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Comparison of *Photorhabdus luminescens* and *Vibrio fischeri lux* Fusions to Study Gene Expression Patterns

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Abstract A comparison of promoter fusions with the *luxCDABE* genes from *Vibrio fischeri* and *Photorhabdus luminescens* was made using promoters from several genes (*katG*, *sodA*, and *pqi*-5) of *E. coli* that are responsive to oxidative damage. The respective characteristics, such as the basal and maximum bioluminescence and the relative bioluminescence, were compared. *E. coli* strains carrying fusions of the promoters to *P. luminescens lux* showed higher basal and maximally induced bioluminescent levels than strains carrying the same promoter fused to the *luxCDABE* genes from *V. fischeri*. The sensitivities between the strains were similar, regardless of the luciferase used, but lower response ratios were seen from strains harboring the *P. luminescens lux* fusions. Furthermore, using the two *katG::lux* fusion strains, the bioluminescence from the *P. luminescens lux* fusion strain, DK1, was stable after reaching a maximum, while that of strain DPD2511 decreased very rapidly due to substrate limitation.

Key words: *P. luminescens*, *V. fischeri*, *luxCDABE*, gene fusions, bioluminescence, oxidative damage

The use of bioluminescence to study gene expression is a widespread practice, because of its application in numerous fields of biology and environmental studies. Bacterial assays allow faster responses and, thus, have attracted increased usage [19]. Since both eukaryotic and prokaryotic luciferases exist, promoter/gene fusions can be studied in both bacterial and eukaryotic cell systems [21, 22, 26]. For bioluminescent reporters, the promoter from the gene of interest is fused to the *luc* gene, the bacterial *luxAB* genes, or the entire *lux* operon, such as *luxCDABE*, and this fusion is then transformed or transfected back into the host cells of interest. Both the *luc* and *luxAB* fusions, however, require the addition of substrate [8, 17, 18] and are, therefore, limited in the scope of their use.

Furthermore, numerous bioluminescent bacteria have been isolated and their *lux* genes were cloned [2, 9, 28]. The more commonly known *lux* operons were purified from the marine bacteria *Vibrio fischeri* and *Vibrio harveyi*. The LuxA and LuxB luciferase subunits encoded by the genes from *Vibrio fischeri* are thermolabile and show a maximum activity at temperature of about 28–30°C [29]. In contrast, the LuxA and LuxB luciferase subunits encoded by the genes from *Vibrio harveyi* and terrestrial bacteria, such as *Photorhabdus luminescens*, are generally more heat stable and have a maximum activity at temperature of around 37°C [9, 29]. This difference in their stabilities was utilized to study the role of the DnaKJ heat-shock proteins in the re-folding of denatured proteins [16]. Nevertheless, a BLASTP analysis of the *V. fischeri* and *P. luminescens* luciferase subunits shows them to be similar; with 67% and 53% identical amino acids, and similarities of 80% and 71% for the LuxA and LuxB proteins, respectively. Furthermore, several studies have shown that fusions of the gene encoding the LuxA and LuxB proteins from different bacterial sources lead to active luciferase formation, but not with all combinations [1, 34].

Although both of these operons have been cloned [6, 9, 33], studied intensively [2, 8, 17, 18], used in numerous gene expression studies to detect chemical inducers and/or the induction of stress responses [3, 15, 20, 23, 25], and have been compared in their responsiveness and sensitivity to other reporter genes [4, 11, 12], only one study briefly looked into the improvement of a *V. fischeri lux* biosensor through the use of a *P. luminescens lux* fusion [5]. Furthermore, high-density immobilized bioluminescent bacterial systems are now being used to study genome-wide transcriptional responses to different chemical and environmental stimuli [30, 32]. To date, however, a direct

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comparison between these two operons, such as thermolabile and thermostabile *lux*, when fused to several of the same promoters, has not been reported. Therefore, in this study, six strains, each having the promoter region from one of three different oxidative damage-responsive genes (*i.e.*, *sodA*, *pqi-5*, and *katG*) fused to one of the operons, were characterized and compared with respect to their basal level, maximally induced bioluminescences, and response kinetics.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

All of the bacterial strains used in this study are listed in Table 1, along with their plasmids. *E. coli* strain RFM443 [rpsL- (StrR), galK2, lacΔ74] was transformed with the *Photorhabdus luminescens* (*PL-lux*) pDK1 (*katG::PL-lux*), pDS1 (*sodA::PL-lux*), and *pSodA::VF-lux* to give strains DK1 [20], DS1, and EBHJ2 [14], respectively. Strain DPD2511 [3], (RFM443 carrying p*katG::luxCDABE*), was a gift from Robert LaRossa at the DuPont Co., U.S.A., and contains a fusion of the *katG* promoter to the *VF-lux* operon from *Vibrio fischeri*. Strains DP1 and EBJM are the *pqi-5::lux*/RFM443 strains (Table 1). The strains were initially grown overnight in 4 ml sterile LB media (pH=7) in a 15 ml test tube at the temperature (30°C or 37°C) that is to be used the following day, in a rotary shaker set at 250 rpm. The media used had 50 μg/ml ampicillin added to it.

For chemical exposure tests, 300 μl of the overnight culture were transferred to 50 ml of sterile LB with ampicillin in a 250-ml flask and grown to an optical density (O.D.) of 0.08 at 600 nm under the same conditions as the seed. At this time, the test chemical was added to the final desired concentration, and optical density and bioluminescence were measured every 20 min for 3 h using a spectrophotometer and a Turner 20-e Luminometer, respectively. The bioluminescence was measured with a 100 μl sample of the culture. Three flasks were used for each condition for error analysis.

Table 1. Strains and plasmids used in this study.

Strain	*E. coli* host	Plasmid	Oxidative species
DPD2511	RFM443[a]	p*KatG::luxCDABE* (Vf[b])	H_2O_2
DK1	RFM443	p*KatG::luxCDABE* (Pl[b])	H_2O_2
EBHJ2	RFM443	p*SodA::luxCDABE* (Vf)	O_2^-
DS1	RFM443	p*SodA::luxCDABE* (Pl)	O_2^-
EBJM	RFM443	p*Pqi-5::luxCDABE* (Vf)	O_2^-
DP1	RFM443	p*Pqi-5::luxCDABE* (Pl)	O_2^-

[a]The genotype of RFM443 is (*rpsL*- (StrR), *galK2*, *lacΔ74*).
[b]V-Thermolabile *lux* from *Vibrio fischeri*. P-Thermostable *lux* from *Photorhabdus luminescens*.

To determine whether the degradation of the bioluminescent signal was caused by a loss of the LuxA and B proteins or the LuxC, D, and E proteins, which are required for substrate production, a 100 μl sample of the culture was mixed with 100 μl mixture of LB and 0.1% dodecyl aldehyde, mixed thoroughly, and the bioluminescence read. The addition of excess exogenous aldehyde overcomes any substrate limitation, resulting from which may be the lack of or degradation of the LuxC, D, and E proteins.

Molecular Cloning Techniques

To compare the promoters when fused to the different *lux* genes, similar regions were amplified using PCR. Furthermore, the plasmids used in this study, both of which are medium-copy number, have the same origin of replication and offer resistance to ampicillin. All DNA modifying enzymes used in this study were purchased from New England Biolabs (NEB), U.S.A. The PCR was performed using the HotStart® kit from Qiagen, U.S.A. The primers used for the PCR of the *sodA* promoter were SA1 - 5'-CCGTTGTC-GAATTCCTGGCAATCACG-3' and SA2 - 5'-CTCATAT-TCGGATCCAGTATTGTCGGG-3', corresponding to the region - 141 bps to +58 bps with respect to the start of transcription. The underlined regions correspond to the *Eco*RI and *Bam*HI sites used to clone the PCR product into pDEW201 [31]. After transformation, the resulting colonies were checked for bioluminescence production, and positive strains were grown and their plasmid DNA, pDS1, was purified. Plasmid pDK1 has been described previously [20]. Briefly, it was constructed with the PCR product for the *katG* promoter from p*katG::luxCDABE* [3] using the following primers: KS1 - 5'-GTCCGGATCCGGACATA-ATCAAAAAAGC-3' and KS2 - 5'-CTGATGGTACCG-**TCA**TTTGCCAGTGGC. The underlined regions correspond to the *Bam*HI and *Kpn*I sites used in cloning, and the bold letters are the artificial stop codon included within the primer which lies within the reading frame of the catalase gene, giving a truncated 15 amino acid polypeptide. This PCR product was then digested with *Bam*HI and *Kpn*I and ligated into pDEW201 [31]. The resulting plasmid, pDK1, was transformed into RFM443, and the colonies were checked for their responsiveness to hydrogen peroxide through the production of bioluminescence. One colony was selected for further experiments and was labeled pDK1/RFM443.

For plasmid pDP1, the primers used were PI1 - 5'-CGCGGAATTCCTCAAACCAAGC-3' and PI2 - 5'-CATTGGATCCTTAATGATGCGC-3', corresponding to the region - 393 bps to +337 bps with respect to the start of transcription. The underlined regions correspond to the *Eco*RI and *Bam*HI sites used to clone the PCR product into pDEW201 [31]. After transformation into DH5α, the resulting colonies were checked for bioluminescence production, and positive strains were grown, their plasmid DNA,

pDS1, purified and transformed into RFM443. The sequences of all PCR products (the *katG*, *sodA*, and *pqi-5* promoters) were checked to confirm that they were correct. The corresponding *VF-lux* strains, EBHJ2 and DPD2511, have been described in other publications [3, 14]. For plasmid *pqi-5::VF-luxCDABE*, the promoter region was amplified through PCR using the following primers: EBPQ1 - 5'-TAGGGATCCTCAAACCAAGC-3' and EBPQ2 - 5'-CTAGAATTCGCACATTGGTA-3'. This product, which corresponds to the region 393 bps to +340 bps with respect to the start of transcription, was digested with *Bam*HI and *Eco*RI (underlined regions) and ligated into plasmid pUCD615 [24]. This plasmid was then transformed into RFM443, giving strain EBJM.

Chemicals

Methyl viologen (paraquat) was purchased from Sigma-Aldrich Chemical Co., U.S.A., and 30% hydrogen peroxide solution was from Merck Chemical Co., U.S.A. The paraquat stock solution was prepared to 100,000 ppm using sterile deionized water. Hydrogen peroxide was diluted directly from the purchased stock. The chemical concentrations used were based upon the maximum relative bioluminescent values (the ratio of the sample's bioluminescence to that of the control) from tests with the *PL-lux* strains within a 96-well luminometer (data not shown). In brief, a concentration of approximately 50 ppm paraquat for DS1 and DP1, and 30 ppm hydrogen peroxide for DK1 resulted in the highest relative bioluminescences. Therefore, these concentrations (100%) and 10-fold dilutions (10% and 1%) were used in this study to characterize the responses from these three strains.

Data Analysis

All samples were performed in triplicate for error analysis. The standard deviations for the results are shown as error bars within the graphs.

Results and Discussion

Basal and Induced Bioluminescence Levels

All of the strains constructed or selected for this study are responsive to oxidative damage, either to the presence of hydrogen peroxide (*katG*) via the OxyRS pathway or the superoxide anion (*sodA* and *pqi-5*) through the SoxRS regulators [7, 10, 27]. Therefore, paraquat and hydrogen peroxide were selected as chemical inducers, and the highest concentrations used were 50 and 30 ppm, respectively. In addition to all strains being constructed with the same *E. coli* background, both promoterless plasmids used to construct these strains offer resistance to ampicillin and have the same origin of replication, thus providing a similar number of copies within the strains. Furthermore,

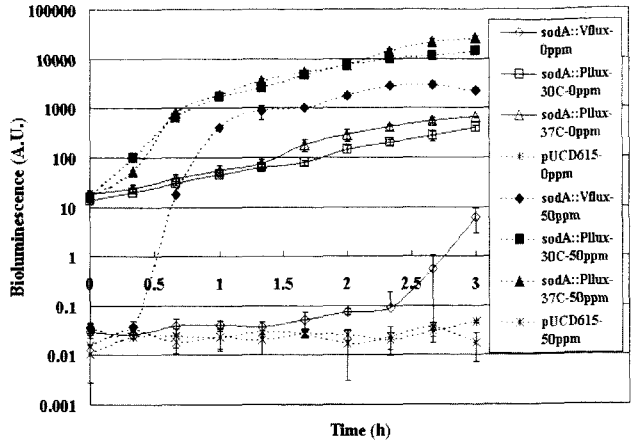

Fig. 1. Response of EBHJ2 and DS1 to the addition of 50 ppm paraquat.
Strain DS1 was tested at both 30°C and 37°C. Open symbols/dashed lines Unexposed culture; Closed symbols/solid lines: 50 ppm paraquat added.

strains transformed with the promoterless plasmids were not bioluminescent (data not shown), while similar regions of the promoters were cloned to guarantee a true comparison.

The bioluminescence (BL) time course plots for the two *sodA* strains, DS1 (*sodA::Pl-lux*) and EBHJ2 (*sodA::Vf-lux*), after exposure to 50 ppm paraquat, are shown in Fig. 1. Although both strains responded very strongly to the addition of paraquat, some distinct differences are apparent. Firstly, the basal level bioluminescence of the *V. fischeri lux* (*Vf-lux*) strain EBHJ2 was about 1,000 times lower on average that that of the *P. luminescens lux* (*Pl-lux*) strain, DS1, even when the *sodA::Pl-lux* strain was cultured at 30°C, the same temperature as used for EBHJ2. Although a slightly lower BL was seen for the *sodA::Pl-lux* strain when grown at this temperature, which might be due to both a lower growth rate and a lower luciferase activity, it was still higher than the *sodA::Vf-lux* strain. Furthermore, this was seen with all the strains tested in this study, with 10~100-fold higher basal BL level with the *sodA-Pl-lux* and *pqi-5-Pl-lux* strains and 1,000-fold higher level from the *katG-Pl-lux* strains, when compared to their *Vf-lux* counterparts.

Degradation of the Bioluminescent Signal

This difference in the bioluminescent signals was studied more indepth using the *katG* fusion strains, DK1 (*katG::Pl-lux*) and DPD2511 (*katG::Vf-lux*), since both of these reached a maximum bioluminescence level within about 1 h, unlike the *sodA-lux* strains, which increased continuously (Fig. 1). Figure 2 shows the bioluminescence values relative to the maximum BL value seen during the test. The bioluminescence level of DPD2511, the *katG::Vf-lux* strain, dropped suddenly after reaching a maximum, and eventually reached a level near that of the control, totaling a loss of more than 99.9%

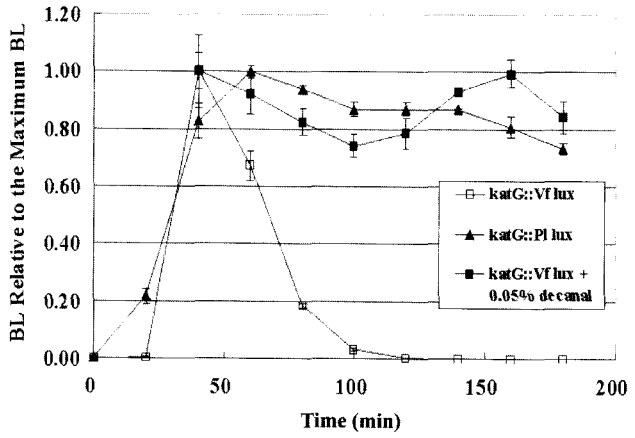

Fig. 2. Degradation of the bioluminescent signal.
Bioluminescent values are shown relative to the maximum BL seen. Samples from the DPD2511 culture were also mixed with 0.1% decanal to supplement the substrate. A total of 30 ppm hydrogen peroxide was added to cultures of DPD2511 and DK1 growing at 30°C and 37°C, respectively.

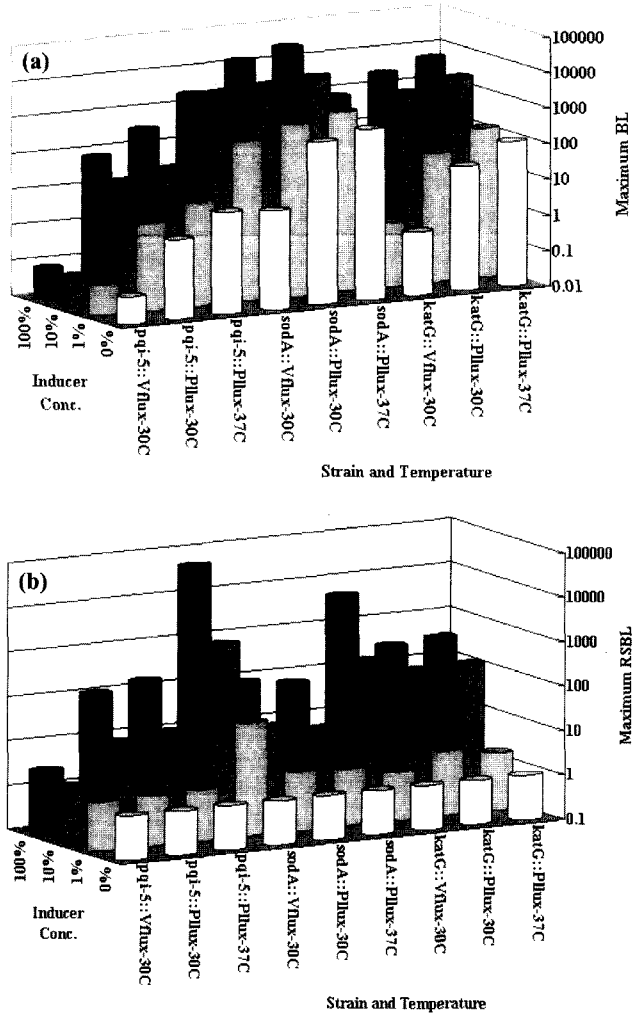

Fig. 3. Maximum (a) bioluminescence and (b) relative specific bioluminescence values for the strains used in this study.

from the maximum value within 2 h. In contrast, the BL from the *katG::Pl-lux* strain remained fairly constant and only decreased by about 25% from the maximum during the same time period. This reduction, however, cannot be attributed to the diffusion of the *lux* proteins into the daughter cells, since the optical density of the *katG::Vf-lux* strain only doubled one hour after its maximum value, yet showed a loss of more than 95% of its BL activity.

Therefore, to determine whether this loss was due to the degradation of the LuxA and B luciferase subunits or possibly the LuxC, D, and E proteins, which are responsible for aldehyde synthesis, the *katG::Vf-lux* culture samples were mixed with 100 µl of 0.1% decanal solution, giving a final concentration of 0.05% decanal or about 25 mM. Under these conditions, if the LuxC, D, or E proteins were being degraded, the excess addition of decanal would supplement the lost production of substrate. This was found to be the case, since the BL of the aldehyde-supplemented samples of the *katG::Vf-lux* strain paralleled that of the *katG::Pl-lux* strain, DK1 (Fig 2). A faster degradation of the bioluminescence, such as that seen with the *katG::Vf-lux* strain, however, provides a clearer image of the gene's expression pattern, since the *Vf-lux* proteins are quickly degraded if the gene is no longer transcribed or is repressed.

Furthermore, the apparent absence of degradation with the *sodA::Vf-lux* strain (Fig 1), EBHJ1, can be explained by the continuous production of the superoxide anion by paraquat [13], which would persist in causing damage and eliciting a stress response within *E. coli.*

Relative Response

Although the *Vf-lux* signal was degraded faster, the maximum induced bioluminescent levels were similar between the strains, as shown in Fig. 1, but was always higher for the *Pl-lux* strains (Fig. 3a). As a result, the high bioluminescent levels from the induced *Vf-lux* strains can be translated into substantial per-cell light levels or specific bioluminescences (SBL), defined as the sample's bioluminescence divided by the culture's optical density at the same time. Furthermore, they can have considerably higher relative specific bioluminescences (RSBL); *i.e.*, the sample's SBL divided by that of the control for the same time point, rather than their *Pl-lux* counterparts, due to the lower basal BL levels of their control (Fig. 3b), with the two strains showing the highest RSBL values being *Vf-lux* strains, EBHJ2 and DPD2511. However, that of the *pqi-5::Vf-lux* strain, EBJM, was lower than DP1, the *pqi-5::Pl-lux* strain, for the same paraquat concentrations. This difference may be attributed to the nature of the *pqi-5* promoter, since the *pqi-5::Vf-lux* strain showed little or no activity above that of RFM443 carrying the promoterless plasmid pUCD615 (Fig. 4).

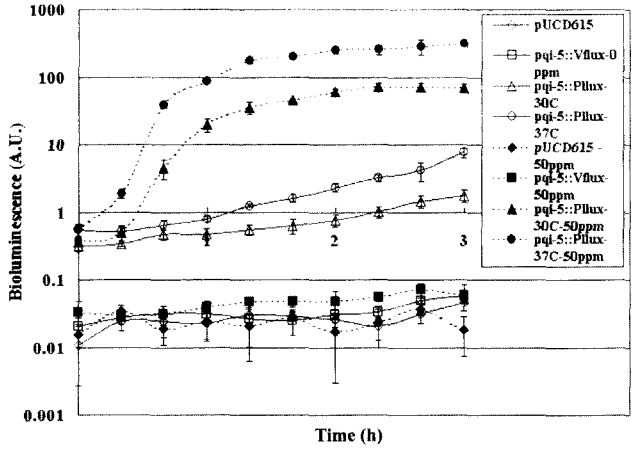

Fig. 4. Response of pUCD615/RFM443, EBJM, and DP1 to the addition of 50 ppm paraquat.
Strain DP1 was tested at both 30°C and 37°C. Open symbols/dashed lines: Unexposed culture; Closed symbols/solid lines: 50 ppm paraquat added.

However, when this plasmid, p*Pqi-5::luxCDABE* (Vf), was transformed into another bacterial host (*i.e.* DH5α), this strain was both bioluminescent and responsive (Data not shown). This apparent lack of activity in RFM443, therefore, is thought to be partly due part to the stability of the *Vf-lux* proteins; *i.e.*, any transcriptional activity from the *pqi-5* promoter, when fused to *Vf-lux*, may be masked by the quick degradation of the *lux* proteins, whereas the stability of the *Pl-lux* proteins allows the strain to produce a discernible response.

Response Time

Table 2 lists the time needed for each of the strains to achieve a two-fold induction or its maximum RSBL. Although very similar, the *Pl-lux* strains tended to be little faster than their *Vf-lux* counterparts in achieving a two-fold induction. Interestingly, they also tended to be slower than the *Vf-lux* strains in reaching a maximum RSBL in response to hydrogen peroxide, but faster for paraquat. The quicker response for paraquat at 37°C may be due to faster production of the superoxide anion at the higher temperature, since the results for the same test done at 30°C were similar to those from the *Vf-lux*. However, the longer time needed for the *Pl-lux* strains to reach a maximum RSBL value with hydrogen peroxide might be the result of a higher rate of denaturing for the *Vf-lux* proteins, which would act first to balance out and then reduce the overall bioluminescent signal. This can be seen in Fig. 2 for the nonsupplemented samples, where the *Pl-lux* strains BL increased smoothly up to the maximum value, while that of the *Vf-lux* strain increased dramatically from 0.5% to 100% within one reading and then the signal proceeded to degrade.

A comparison between strains carrying oxidative-stress promoter fusions with the *lux* operon from either *Vibrio fischeri* or *Photorhabdus luminescens* was performed. The basal BL expression levels from the *Vf-lux* strains were much lower, about 10- 1,000-fold, than the strains carrying the same promoter fused to *Pl-lux*. In addition, although the strains were similar in their induced BLs, tests with various concentrations of chemical inducers found the *Pl-lux* strains to consistently give higher BL values. Due to the *Vf-lux* strains, inherently lower basal BL levels, however, the RSBL values from these strains were generally much higher. One benefit of using a *Pl-lux* fusion is the ability of *Pl-lux* to respond even when fused to weakly expressed promoters, such as the *pqi-5* promoter. Based upon the responses of strain EBJM, shown in Fig. 4, it would be difficult to analyze the transcriptional response from the *pqi-5* promoter, when *E. coli* was exposed to paraquat. However, when the same promoter was fused to *Pl-lux*, the response was easily seen. The reason for this is the stability

Table 2. Growth rate and time, in minutes, to achieve a two-fold and maximum RSBL value. Percentages are relative to the maximum concentration tested, *i.e.*, 30 ppm hydrogen peroxide and 50 ppm paraquat. The strains are described in Table 1.

Strain	Temp.	μ[a]	Chemical	2-Fold			Max. RSBL		
				1%	10%	100%	1%	10%	100%
DPD2511	30	1.02	Hydrogen peroxide	NSR[b]	20	20	NSR	40	40
DK1	30	0.89	Hydrogen peroxide	40	20	20	60	60	60
DK1	37	1.26	Hydrogen peroxide	60	20	20	80	80	80
EBHJ2	30	0.86	Paraquat	80	40	40	140	140	140
DS1	30	1.01	Paraquat	80	40	20	160	180	100
DS1	37	1.13	Paraquat	80[c]	40	20	140[c]	80	80
EBJM	30	1.02	Paraquat	NSR	100[c]	100	NSR	100[c]	100
DP1	30	1.00	Paraquat	NSR	40	40	NSR	160	120
DP1	37	1.26	Paraquat	NSR	40	20	NSR	120	120

[a]Growth rate (h^{-1}).
[b]NSR: No significant response (The RSBL value was below 2-fold).
[c]The maximum RSBL value was less than 2 but not significantly different ($p>0.1$).

of the *Pl-lux* proteins, which allowed generation of a bioluminescent signal, while turnover of the *Vf-lux* proteins presumably occluded the transcriptional response.

In conclusion, based upon its higher basal levels, capability of being used at both 30°C and 37°C, similar sensitivities with *Vf-lux* strains, and the stability of the induced response, *P. luminescens* luciferase fusions by and large appear to be more beneficial for uses in gene expression studies.

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