Construction and characterization of a lux-marked phenanthrene degrading bacterium

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Abstract
Construction of a luminescent microbial biosensor as a sensitive and rapid biotechnological tool for monitoring survival and activity of genetically engineered microorganisms (GEMs) in the contaminated environment has been the main focus of this study. Four rifampicin resistant phenanthrene-degrading bacteria viz., Comamonas testosteroni GZ38A, C. testosteroni GZ39, Pseudomonas putida GZ44 and P. stutzeri P16 were made using gradient plate techniques. All resistant strains were transformed with the plasmid harboring the mini-Tn5-tet transposon cassette, containing the luxAB genes to confer stability. C. testosteroni GZ38A and P. stutzeri P16 were successfully lux-marked in this manner. It was found that lux-marked strains of C. testosteroni GZ38A were not able to degrade phenanthrene. Although the maximum growth rate of lux-marked strain was significantly lower (P < 0.05) than that of the wild type P. stutzeri P16, the P. stutzeri P16-luxAB was selected because it has the following criteria: the stability of expression of tet-resistance over 180 generations, phenanthrene - degradability and high level of luminescence light output in the absence and presence of phenanthrene. The addition of 1 litter (0.5 % v/v) n-decyl aldehyde produced consistently high levels of luminescence at various stages of selected strain. Results clearly indicated that lux-marked strain was appropriately constructed and it is a novel biodegradative luminescent biosensor which enables us to monitor the fate of a phenanthrene bacterial degrader genetically or non-genetically made within a polluted environment.

Keywords: Lux AB gene; Phenanthrene; Molecular Marker and Degradative bacteria.

INTRODUCTION
Large amounts of toxic chemicals are released into the natural environment every year through catastrophic events such as cargo breakage, oil tank explosions and industrial waste disposal. Microorganisms, because of their wide range of substrates utilisation, have been considered as useful tools for degradation of such pollutants (Bedard and Quensen III, 1995; Bennet and Faison 1997; Bossert and Compeau, 1995; Semple et al., 1999). In general, however, a single microbe is not able to degrade complex compounds and or application of consortia of microbes may also not result in complete degradation, as microbes within the consortia may compete with each other (Dwyer et al., 1988). Genetic engineering methods can be used to improve the metabolic activity of introduced biodegradative microorganisms (Ensley and Deflaun, 1995; Garbisu and Alkorta, 1997; Shaw et al., 1992). There are several barriers for application of Genetically Engineered Microorganisms (GEMs) in the environment (Smit et al., 1992). Monitoring of GEMs is one of these problems which should be considered before their release. Several genes have been used for tracking GEMs in soil and water, such as lacZ, xylE, gas, gap and lux (Brandt et al., 2002 ; Haddix et al., 2004 and Lindow, 1995). The bioluminescence marker system has been shown to be a good marker system for monitoring the survival and activity of bacteria in the environment (Hollis et al., 2000; Grant et al., 1991; Ritchie et al., 2003; Shao et al., 2002 and Shaw et al., 1992). lux marking of bacteria which can degrade two-ring Polycyclic Aromatic Hydrocarbons (PAHs) has been reported (King et al., 1990) but, to date, lux marking of strains capable of degrading PAHs with three or more
rings has not been reported. In this study, the phenanthrene degrading bacteria, *Pseudomonas stutzeri* P16, *Comamonas testosteroni* GZ38A, *C. testosteroni* GZ39 and *P. putida* GZ44 were chosen, as phenanthrene, a tricyclic aromatic hydrocarbon, has been used as a model for degradation of high molecular weight and carcinogenic PAH (Boldrin et al., 1993; Narro et al., 1992). Furthermore, *P. stutzeri* P16 was selected, because it as this strain is a natural soil isolate (Stringfellow and Aitken, 1994; Goyal and Zylstra, 1994). Mini-Tn5 lux-transposon carrying luxAB genes was used for lux-marking the above strains because of resulting in high light output, and less effective on wild type genes.

**MATERIALS AND METHODS**

**Bacterial strains used in this study:** *C. testosteroni* GZ38A, *C. testosteroni* GZ39 and *P. Putida* GZ44 were provided by Dr. GJ Zylstra (Rutgers University, New Brunswick, USA). *Pseudomonas stutzeri* P16 was donated by Prof. MD Aitken (University of North Carolina, North Carolina, USA) and *Escherichia coli* SM10 (λ, pir) containing the mini-Tn5 transposons on the plasmid PUT (Herrero et al., 1990) was provided by Dr LA Glover (University of Aberdeen, Aberdeen, UK).

**Growth conditions:** All strains were initially grown in either Luria-Bertani (LB) broth (Oxoid, Ltd) (tryptone: 10 g. Yeast extract agar: 5 g, NaCl: 10 g, distilled water: 1 litter). They were also grown in Minimal Base Medium (MBM) [(NH₄)cl: 0.5 g, NaH₂PO₄, H₂O: 0.5 g, KH₂PO₄: 0.5 g, MgSO₄, 7H₂O: 0.5 g, NaCl: 4 g, trace element solution: 1 ml, Distilled water: 1 litter, pH 7) (trace element solution was composed of: CuSO₄, 5H₂O: 0.64 g, FeSO₄,7H₂O:0.11 g, MnCl₂,4H₂O: 0.79 g ZnSO₄, 7H₂O: 0.15 g and distilled water: 100 ml)] or modified Hutner’s base medium (Cohen-Bazire 1957 and Hutner, 1950) supplemented with phenanthrene as sole carbon source. All strains grown at 30°C except *E. coli* SM10 which was grown at 37°C.

LB agar plates containing rifampicin in gradients from 0→12.5 µg ml⁻¹, 12.5 µg ml⁻¹→25 µg ml⁻¹, 25 µg ml⁻¹→50 µg ml⁻¹, 50 µg ml⁻¹→75 µg ml⁻¹, and 75 µg ml⁻¹→100 µg ml⁻¹ were made as previously described (Cappuccino and Sherman, 1996). Phenanthrene degrading strains were either streaked on the surface of agar plates or added as a droplet.

**Samples of an transposon produce:** Overnight culture of *E. coli* SM10 (λ, pir) (100 µl) were mixed with each of the phenanthrene degrading strains (50 µl) in 5 ml of sterile NaCl (0.85% w/v), and filtered through a 45 mm diameter, 0.47 µm nitrocellulose filter (Whatman Ltd.). Filters were then placed on the surface of LB agar plates and incubated overnight at 30°C. The colonies grown on the filter surface were suspended in sterile phosphate buffer (0.01 M, pH 7) and spread on LB agar containing tetracycline (12.5 µg ml⁻¹) and rifampicin (25 µg ml⁻¹).

Before and after transposon mutagenesis, overnight cultures of the above strains were transferred into minimal basal medium agar plates as previously described (Kioyhora et al., 1982). Phenanthrene (10% w/v) was uniformly sprayed over the surface of the agar plates by a spray gun (Richard Kohnsten Ltd.). Phenanthrene utilisation by *E. coli* and wild type, rifampicin resistant and luminescent strains of *P. stutzeri* P16 were also checked by growing them in TEP medium (Na₂HPO₄ 7H₂O: 1.5 g, KH₂PO₄: 1g, NH₄Cl: 2g, Na₃S₂O₃.5H₂O: 0.02 g, tap water: 1 litter, Phenanthrene 2 g l⁻¹, PEP medium (peptone: 5 g, distilled water: 1 liter, phenanthrene: 2 g l⁻¹) and PW medium (peptone: 5 g, distilled water: 1 litter). Phenanthrene was sterilized before use by filtration (Stringfellow and Aitken, 1994).

The stability of luxAB genes was assessed by repeated subculturing in non-selective LB medium at 2 day intervals, over 14 days. Insert stability was calculated as the percentage of total cells (plated on LB) expressing resistance to tetracycline (plated on LB containing tetracycline).

**Bioluminescence measurement:** Optimal conditions for maximal light output from lux-marked *P. stutzeri* P16 luxAB4 were determined by incubating 1 ml samples of early exponential (OD₆₀₀= 0.03), mid exponential (OD₆₀₀= 0.3) and late exponential phase (OD₆₀₀= 0.7) at 30°C. Luminescence measurement were carried out at 25°C using an automated temperature controlled luminometer LKB125 following incubation of 1 ml batch culture sample with 1 µl n-decyl aldehyde (0.55 v/v) for 5 minutes at room temperature. Luminescence values were recorded as an average of triplicate readings and expressed in relative light units (RLU: 1 RLU is equivalent to 1 mV 10 S⁻¹) (Amini-Hanjani et al., 1993). Colonies could also be detected using a nitrogen cooled, slow scanning type charge couple device (CCD) camera. The camera was encased in a light-tight box. Images were stored on a Dell system 310 computer. A 0.02 S exposure was required for bright field images, and a 2 min exposure for dark field images. Culturable counts were determined by the drop plate technique (Hoben and Somasegaran, 1982).
RESULTS

Growth and mineralisation of phenanthrene was detected as the formation of colonies surrounded by transparent (clear) zones on phenanthrene sprayed agar plates. *C. testosteroni* GZ39 and *P. putida* GZ44 did not form a clear zone when strains were streaked on minimal base medium (using tooth picks). However, they grew when a drop of overnight culture was transferred to minimal base medium, and as growth increased, clearer zones developed by all four strains (Table 1). The rifampicin resistant mutants that grew on the highest concentration of rifampicin, were selected and from these strains of *C. testosteroni*, lux-marked cells of strain GZ39A were detected on tetracycline resistant agar plates. Three lux-marked GZ38A strains: *C. testosteroni* GZ38A luxAB5, *C. testosteroni* GZ38A luxAB9 and *C. testosteroni* GZ38A luxAB12 were checked for phenanthrene degradation, but none of these could grow on phenanthrene and produce clear zone on agar plates (Table 2). From 12 lux-marked colonies obtained from *P. stutzeri* P16, mutants 3, 4 and 8 gave higher luminescence than others. *P. stutzeri* P16 luxAB3, *P. stutzeri* P16 luxAB4, *P. stutzeri* P16 luxAB8, rifampicin resistant mutant and wild type strains of *P. stutzeri* P16 could all grow on PW medium (peptone + tap water), PEP medium (peptone + phenanthrene), and TAP medium (tap water + phenanthrene) while *E. coli* SM10 (λ pir) grew just on PEP and PW medium (Table 3). From the above three lux-marked strains of *P. stutzeri* P16, *P. stutzeri* P16 luxAB4 was selected for further characterization, because the percentage of cells expressing tet resistance with each successive samples was stably maintained (Fig. 1). Ability of *P. stutzeri* P16 luxAB4 to degrade phenanthrene was confirmed on solid medium and visualized in the dark by CCD camera (Fig. 2).

The concentration of aldehyde required for optimal

![Image](https://example.com/image.png)

Table 1. Growth of phenanthrene-degrading strains on mineral base agar plates supplemented with phenanthrene inoculated either by tooth stick streaking or adding droplet. (+) is a symbol for the density of culture. More (+) indicates denser culture and better growth.

<table>
<thead>
<tr>
<th>Strain</th>
<th>streaking ≤2 days</th>
<th>&gt;2 days</th>
<th>droplet ≤2 days</th>
<th>&gt;2 days</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. testosteroni</em> GZ38A</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><em>C. testosteroni</em> GZ39</td>
<td>_</td>
<td>_</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. putida</em> GZ44</td>
<td>_</td>
<td>_</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. stutzeri</em> P16</td>
<td>+++</td>
<td>+++++</td>
<td>++++</td>
<td>++++</td>
</tr>
</tbody>
</table>

Table 2. Growth of wild type, rifampicin resistant and lux-marked strains of *C. testosteroni* GZ38A on different medium supplemented with appropriate antibiotics. More (+) indicates denser culture and better growth.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MBM + phen</td>
</tr>
<tr>
<td><em>C. testosteroni</em> GZ38A (wt)</td>
<td>+</td>
</tr>
<tr>
<td><em>C. testosteroni</em> GZ38A (Rif*)</td>
<td>+</td>
</tr>
<tr>
<td><em>C. testosteroni</em> GZ38A luxAB5</td>
<td>_</td>
</tr>
<tr>
<td><em>C. testosteroni</em> GZ38A luxAB9</td>
<td>_</td>
</tr>
<tr>
<td><em>C. testosteroni</em> GZ38A luxAB12</td>
<td>_</td>
</tr>
</tbody>
</table>

MBM = minimal base medium, rif = rifampicin, tet = tetracycline, Rif* = rifampicin resistant, wt = wild type, phen = phenanthrene.
levels of light output from \emph{P. stutzeri} P16 luxAB4 was determined by incubating cells for varying times in the presence of a range of concentrations of n-decyl aldehyde and the substrate. This showed that the addition of $1 \mu\text{l} (0.5 \% \text{ v/v})$ n-decyl aldehyde produced consistently high levels of luminescence at various stages of growth (Fig. 3).

Growth and bioluminescence profiles for \emph{P. stutzeri} luxAB in LB medium were monitored with time. Light output per unit biomass increased during growth, as illustrated in figure 4. Plots of light output against biomass concentration were linear (Fig. 5) confirming that light output was directly proportional to cell density during growth. The lower limit of detection for cells of \emph{P. stutzeri} P16 luxAB4 was approximately $28.3 \times 10^3$ cells ml$^{-1}$.

Figure 6 illustrates the growth curve for the wild type and \emph{lux}-marked \emph{P. stutzeri} P16 luxAB4. The mean maximum growth rate of \emph{lux}-marked strain was 0.49 h$^{-1}$, significantly lower ($P < 0.05$) than the mean maximum growth rate of the wild type \emph{P. stutzeri} P16.

**DISCUSSION**

Advantages of bioluminescence-based marker systems for monitoring the activity of GEMs have often been reported in the literature (Meikle \textit{et al.}, 1992; Prosser, 1994; Prosser \textit{et al.}, 1996 and Rattray \textit{et al.}, 1990). Such a this marker systems, was chosen in our studies to trace phenanthrene degrading strains. Although the rate of phenanthrene degradation varied greatly in the
four wild type strains selected for this study. These strains were generally able to degrade phenanthrene in less than 2 days.

The gradient plate technique was found to be a good approach for obtaining rifampicin resistant mutants. Spreading of inocula on the surface of agar plates gave better results than streaking. The reason for this is unclear, but may be due to a higher number of introduced cells using the spread technique, resulting in a higher chance of obtaining an antibiotic resistant mutant.

Lux-marking of *C. testosteroni* GZ39 and *P. putida* GZ44 was unsuccessful, possibly because of instability, degradation of inserted genes within the recipient strains (*C. testosteroni* GZ39 and *P. putida* GZ44), or unsuitability of the vector that would prevent them receiving the plasmid.

Despite many advantages of mini-tn5 transposons for DNA insertion into Gram-negative bacteria, there are some problems associated with this DNA introductory system (de Lorenzo *et al.*, 1998). For example, disruption of chromosomal sequence causing disruption of cell metabolism is possible probably due to disruption of genes in the phenanthrene degrading pathway. Therefore All 3 lux-marked *C. testosteroni* GZ38A (*luxAB5, luxAB9, luxAB12*) were not able to utilize phenanthrene sprayed over the minimal base medium, therefore they were discarded. However, *lux*-marked
strains of P. stutzeri P16 which could produce a clear zone on agar plate covered with phenanthrene, showed that insertion of the genes did not effect degradation ability of these strains. This supports pervious studies (Rattray et al., 1992; Ritchie et al., 2003 and Van Dyke et al., 1996) on the advantage of the use of luxAB gene for monitoring the biodegradative GEMs in the environment.

Despite high light output from two of the lux-marked strains, P. stutzeri luxAB3 and P. stutzeri luxAB8, these constructs were not stable in the absence of selection pressure (tetracycline), and therefore, were also discarded. The luxAB tet cassette was stable over 180 generations in P. stutzeri P16 luxAB4. A lux-marked Aeromonas salmonicida showed similar luxAB gene stability over 140 generations (Ferguson et al., 1995). Significant differences were observed between growth rates of lux-marked and wild-type strains of P. stutzeri P16 luxAB4, and also between chromosomally lux-marked strains of Aeromonas salmonicida and wild-type the strain. However, no significant differences in the responses of these two A. salmonicida strains to starvation stress were found (Ferguson et al., 1995). Ferguson et al. (1998) also provided an assessment of the use of lux-based marker system in investigating the survival and activity of A. salmonicida in aquatic environment.

In conclusion assessment of construct stability of P. stutzeri P16 luxAB4 under non-selective conditions showed that the lux genes were stable. Light output was proportional to cell concentration and optical density over several orders of magnitude. Although there was a significant difference between the specific growth rate of wild-type and lux-marked strains of P. stutzeri P16, this may have been due to the increased metabolic burden of replicating the foreign DNA. Degradation of phenanthrene by this strain was not affected by insertion of foreign DNA. Therefore this strain can be used for further studies in monitoring biodegradative GEMs in the environment.

References


