained 5 g/l yeast extract (Difco), and 8 g/l nutrient broth (Difco) or 23 g/l nutrient agar (Difco), respectively. Yeast extract-mannitol broth (YMB) was as described by Nelson and Child (1981) and was solidified when required by addition of 15 g/l agar to yield yeast extract-mannitol agar (YMA). Yeast extract-adonitol agar (YAA) was simi-

lar in composition to YMA except that mannitol was replaced with adonitol (10 g/l). When required, antibiotics were added at the following concentrations: 10 µg/ml tetracycline, 25 µg/ml chloramphenicol (Cm), 40 µg/ml ampicillin (Ap), 50 µg/ml bleomycin (Bl), rifampicin (Rif), or streptomycin (Sm) and 100 µg/ml kanamycin (Km). The medium used for expression of bioluminescence, named bioluminescence medium, consisted of filtered YMB medium containing 1 ppm n-decanal (n-decyl aldehyde) and was prepared as follows: YMB was filtered through two layers of Whatman No. 1 filter paper, autoclaved, and 1/10 volume of the same medium that had been saturated with 10 ppm n-decanal was then added. This saturation had been achieved by rotary shaking at 175 rpm at 27° C for 1 h in a closed container and autoclaving. The components of the bioluminescence medium were stored in the dark at 4° C and mixed only before use to minimize degradation and chemiluminescence.

Extraction and manipulation of plasmid DNA. Extraction of plasmid DNA was performed by the preparative method of Birnboim (1983) modified as previously described (Boivin et al. 1987). Digestion of plasmid DNA with restriction endonucleases and S1 exonuclease was done according to Maniatis et al. (1982). Gel electrophoresis was carried out on horizontal 0.7% agarose slab gels. Ligation conditions and procedures were essentially as described previously (Boivin et al. 1987). Transformation followed the general procedure of Gross and Vidaver (1981) with some modifications (Boivin et al. 1987).

Bacterial matings. The donor strains, *E. coli* WA803 (pDB30) or WA803 (pGS9), were grown in YNB containing Cm for 16 h at 27° C. The recipient strains were grown at 27° C in YNB (*E. coli* HB101, *Pseudomonas* and *Agrobacterium* spp.) or YMB (*Rhizobium* sp.) for 12 to 36 h depending on growth rate. Mating between ca. 10^9 colony forming units (cfu) of donor and recipient was done on membrane filters placed at the surface of a YNA plate when recipients were *Pseudomonas* and *Agrobacterium* spp., or a YMA plate for *Rhizobium* sp. recipients. After 16 h at 27° C, bacteria were resuspended in saline and 1/50, or 1/1000 in the case of *Pseudomonas* sp. RB100C, of the suspension was plated on the appropriate selective medium containing Km. For matings between *E. coli* strains, incubation was for 4 h at 34° C and 1/50000 of the suspension was plated on the selective medium. Different strategies were used to counterselect the donor; *Pseudomonas* transconjugants were selected on minimal medium, while selection was done in the presence of streptomycin for *E. coli* HB101, rifampicin for *R. meliloti* A3RIF, or on the basis of adonitol utilization for the other strains.

Expression of bioluminescence by free-living bacteria. Expression of bioluminescence was detected either visually on plates or in liquid medium with an electronic scintillation counter. Visual observation on plates was used to identify bioluminescent colonies and involved placing a drop of n-decanal on the inside of the petri dish lid and viewing in the dark. To estimate bioluminescence in liquid medium, cells were prepared by growth to mid-exponential phase in filtered YMB medium. Bacterial cultures were diluted in saline and plated on YNA (*Pseudomonas* and *Agrobacterium* spp.) or YMA (*Rhizobium* spp.) to estimate cell density in cfu. The same cultures that were used to estimate density were diluted 100-fold, and 2–10 µl of the dilution was added to 15 ml bioluminescence medium in a glass scintillation vial. The vial was shaken immediately and, after a standardized time of 5 min, was introduced in the counting chamber of a liquid scintillation counter (LKB model 1217) operating in the chemiluminescence mode. Background chemiluminescence was substracted from the values obtained. In each individual experiment, bioluminescence yielded by a given strain was estimated from the cpm measurements obtained with suspensions diluted to an approximate cell density of 10^3 cfu/ml (from about $0.5–2.0 \times 10^3$ cfu/ml). Bioluminescence values normalized to a cell density of 10^3 cfu/ml were obtained in 5 independent experiments for each strain.

Plant culture and recovery of bioluminescence from nodules. Three-day-old cultures of *R. leguminosarum* biovar *viceae* strains 175B2 L, 175F9 L and 175F19 L were inoculated on surface-sterilized seeds of pea (*Pisum sativum* L.) cv. Homesteader and faba bean (*Vicia faba* L.) cv. Outlook, sown in sterilized, modified Leonard Jar assemblies as described by Chalifour and Nelson (1987). The plants were supplied with the nitrogen (N)-free nutrient solution of Chalifour and Nelson (1988). The same procedure was followed for inoculation of *R. meliloti* strains A3RIF L and 3Doa20a L on seeds of alfalfa (*Medicago sativa* L.) cv. Saranac. Plants were grown at room temperature under fluorescent lights at a photosynthetic photon flux density of 300 µmol/m^2 per s with a 16 h photoperiod. Twenty four days after sowing, plants were harvested one at a time, and 5 nodules selected randomly were collected from each root system and weighed without delay. The nodules were crushed for 30 sec with a glass rod, while soaking in a drop of N-free solution; then, 10 ml of N-free solution was added and used to rinse the glass rod. Ten microliters of the suspension was added to 15 ml bioluminescence medium and bioluminescence was measured as described above.

Chemicals and enzymes. Antibiotics and n-decanal were purchased from Sigma Chemical Co. Restriction endonucleases, S1 exonuclease and T4 DNA ligase were obtained from Boehringer Mannheim Biochemicals.

Results

Construction of Tn5-Lux and replacement on a transposition vector

The various steps in construction of Tn*5*-Lux were as follows (see also Fig. 1). In pDB20, a DNA segment has been deleted from pBR322 to remove the unique restriction sites for *Hin*dIII, *Sal*I, *Bam*HI, and *Ava*I. Insertion of Tn*5* on pDB20 to yield pDB25 was achieved by introduction of pGS9 in HB101 (pDB20) by mating, subculturing with selection for both plasmids and then selection for Ap^r only to allow the loss of pGS9. The culture was finally diluted and plated on YNA containing Ap and Km. One of the Km^r, Cm^s colonies contained a plasmid, pDB25, with a single Tn*5* insertion and was very stable in its monomer form, in contrast with other pBR322 deletion derivatives carrying Tn*5* that have been obtained in this work, or described by De Vos et al. (1986). Restriction analysis of pDB25 indicated that Tn*5* had inserted between the Ap^r gene and the replication origin of pDB20, at position 1.5 kb starting from the *Eco*RI site. In pDB25, the only sites for *Hin*dIII, *Bgl*II, *Sal*I, *Bam*HI, *Sma*I and *Ava*I were

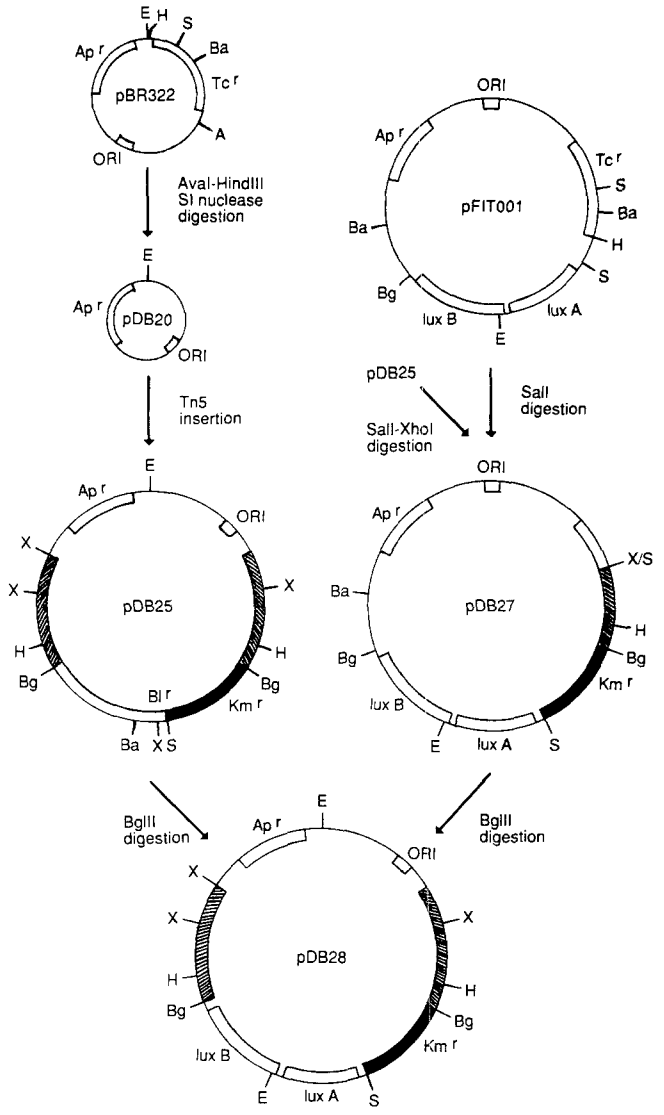

Fig. 1. Construction of Tn*5*-Lux. Methods for extraction and manipulation of plasmid DNA have been described previously (Boivin et al. 1987). *Black* and *hatched* segments represent the kanamycin resistance gene (Km^r) and insertion sequences, respectively. Genetic mapping has been done according to the known structure of pBR322 (Bolivar et al. 1977) and Tn*5* (Rothstein et al. 1981) and to molecular weights calculated by computer analysis of data obtained by gel electrophoresis and determined against a standard of *Hin*dIII cut lambda DNA. Origins of replication are indicated as ORI. *Ava*I site is only shown in pBR322. X/S indicates the ligation site between the *Xho*I end of the pDB25 fragment and the *Sal*I end of pFIT001. Abbreviations for endonuclease cleavage sites: Av, *Ava*I; Ba, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; X, *Xho*I. Ap^r, ampicillin resistance gene; Tc^r, tetracycline resistance gene; *lux* A, B; bioluminescence genes

those present in Tn*5*. A new *Xho*I site was created on the right side of Tn*5* after its insertion on pDB20. This new site may have been a consequence of the duplication of a few base pairs associated with the process of Tn*5* insertion.

To build pDB27, the Km^r gene of Tn*5*, carried on a 2.2-kb *Sal*I–*Xho*I fragment of pDB25, was fused to the *lux*AB genes by replacement of a *Sal*I fragment of pFIT001 bearing part of the Tc^r gene. In pDB27, expression of *lux*AB came under the control of the *neo* promoter of the Km^r gene. This could be inferred since ligation of the *Sal*I–*Xho*I fragment in the opposite orientation did not yield bioluminescent transformants (data not shown). To build pDB28, the 3.6-kb *Bgl*II fragment from pDB27 containing the Km^r-*lux*AB gene fusion, but not the *neo* promoter, was used to replace the central *Bgl*II fragment of Tn*5* on pDB25. This last step gave rise to a new 6.6-kb transposon derivative, Tn*5*-Lux, which is composed of two insertion sequences flanking a central unique region. The genes of the central region, Km^r and *lux*AB, are expressed from the *neo* promoter of the left insertion sequence as in Tn*5*. The restriction sites in the unique central region of Tn*5*-Lux are slightly different from those in Tn*5*, since the *Bam*HI and *Xho*I sites are no longer present and there is one *Eco*RI site in the derivative. The portion of Tn*5* downstream of the *Sal*I site, including the entire coding region for Sm^r and the end of coding region for Bl^r (Collis and Hall 1985), is no longer present in the transposon derivative. The modification in the Bl^r phenotype was used to screen for in situ replacement of Tn*5* on pGS9, a broad-host-range suicide vehicle (Selvaraj and Iyer 1983), by Tn*5*-Lux to yield pDB30 (Table 1), the transposition vector carrying Tn*5*-Lux. The replacement was achieved by transformation of pDB28 in WA803 (pGS9), plasmid extraction from transformants carrying both plasmids and the use of this plasmid preparation to transform WA803 to Cm^r. A colony with Lux^+, Ap^s and Bl^s phenotype contained a plasmid, pDB30, that was of about the same size as pGS9, as seen by gel electrophoresis. Restriction analysis with *Bgl*II and *Hin*dIII demonstrated that an exact replacement of Tn*5* by Tn*5*-Lux had occurred.

Transfer of Tn5-Lux using the suicide plasmid vehicle pDB30

The transfer frequencies of the original Tn*5* and the Tn*5*-Lux transposon derivative were compared using *Pseudomonas* sp. RB100C as the recipient bacterium. The frequency of transconjugants per recipient cell was 10^{-5} for Tn*5*, and 10^{-6} for the derivative. The decrease in transfer frequency was assumed to result from alteration of transposition and not of vector transfer functions. Indeed, the structures of pDB30 and pGS9 were found to differ only in the transposon itself (see above). The difference in size between Tn*5* and its derivative is small (0.85 kb) and is probably not responsible for the difference in the Km^r transfer frequency. However, it appears likely that a deletion of 15 nucleotides in the right insertion sequence, left of position 1,520, has led to lower transposition efficiency. This deletion includes the left inverted repeat and the last two bases of the translational stop codon of the transposase coding region (Johnson et al. 1982).

Different strategies were used to obtain bioluminescent transconjugants from the various strains used in this study (Table 2). The simplest of these strategies was to use prototrophy of the recipient and thus counterselect the donor on minimal medium containing kanamycin. This was achieved with *Pseudomonas* sp. RB100C (see above) and *P. putida* CH183, but not with *Agrobacterium* strains, which exhibited too high endogenous level of Km^r on minimal medium. YAA containing Km was found to be more selective for the *Agrobacterium* transconjugants, while the sugar in this medium, adonitol, was a carbon source not utilized by *E. coli*. This medium was also suitable for *Rhizobium*, and YAA with kanamycin was thus used for selection of

Agrobacterium and *Rhizobium* spp. bioluminescent transconjugants. Colonies of spontaneous adonitol-utilizing WA803 sometimes appeared on the selective medium, but they could be distinguished easily on the basis of aspect and high bioluminescence intensity.

Expression of bioluminescence

Once the expression of bioluminescence had been verified on solid medium, a system was devised to monitor bioluminescence precisely in liquid medium. After filtration, the YMB medium was suitable for the determination of bioluminescence, since it was almost perfectly clear and allowed adequate growth of all the bacteria studied in this work. In previous studies, exogenous aldehyde had usually been added as vapor or as a sonicated suspension to liquid medium, since n-decanal is generally considered immiscible. This practice is not likely to allow good distribution and availability of substrate in liquid medium. In this study, it was found possible to include the aldehyde substrate directly in the bioluminescence medium, thus obtaining relatively strong and reproducible signals. Concentrations higher than 1 ppm n-decanal had a toxic effect on bacteria, while lowering the concentration below 1 ppm resulted in a decreased signal. In each individual experiment, readings of bioluminescence were linearly proportional to the number of cells in the dilute suspensions used. Intensity of bioluminescence produced in liquid medium varied between the different bacteria (Table 2). Only the bioluminescence yielded by *R. leguminosarum* 175F9 L and *A. rhizogenes* 15834 L was equivalent to that of the *Pseudomonas* strains RB100C L and CH183 L. Lower values were obtained for the other *Rhizobium* and *Agrobacterium* strains. Different results were obtained for different strains of a given species. For example, among the *Agrobacterium* representatives, *A. rhizogenes* 15834 L and A4 L gave the strongest and weakest signal, respectively. However, in spite of these variations, the level of bioluminescence produced by the pseudomonads, agrobacteria and rhizobia tested differed by less than 1 order of magnitude (Table 2). In contrast, expression of bioluminescence by *E. coli* WA803 (pDB30) was much higher than for the other strains; it was also more variable, with a coefficient of variation of 60% (data not shown). Such variability is likely to arise from a gene dosage effect, since WA803 is the only strain, among those studied here, where Tn*5*-Lux is located on an unstable plasmid. Moreover, the antibiotic used to maintain the plasmid during growth, Cm, is known to alter DNA replication and plasmid copy-number. For the other recipient bacteria, Tn*5*-Lux had inserted either in the chromosome or else in stable, indigenous plasmids. Shaw and Kado (1986) also obtained more intense bioluminescence with enterobacteria than with other bacteria and they assumed this phenomenon to be associated with higher copy-number of their bioluminescence plasmid vector in enterobacteria.

The bioluminescent transconjugants of *R. meliloti* and *R. leguminosarum* biovar *viceae* had retained their capacity to produce effective N_2-fixing symbioses with their respective hosts. The nodules initiated by *R. leguminosarum* biovar *viceae* strains 175B2 L, 175F9 L and 175F19 L, were further analysed for expression of bioluminescence. No bioluminescence could be observed from those nodules by visual examination or photography as long as their cortical layers remained intact (data not shown). However, crushing of nodules allowed detection of bioluminescence by both techniques, presumably because destruction of permeability barriers increased the availability of oxygen and the luciferase substrate to the bacteroids. Recovery of bioluminescence from nodule extracts could be quantified as before and was proportional to nodule fresh weight for each host-strain combination (not shown). Intensity of bioluminescence recovered from nodules (Table 3) was correlated with the level of expression of bioluminescence by the free-living rhizobia grown in liquid medium (Table 2). Pea and faba bean nodules initiated by strain 175B2 L produced equivalent bioluminescence per mg fresh weight, and the results with strain 175F9 L were similar. However, intensity of bioluminescence per unit fresh weight of nodules initiated by strain 175F19 L was lower on faba bean than on pea (Table 3). This could be related to more extensive development of cortical tissue in nodules initiated by strain 175F19 L on faba bean.

Table 3. Bioluminescence recovered from nodules initiated on pea and faba bean by bioluminescent strains of *Rhizobium leguminosarum*

Host	Strain	Mean fresh weight of nodules (mg)	Bioluminescence per mg nodule (relative light units)[a]
Pisum sativum L.	175B2 L	4.83 ± 1.05	6.84 ± 0.49
	175F9 L	4.20 ± 0.70	10.38 ± 0.74
	175F19 L	4.78 ± 0.54	4.06 ± 0.53
Vicia faba L.	175B2 L	5.53 ± 1.40	6.61 ± 0.18
	175F9 L	8.44 ± 0.64	11.23 ± 0.33
	175F19 L	9.40 ± 0.99	1.09 ± 0.15

[a] Relative light units expressed as total cpm obtained per mg nodule fresh weight divided by 10^4. Values are means of 5 replicates ± standard errors

Discussion

The construction of Tn*5*-Lux shows a *Sal*I – *Bgl*II fragment of 2.4 kb, harboring the *lux*A and *lux*B genes, to contain all the structural genes required for expression of the bioluminescence character. Moreover, the fusion of this fragment to the left part of Tn*5*, downstream of the *Sal*I site, allows constitutive expression of both Km^r and bioluminescence characters. Even with an altered right insertion sequence, the Tn*5* derivative efficiently transposes in gram-negative bacteria, although at a reduced frequency. In our opinion, working with only two of the *lux* genes and using exogenous substrate is a very sensitive and convenient method. First, the *lux*AB cluster (ca. 2.1 kb) is smaller than the *lux*ABCDE cluster (ca. 7.5 kb), and thus is more likely to be integrated in a transposon without altering its functions. Moreover, relying on an exogenous substrate eliminates loss of energy through the fatty acid reductase pathway and constant production of bioluminescence. Finally, expression of bioluminescence becomes independent of endogenous aldehyde production which is related to fatty acid concentration and nutritional factors.

Comparisons between the structures of the suicide plasmid vehicles pGS9 and pDB30 suggest that Tn*5*-Lux could be introduced, by means of pDB30, in any strain amenable to insertion mutagenesis with the original transposon vector. Furthermore, the levels of expression of bioluminescence

cence by strains mutagenised with Tn*5*-Lux are fairly comparable among representatives of the *Pseudomonas, Agrobacterium,* and *Rhizobium* genera. Use of the transposon should also allow stable integration of the *lux* genes in indigenous replicons of the recipient bacteria. In contrast, variable expression of *lux* genes, that might be related to gene dosage effect, has been noticed here with WA803 (pDB30) harboring *lux* genes on a plasmid. Working with bacteria other than those studied here, Shaw and Kado (1986) have obtained wide variation in expression of the *lux* genes carried on the broad-host-range plasmid pUCD607. Problems of genetic instability are often associated with broad-host-range plasmid vectors in the gram-negative bacteria (Krishnapillai et al. 1986).

The properties of uniform expression in different bacteria and of stability of the bioluminescence phenotype, which are conferred by the Tn*5*-Lux transposon, are likely to prove useful in ecological studies on colonisation of natural environments by bacteria harboring the bioluminescence character. In spite of this, the physiological state of the bacteria remains a major source of variability in expression of bioluminescence (Shaw et al. 1987). Enumeration of bacteria based on bioluminescence measurement is likely to be possible only if the physiological state and other factors such as temperature, oxygen and substrate availability can be standardized. Under such defined conditions, the high levels of bioluminescence obtained in this study suggest that the Tn*5*-Lux system could be readily used to measure low bacterial cell densities. Additionally, it was shown for three strains of *R. leguminosarum,* that both nodule bacteroids and free-living cells had the capacity to express bioluminescence genes encoded by the transposon derivative. Expression of bioluminescence in dinitrogen-fixing nodules can be useful for confirming in a very simple way the presence of an inoculated rhizobial strain, without the need for antibiotic or serological typing.

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