POTENTIAL LUMINESCENCE AS AN INDICATOR OF ACTIVATION OF GENETICALLY-MODIFIED PSEUDOMONAS FLUORESCENS IN LIQUID CULTURE AND IN SOIL

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Summary—Potential luminescence was used to assess the ability of luminescence-marked cells of Pseudomonas fluorescens to regain activity following starvation in soil or in liquid culture. In soil, potential luminescence was measured by determining luminescence periodically during incubation of samples of inoculated soil with double-strength complex medium supplemented with sodium citrate. Luminescence increased to a maximum during incubation. Following starvation of cells in soil, maximum luminescence decreased and the time taken to reach the maximum increased. Activity measured by dehydrogenase assays did not vary during the incubation and was not significantly affected by starvation of the cells. Viable cell concentration correlated well with final potential luminescence values and with luminescence in the absence of nutrient amendment, but not with potential dehydrogenase activity. Growth of P. fluorescens, previously starved in liquid medium, was preceded by a lag which increased in length as the duration of the starvation period increased. Although luminescence of the starved cultures decreased with increased starvation period, there was no detectable lag in luminescence following addition of nutrients. Potential luminescence therefore enables rapid, non-extractive and selective determination of changes in activity of luminescence-marked microorganisms in soil, the size of the active population and the time taken to recover from periods of starvation.

INTRODUCTION

A major technical development in the detection of microorganisms in natural environments has been the tagging of particular strains with specific marker genes, notably genes for antibiotic or heavy metal resistance (Bale et al., 1987), lacZY (Drahos et al., 1986) and xylE (Winstanley et al., 1989). Detection of organisms expressing these genes requires extraction of cells from the environment, followed by growth and enumeration on laboratory media. Detection of organisms bearing genes encoding resistance to antibiotics or heavy metals employs selective media containing these compounds. LacZY-marked organisms are detected on media containing the chromogenic substrate X-gal, while colonies of xylE-marked organisms are detected by the appearance of a coloured product after spraying with catechol. Marker genes themselves may also be detected using nucleic acid probes, with sensitivity increased by PCR amplification, following extraction of DNA from extracted cells or direct DNA extraction from the environment (Steffan and Atlas, 1991). Currently, these techniques provide only qualitative, rather than quantitative data on cell concentrations due to problems of extraction and purification of DNA and uncertainties regarding gene copy number and the extent of gene transfer. Extraction of DNA from soil in a form suitable for probing and PCR amplification is particularly difficult because of problems of contamination by soil clay and humic components.

Bioluminescence-based marker systems involve the introduction of genes for luminescence, originally cloned from the marine bacteria Vibrio fischeri or Vibrio harveyi. Eight genes are involved, located on two operons (Meighen, 1991). The structural genes, luxA and B, encode luciferase, luxC, D and E code for the synthesis and recycling of the aldehyde substrate, luxI and R genes are involved in regulation of luciferase production (Nealson et al., 1970) via a positive feed-back mechanism and function of the luxG gene is unknown (Meighen, 1991). Light production is catalysed by luciferase, which requires oxygen, a long-chain fatty aldehyde as a substrate, and a source of reducing equivalents, usually reduced flavin mononucleotide (FMNH2) (Hastings et al., 1985; Meighen, 1988). In strains lacking some or all of the luxC, D and E genes, light emission requires exogenous addition of the substrate n-decyl aldehyde.

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Luminescence-based systems possess many of the advantages of other marker systems. Viable cell enumeration of extracted cells is possible against an indigenous background population by visual detection of luminescent colonies (Grant et al., 1991) and may be combined with use of selective media to increase sensitivity. Individual luminescing cells and microcolonies may be detected by charge-coupled device (CCD) image enhanced microscopy (Silcock et al., 1992; Waterhouse et al., 1994) and extracted DNA may be probed for the lux genes.

The major and unique advantage of luminescence-based systems is their ability to detect marked cells by luminometry, without the need for cell or DNA extraction. Luminometry of soil sampled within 1 h of inoculation of cells of luminescent E. coli strains enables detection of 200-6000 cells g soil\(^{-1}\), depending on the strain used (Rattray et al., 1990). Luminometry is a measure of population activity and prolonged incubation leads to a decline in light output to a basal level, reflecting the metabolic activity of the marked population. Luminescence therefore provides a measure of instantaneous or actual activity, which is approximately three orders of magnitude more sensitive than traditional measures (e.g. measurement of enzyme activity), and is selective for the marked strain (Meikle et al., 1992). In addition, the technique is rapid, providing results within 5 min of sampling.

Several traditional methods for determination of microbial biomass in the soil involve activation by substrate addition, followed by measurement of metabolic activity. The most commonly used is the substrate induced respiration (SIR) method of Anderson and Domsch (1978) in which respiration is assessed following addition of excess glucose. Adoption of a similar approach to measurement of light output (i.e. determination of potential luminescence) potentially provides a further advantage to luminescence-based marker systems, enabling non-extractive determination of both the time required for activation of the marked population and the size of the population capable of activation. Here we describe optimization of the conditions to enable such measurements and their testing following inoculation of a luminescent strain of P. fluorescens into sterile or non sterile soil. We also describe studies on activation and resuscitation of P. fluorescens following starvation in liquid culture.

**MATERIALS AND METHODS**

**Bacterial strain and culture conditions**

A strain of *P. fluorescens*, 10586s/FAC510, genetically modified to express genes for luminescence, was used in all studies. Details of its construction and light output characteristics are provided by Amin-Hanjani et al. (1993). Luciferase is produced constitutively by this strain, which bears chromosomally integrated luxA and B genes, the structural genes for luciferase. Light output was determined after addition of exogenous aldehyde substrate, as the construct lacks functional luxC, D and E genes. During growth in batch culture light output was directly proportional to biomass concentration. Routine growth and maintenance of cultures was carried out at 30°C in L broth (tryptone, 10 g; NaCl, 5 g; yeast extract, 5 g; glucose, 1 g; distilled water, 1 litre; pH 7).

**Soil microcosms**

Soil microcosms consisted of 5 g sterile Craibstone soil [Countesswells series; sandy loam; cation exchange capacity, 7.4 cmol kg\(^{-1}\); organic matter, 4.25%; pH 7, following amendment with Ca(OH)\(_2\)] in Universal bottles. For experiments requiring sterile soil, microcosms were autoclaved at 121°C for 1 h on three separate occasions. The matric potential of the soil was adjusted to \(-30\) kPa by even dispersal of the appropriate volume of inoculum cell suspension and nutrient or buffer amendment. The volume was determined by the antecedent (i.e. prior to inoculation) matric potential and with reference to the soil moisture release characteristic. Details of nutrient amendments are given in the Results section. Control microcosms were amended with 15 mM phosphate buffer (pH 7), also to \(-30\) kPa.

Inocula were prepared by harvesting exponentially growing cells of *P. fluorescens* 10586s/FAC510 from batch cultures grown in L broth at 30°C. Cells were washed three times in 15 mM phosphate buffer (pH 7) and resuspended in a volume of buffer sufficient to give the required cell concentration. Following inoculation, microcosms were kept overnight at 25°C, after which they were destructively sampled and analysed. Viable cell concentration was determined by dilution plate counting. A decimal dilution series of a soil suspension was prepared in 15 mM phosphate buffer (pH 7) and samples were plated, in triplicate, on L broth solidified by addition of 1.5% (w/v) Technical No. 3 agar (Oxoid). Colonies were counted after incubation of plates for 48 h at 30°C.

Luminescence was measured in suspensions of soil in phosphate buffer, following addition of 1 \(\mu l\) n-decyl aldehyde, using an LKB 1251 Luminometer, as described by Amin-Hanjani et al. (1993). Light output was integrated over a period of 10 s and expressed as relative light units (RLU). Measurements were carried out in triplicate for samples from each of three microcosms. To determine potential luminescence, 0.5 g soil was shaken continuously with 1 ml of test solution in a luminometer cuvette. Luminescence was measured as described above periodically during incubation at room temperature.

To determine the effect of starvation period on potential luminescence, microcosms consisted of 50 g sterile soil in 250 ml Erlenmeyer flasks, inoculated as described above to give a matric potential of \(-30\) kPa. Triplicate microcosms were sampled destructively for determination of dehydrogenase activity and viable cell concentration as described...
above. Potential luminescence was measured as described above, except that a 10⁻³ dilution of a 2 g soil sample in phosphate buffer was incubated with double strength 523 medium amended with 10 mg ml⁻¹ sodium citrate. Potential dehydrogenase activity was determined by measurement of dehydrogenase activity in triplicate 2 g soil samples incubated for 2 h in sterile glass Universal bottles with double strength 523 medium (Kado et al., 1972) amended with 10 mg ml⁻¹ sodium citrate. Single strength 523 medium contains, 1.7 distilled water, sucrose, 10 g; casamino acids, 8 g; K₂HPO₄, 3H₂O, 3.93 g; MgSO₄·7H₂O, 0.3 g; yeast extract, 4 g.

Dehydrogenase assays

Dehydrogenase activity was determined by measurement of formazan produced following incubation of samples (2 g) from each of triplicate microcosms with p-iodo nitrophenyl tetrazolium chloride for 6 h (Benefield et al., 1977), modified as described by Meikle et al. (1992).

Starvation and resuscitation in liquid medium

Stationary phase cells of P. fluorescens were starved by harvesting and washing as previously described and resuspending in 15 mm phosphate buffer (pH 7). The suspension was kept, without shaking, for 29 days at 30°C. Samples (1 ml) were taken at regular intervals, inoculated into triplicate 250 ml Erlenmeyer flasks containing 100 ml L broth. Flasks were incubated, with shaking, at 30°C until stationary phase was reached. Samples were removed periodically for measurement of biomass concentration (A₆₀₀) and luminescence.

RESULTS AND DISCUSSION

Optimization of potential luminescence in soil

Luminescence was measured following amendment with L broth and 523 medium, with or without supplementation with sodium acetate or sodium citrate. These were chosen as ideal media for P. fluorescens growth in liquid culture. Best results were obtained with 523 medium, and highest luminescence was achieved following amendment with citrate. The concentration of citrate was, however, critical. Amendment of inoculated sterile soil with 523 medium containing 10 mg l⁻¹ sodium citrate [Fig. 1(a)] gave maximum luminescence, after 0.4 h. Maximum luminescence was less following amendment with 25 mg l⁻¹ citrate but values after 1.2 h were similar [Fig. 1(b)]. At concentrations of 50 and 100 mg l⁻¹ citrate luminescence was inhibited [Fig. 1(c), (d)]. Figure 1 also illustrates typical kinetics for potential luminescence. Ideally, luminescence should rise immediately following substrate addition to a constant value, which is then maintained throughout the incubation period. Delays in achieving maximum luminescence will result from a lag in activation of the population and activity will fall when nutrients are fully utilized or when factors such as oxygen supply limited activity. Repeated experiments with freshly-inoculated cultures and cultures which had been starved for short periods led to a choice of an incubation period of 2 h. This was found to be optimal for activation of populations and for characterization of kinetics of luminescence. Maximum luminescence occurred during this period, and longer periods led, in some cases, to exponential increases in luminescence through growth.

The amount of substrate required for optimal and consistent luminescence depends on the size of the active microbial population. L broth and 523 medium are routinely used for growth in liquid culture with initial cell concentrations in the order of 10⁷ cells ml⁻¹. In non-sterile soil, initial indigenous cell concentrations will be in the order of 10⁶ g⁻¹. The amount of substrate available per cell following amendment of 0.5 g soil with 1 ml 523 medium will consequently be ca 1% that in typical liquid culture and substrate will be fully utilized rapidly. This problem is exacerbated in non-sterile soil, in which the indigenous population will compete for substrate. Figure 1(a)-(d) demonstrates the effect of indigenous microflora on potential luminescence. Despite similar concentrations of luminescent cells in all microcosms, luminescence was significantly lower in non-sterile soil compared to sterile soil. 2-way analysis of variance (ANOVA) on each set of data gave probabilities of 0.999, 0.997, 0.997 and 0.987 for addition of 10, 25, 50 and 100 mg sodium citrate l⁻¹. Dilution of soil prior to incubation reduced the differences between luminescence in sterile and non-sterile soil.

As a result of these experiments, standard conditions for measurement of potential luminescence were defined: microcosms were amended with double-strength 523 medium supplemented with 10 mg ml⁻¹ sodium citrate, and luminescence was measured over 2 h.

Effect of length of starvation period in soil on potential luminescence

To determine the relationship between viable cell concentration and potential luminescence during short-term starvation in the soil, inoculated microcosms containing autoclaved soil were incubated for 15 days. Potential luminescence was determined by addition of double-strength 523 medium supplemented with 10 mg sodium citrate ml⁻¹ and incubation for up to 2 h [Fig. 2(a)]. 1-way ANOVA was performed on each set of data to determine if changes in luminescence during the incubation were significant. All changes during the 2 h period were significant, except in samples taken on day 15 (see later). In samples taken on days 0 and 2, an initial increase in luminescence, for 0.5–1 h, was followed by relatively constant levels. Luminescence from soil samples taken at day 1 increased in a similar manner, after an initial decrease between 0 and 0.33 h. Samples taken at 4 and 11 days showed more prolonged increases in
Fig. 1. Changes in luminescence in samples of sterile (O) and non-sterile (●) soil inoculated with P. fluorescens. Samples were amended with 523 medium supplemented with (a) 10, (b) 25, (c) 50 and (d) 100 mg ml⁻¹ sodium citrate and incubated for 2 h at 30°C.
Measurement of activation of *P. fluorescens* by luminescence

**Fig. 2.** Changes in luminescence (a) and dehydrogenase activity (b) in samples of sterile soil inoculated with *P. fluorescens* and sampled at 0 (○), 1 (●), 2 (□), 4 (△), 8 (■), 11 (■) and 15 (▲) days. Samples were amended with 523 medium supplemented with 10 mg ml⁻¹ sodium citrate and incubated for 2 h at 30°C.
Fig. 3. (a) Changes in viable cell concentration (○), luminescence of unamended samples (AL) (●), luminescence immediately following nutrient amendment (PL,) ([■]), final potential luminescence (PL,) (▲) and final potential dehydrogenase activity (DH) (○) following inoculation of sterile soil with \textit{P. fluorescens}. (b) Changes in AL (●), PL, ([■]), (PL,) (▲) and (DH) (○) per viable cell.
luminescence and were only approaching constant values at the end of the incubation. The sample taken at day 8 showed a similar initial increase but luminescence fell after 0.5 h. Luminescence in soil sampled at 15 days did not vary significantly (P = 0.28) during incubation for 2 h, but increased during incubation for a further 3 h (P = 0.006) (data not presented).

In addition to luminescence measurements, dehydrogenase activity was determined following amendment of soil with double-strength 523 medium supplemented with sodium citrate, using the standard 6 h incubation period. Changes in a activity are illustrated in Fig. 2(b) in samples taken between 0 and 15 days. Dehydrogenase values were independent of incubation time, except for samples taken on day 1. 1-way ANOVA for other sample times gave probabilities ranging from 0.101 to 0.964. Activity in the sample taken on day 1 decreased significantly (P = 0.996) during the incubation. Dehydrogenase activity did not vary significantly with the time at which samples were taken (P = 0.673, 2-way ANOVA calculated over all incubation periods).

Table 1. Pearson correlation coefficients between viable cell concentration (VCC), luminescence of unamended samples (AL), luminescence immediately following nutrient amendment (PL), and the end of the incubation period (PLT) and dehydrogenase activity at the end of the incubation period following nutrient amendment (PD) following inoculation of sterile soil with P. fluorescens

<table>
<thead>
<tr>
<th></th>
<th>VCC</th>
<th>AL</th>
<th>PL</th>
<th>PLT</th>
<th>PD</th>
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<tr>
<td>VCC</td>
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<td>0.833*</td>
<td>1</td>
<td>0.603</td>
<td>0.951*</td>
</tr>
<tr>
<td>AL</td>
<td>0.833*</td>
<td>1</td>
<td>0.851*</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>PL</td>
<td>0.603</td>
<td>0.851*</td>
<td>1</td>
<td></td>
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<tr>
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<td>0.700</td>
<td>1</td>
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<tr>
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<td>0.157</td>
<td>0.470</td>
<td>-0.009</td>
<td>1</td>
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</table>

*Indicates significant correlation at the 5% level of significance.

Luminescence measured without nutrient amendment (AL) has been shown to be equivalent to metabolic activity (Meikle et al., 1992) and correlated significantly with PLT, as expected. Correlation of AL with viable cell concentration was less strong, being significant at the 10%, but not the 5% level of significance. This weaker correlation was due to an approximate 3-fold increase in AL per viable cell between days 0 and 2. An increase in in situ cell activity may have resulted from, during this period, substrates released during autoclaving of soil, although this was not reflected in an increase in viable cell concentration [Fig. 3(a)]. Alternatively, the reduction in viable cell concentration following inoculation may have been due to adsorption of cells to soil particles, leading to a reduction in extraction efficiency. Between 2 and 15 days, AL per viable cell varied little. Thus, during starvation in soil between 2 and 15 days, the activity of the inoculum was directly related to the concentration of extractable, culturable cells, suggesting that cells were not entering a non-culturable state. A similar effect was seen with potential luminescence values (PLT). An initial 3–4-fold increase in PLT per viable cell between 0 and 2 days was followed by relatively constant values, with the exception of a decrease at day 8. The high correlation between potential luminescence (PLT) and viable cell concentration confirms its utility as a measure of active soil biomass, with a similar basis to the Anderson and Domsch (1978) substrate-induced respiration technique. A major distinction, however, is the specificity of the luminescence technique, in determining biomass of the marked organism only, in the presence of the indigenous microflora. In addition, measurement of potential luminescence is more rapid, with results after incubation for only 2 h. Luminescence is easier to measure, while characterization of the kinetics of luminescence during incubation provides information on the time required for activation of the population. The correlation of PLT with viable cell concentration was much greater than that with AL but PLT values were also much higher, increasing sensitivity by approximately one order of magnitude. Combined use of both techniques would be of particular advantage where the activity of marked cells is reduced by inability to utilise substrates available within the soil or by competition with the indigenous microflora.

Starvation and resuscitation in liquid medium

Resuscitation of cells by inoculation into L broth, following starvation in phosphate buffer, was investigated by measuring increases in absorbance and luminescence. Changes in absorbance indicated an increasing lag period with increased starvation (Table 2) as found by other workers (Amy et al., 1983). The duration of the lag was calculated by extrapolation of semilogarithmic plots to the initial biomass concentration and increased from 2.77 h immediately after suspension in phosphate buffer to
Table 2. Lag periods prior to increases in luminescence, growth and achievement of maximum luminescence per unit biomass following inoculation of L broth with P. fluorescens starved in phosphate buffer. Values in parentheses are standard errors of triplicate measurements (ND—not detected)

<table>
<thead>
<tr>
<th>Starvation period (days)</th>
<th>Lag phase prior to growth (h)</th>
<th>Lag phase prior to increase in luminescence (h)</th>
<th>Time required for achievement of maximum luminescence per unit biomass (h)</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>2.77 (0.13)</td>
<td>&lt;1</td>
<td>&lt;1</td>
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<tr>
<td>1</td>
<td>14.2 (0.046)</td>
<td>&lt;1</td>
<td>1</td>
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<tr>
<td>2</td>
<td>7.21 (0.024)</td>
<td>&lt;1</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>8.21 (0.076)</td>
<td>&lt;2</td>
<td>3.67</td>
</tr>
<tr>
<td>7</td>
<td>8.45 (0.152)</td>
<td>&lt;2</td>
<td>4</td>
</tr>
<tr>
<td>14</td>
<td>7.86 (0.092)</td>
<td>&lt;2.5</td>
<td>4</td>
</tr>
<tr>
<td>19</td>
<td>10.3 (0.095)</td>
<td>&lt;2</td>
<td>4</td>
</tr>
<tr>
<td>29</td>
<td>13.2 (0.097)</td>
<td>&lt;1.5</td>
<td>ND</td>
</tr>
</tbody>
</table>

13.2 h after starvation of cells for 29 days. Luminescence values in samples taken immediately after inoculation into L broth decreased with increasing starvation period but always increased significantly by the subsequent sampling interval (Fig. 4, Table 2). A lag period prior to increase in activity measured by luminescence was, therefore, not detected and cells became activated several hours before growth was detected. This applied even after starvation for 29 days, when luminescence increased prior to the second sampling interval (1.5 h) while the lag period prior to growth was 13.2 h. Although luminescence showed no lag period, starvation increased the time required for attainment of maximum activity per unit biomass (Table 2). In all samples except those taken after starvation for 29 days, maximum luminescence per unit biomass was reached prior to growth. In cells starved for 29 days, luminescence activity increased throughout the lag period and may not have reached a maximum by 16 h, when sampling was discontinued. The observation that luminescence activities of cells starved for 29 days were similar to those of non-starved cells indicates that starvation did not induce physiological changes affecting luminescence following activation of cells.

Although recovery of starved cells in liquid and soil was achieved by different means, the former experiments indicate the distinction between activity and growth. Luminescence activity increased in all cases during the lag period for growth. In the soil, it is important for starving populations to utilize substrate as soon as it becomes available, to prevent its acquisition by competitors. This is particularly important when assessing the likely significance of risks associated with activity of a genetically-modified microorganism. If such an organism is able to respond to nutrient supply more quickly than the indigenous microflora it will have a competitive advantage with potential consequences in terms of environmental effect, persistence and gene transfer.
Potential luminescence therefore provides a means of assessing the biomass of marked cells capable of activation by appropriate nutrients. The technique is applicable to any luminescence-marked strains, although the nutrient amendment and incubation conditions may vary. The major advantages of the technique are sensitivity, selectivity and the lack of requirement for cell extraction. It is therefore particularly applicable to detection and risk assessment of genetically-modified microorganisms but is also of considerable value in starvation survival studies of particular organisms in natural environments. Under the conditions used in our study, potential luminescence in soil samples correlated well with viable cell concentration, indicating an absence of non-culturable cells. More extensive studies would be required for confirmation, but activation of non-culturable cells measured by potential luminescence, coupled to viable cell enumeration, could enable distinction between, and quantification of, these two populations.

In addition to assessing biomass concentration, potential luminescence measures the time required for activation of cells following periods of starvation. After starvation in soil for 11 days, activation occurred within the 2 h incubation period but activation following starvation for 15 days required longer incubation for detection of activation. Liquid culture studies demonstrated the differences in lag period prior to achievement of maximum luminescence activity and prior to growth. Choice of the optimal incubation period involves a balance between the convenience of a short assay period, and the danger of growth occurring if activation is rapid, and the requirement for longer incubations for cells which require more time for activation following longer periods of starvation.

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