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Improved Sensitivity of the *in vivo lux* Reporter System in Measuring Reactive Oxygen Species during Antibiotic Treatment of *Escherichia coli*

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Reactive oxygen species (ROS) in *Escherichia coli* can be produced through aerobic respiration as well as through environmental stressors and has been shown to cause direct damage to various cell components. Measurements of internal ROS production have previously been performed through the use of a *lux* operon with *luxCDABE* genes under the control of the *katG* promoter. This study tested the sensitivity and effectiveness of two split *lux* systems (*luxAB* and *luxAB+luxCDE*) for measurement of internal ROS enhanced through the external substrate, nonanal, and induced through the antibiotics, tobramycin and norfloxacin, compared to the sensitivity of the full *luxCDABE* reporter system. The split *lux* system was found to have an enhanced luminescence response to antibiotic treatment, where *luxAB* and *luxAB+luxCDE* produced 2.8 times and 1.5 times higher signal than the full *luxCDABE* system, respectively. The response ratio of the split system containing only *luxAB* was the highest at 5.5 times and 30 times larger than the full *luxCDABE* and split *luxAB+luxCDE* systems, respectively. Through calculations of EC_{200} and the detection limit, the split *luxAB+luxCDE* system was shown to be the most sensitive in detecting ROS. Variation within the data suggests that other factors may contribute to the effectiveness and sensitivity of the *lux* systems, possibly competition between external and internal substrates in the *luxAB+luxCDE* system. Overall it was determined that the split *lux* reporter systems were more sensitive to ROS than the full *luxCDABE* system, and also produced a stronger luminescence signal.

Reactive oxygen species (ROS) are highly reactive molecules that are present as a by product of aerobic respiration in bacterial cells and can also be produced through environmental stressors (1). ROS can cause direct damage to the cell by reacting with the DNA, RNA, proteins and the membrane of the cell (1). The bacterial cell, therefore, has developed various mechanisms to deal with the ROS produced when the ROS concentration exceeds the cell's normal capabilities to defend against it, termed as oxidative stress. The OxyR regulon in *Escherichia coli* is one such defense mechanism against peroxides (1). A peroxidase - HPI is coded by *kat*G and is transcribed when the oxidised form of OxyR is bound to the promoter of *kat*G (2). Cells treated with Bactericidal antibiotics have previously been shown to have increased ROS, mainly hydroxyl radicals (3, 4), production.

A reporter construct under the control of the *kat*G promoter has been previously used to measure the ROS within a cell. For example, the *kat*G::*lux* fusion used by Belkin *et. al*. (5) to detect oxidative stress in *E.coli*. The *lux* operon consisted of *luxCDABE*, where *luxAB* codes for the enzyme complex – luciferase and *luxCDE* codes for the fatty acid reductase complex that is needed to produce the aldehyde substrate on which luciferase acts to create the luminescence (6). Traditionally, the *lux* reporter system consists of *luxCDABE* genes under the control of an inducible promoter (8, 7). This system, however, was not sufficiently sensitive to reliably quantify the stressor inducing the oxidative stress (4, 10). Yagur-Kroll and Belkin (9) discovered that reporter systems with a split *lux* system in which the *luxAB* genes are under the control of an inducible promoter and the *luxCDE* genes are under the control of a constitutive promoter, or any such combination of inducible and constitutive promoters, produced a signal on induction that was far greater than that from a reporter system consisting of *luxCDABE* under the control of a single inducible promoter. Determining a sensitive system to reliably measure an environmental stressor would be an incredibly useful tool to measure potential small changes in the bacterial environment and can be potentially used to measure similar small changes in the larger environment to help circumvent and prepare for any negative changes, as shown by Belkin (11).

The purpose of this experiment was to determine if split *lux* systems, derived from *P. luminescens* (9), (either *luxCDE+luxAB* or just *luxAB)* were more effective and sensitive reporters than the full *lux* operon system for accurately measuring potential oxidative stress, formed by the antibiotics tobramycin and norfloxacin (4). From our experiments, the split *lux* systems had a better signal than the full operon system, however, the system containing only *luxAB* had the higher signal of the two.

MATERIALS AND METHODS

Chemicals. All chemicals used were of the highest analytical grade. Nonanal, norfloxacin, tobramycin, hydrogen peroxide (H_2O_2), dimethylformamide (DMF), and paraquat (methyl viologen) were obtained from Sigma Aldrich. Bacterial strain *E. coli* K12 strain BW25113 (The University of British Columbia, Vancouver, BC, Canada) was used for activity assays, and the plasmids from S. Yagur-Kroll and S. Belkin (The Hebrew University, Jerusalem, Israel), p*katG::luxAB*, p*katG::luxCDE* and p*katG::luxCDABE,* were received in strains MG-I, AG-I and RFM1 respectively (9). M9 minimal media was prepared with 0.05% NaCl (Fisher), 0.7% Na_2HPO_4 (ACS), 0.3% KH_2PO_4 (EM Science), 0.1% NH_4Cl (BDH Chemicals), 0.02% $MgSO_4 \cdot 7H_20$ (Gibco), and 0.2% glycerol (Sigma Aldrich).

Strain preparation. Each bacterial strain received from Yagur-Kroll and Belkin was grown in LB broth at 37°C to an OD_{600} of 0.6. Plasmid isolation was performed using the GeneJET Plasmid Miniprep Kit (Thermo Scientific). The isolated plasmids, p*katG::luxAB*, p*katG::luxCDE*, and p*katG::luxCDABE*, were transformed into competent wild-type *E. coli* (BW25113) as outlined in (12). Transformants were recovered by adding LB broth (Fisher) and incubating at 37°C for 45 min. Antibiotic selection was carried out using LB agar (Fisher) plates containing 100 µg/ml ampicillin, 50 µg/ml chloramphenicol, or both. Frozen stock was created by mixing 500 µl of 40% glycerol (Fisher) in dH2O and 500 µl of bacterial culture in a 1.0 ml cryogenic vial stored at -80°C. The strains generated include: the full *luxCDABE* system (CLYV13W-1), the split *luxCDE+luxAB* system (CLYV13W-2), and the split *luxAB* system (CLYV13W-3) as listed in Table 1.

TABLE 1. Genotypes of each strain.

Strain	Plasmid with inducible gene inserted	Transformants	Antibiotic selection
E.coli BW25113	*luxAB*	CLYV13W-3	ampicillin
E.coli BW25113	*luxCDE* and *luxAB*	CLYV13W-2	ampicillin and chloramphenicol
E.coli BW25113	*luxCDABE*	CLYV13W-1	chloramphenicol
E.coli BW25113	N/A (Wildtype)	N/A	none

Minimum inhibitory concentration (MIC). In a 96-well plate, concentrations of 2.0, 1.0, 0.5, 0.25, 0.125, 0.0625 µg/ml of tobramycin and norfloxacin were prepared using M9 minimal media supplemented with tobramycin and norfloxacin stocks, and bacterial cells ($1.0x10^7$ cells). Each of the four strains was incubated with tobramycin and norfloxacin, separately. The positive control was M9 media without antibiotics and *E. coli* culture; negative control was M9 media without any antibiotics or culture. The colonies were incubated in 37°C overnight. The colonies were visually observed to check for no growth.

Growth rate. Freshly inoculated cultures of each strain (Table 1) was grown in M9 minimal media with aeration at 37°C and measured for turbidity at 600 nm.

Paraquat experiment. Paraquat, a known chemical that generates reactive oxygen species, was used to optimize the concentration of the substrate, duration of reactive oxides generation, and the sensitivity of the luminometer. Each stain was grown until it reached OD_{600} of 0.8 (13). Dilutions of paraquat were prepared in concentrations of 200, 100, 50, 20, 10, and 1 mg/l. Luciferase activity for paraquat treated cells was reported in RLU using a Turner Biosystems Luminometer TD-20/20 at default sensitivity of 60%. RLU was measured at t = 0 until 60 min at 10 min intervals. The optimal sensitivity setting of the luminometer was tested, and adjustments were made so that the entire range of sample data can be measured.

Nonanal experiment. Nonanal was used as a substrate to produce luminescence in the activity assay, and the concentration to be used was optimized. The substrate (96% nonanal) was dissolved in DMF to create a 1 mg/ml stock of nonanal. CLYV13W-3 strain was grown until the pre-determined OD_{600} of 0.6. Culture was supplemented with two concentrations below the MIC of tobramycin (1.0 µg/ml and 0.5 µg/ml) and norfloxacin (0.125 µg/ml and 0.063 µg/ml). At every 25 min, 1 ml of culture was removed and a varied amount of nonanal was added to make final concentrations of 50, 30, 20, 10, and 1 µg/ml. RLU was reported at 40.2% sensitivity, 3-second delay, and measured through 10 seconds.

Experimental conditions and data analysis. Each strain was grown in 200 ml of M9 minimal broth supplemented with the proper antibiotics (Table 1) overnight at 37°C to an OD_{600} of 0.6. Nonanal was added to sampling cuvettes prior to the addition of culture. Strains were treated with three different concentrations of tobramycin (2.0, 1.0, and 0.5 µg/ml) or norfloxacin (0.25, 0.0125, and 0.0625 µg/ml) at time zero. One ml of culture was added to the sampling cuvette. Luminescence was reported every 20 min for one hour. Untransformed parental strain BW25113 treated with antibiotic was the negative control. Samples without antibiotic were untreated controls for sensitivity measurements. Background signal was reported using the samples of each treatment without nonanal. Response ratio is calculated as [ΔRLU treated - ΔRLU untreated] ÷ ΔRLU untreated, where ΔRLU is Relative Light Units minus the background. Raw RLU measurements were used to calculate the EC_{200} and detection limit for CLYV13W-3, CLYV13W-2, and CLYV13W-1. The EC_{200} was calculated as the antibiotic concentration resulting in a two-fold increase in RLU compared to the untreated controls as seen in (14). Detection limit was calculated as the antibiotic concentration resulting in a RLU three standard deviations above the background (15). The lower the EC_{200} and detection limit, the more sensitive the system (14, 15).

RESULTS

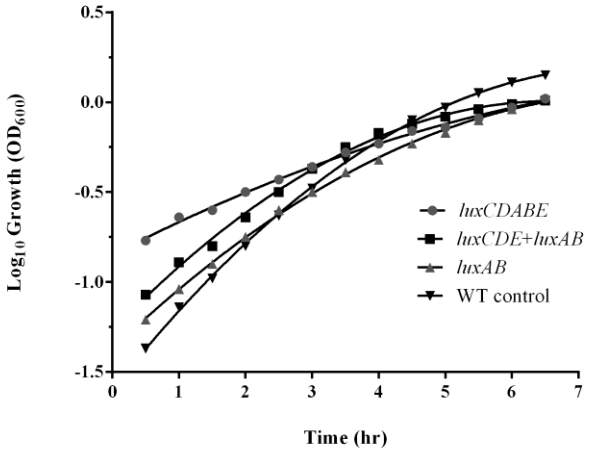

FIG 1. Difference in growth across *E. coli* harboring the split *lux*, full *lux*, or wild-type control system. CLYV13W-1, *katG::luxCDABE*; CLYV13W-2, *katG::luxCDE+katG::luxAB*; CLYV13W-3, *katG::luxAB*; BW25113, wild-type negative control.

The rate of growth for all strains in M9-minimal media. The data points from the growth experiment were plotted on a semi-log graph (Fig. 1). All four cultures showed log growth till an OD_{600} of 0.5, reached at approximately 3.5 hr. The WT BW25113 strain had the highest growth rate, showing the steepest curve, followed by strains CLYV13W-3, CLYV13W-2 and finally CLYV13W-1 which showed the slowest growth rate. The growth in minimal media showed that the *lux*CDABE plasmid in strain CLYV13W-1 impacted growth. There

were small differences in the initial growth rate of the other constructs, but had rates comparable to the WT BW25113 strain lacking the plasmid. As the turbidity increased, the rate of growth for the strains decreased, with the strain CLYV13W-2 showing the most significant decrease. Thus in order to lessen the impact of the slowed growth, the experiment was done around the concentration of 0.5.

The response of all strains to paraquat. The highest RLU was reported for strain CLYV13W-2 representing the split *lux* system, and basal level signal was generated by wildtype BW25113 strain (data not shown). There was no dose-dependent response to paraquat; however, a time-dependent trend was observed. The optimal time for induction was determined at 60 min, as signals were highest for all strains. Sensitivity of 60% was used for the luminometer, and it was determined that an optimal sensitivity was 40.2%, with which all signals were measurable.

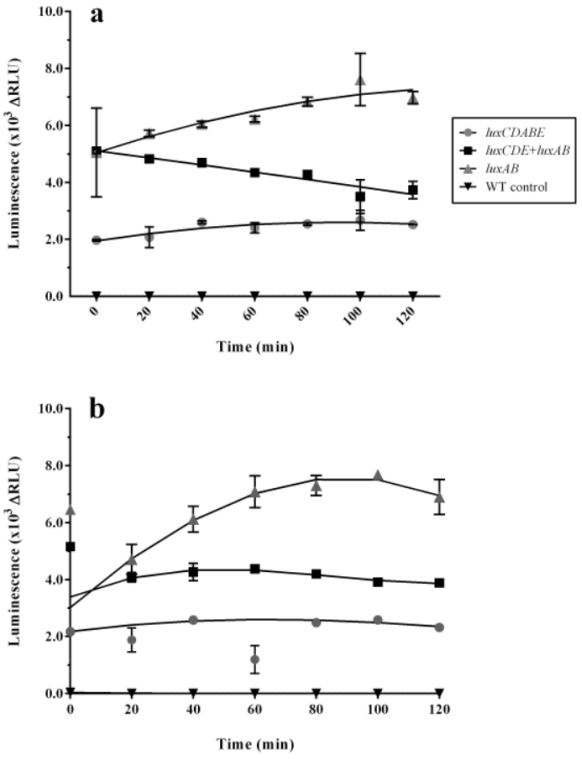

FIG. 2. Effect of antibiotic treatment on the luminescence response of *E. coli* containing the full *lux* or split *lux* system. a) response to 1.0 µg/ml of tobramycin; b) response to 0.25 µg/ml of norfloxacin. CLYV13W-1, *katG::luxCDABE*; CLYV13W-2, *katG::luxCDE+katG::luxAB*; CLYV13W-3, *katG::luxAB*; BW25113, wild-type negative control. Luminescence is measured in Relative Light Units (ΔRLU) where background RLU is subtracted for each datum point. n=2. Error bars represent standard deviation. Trend lines are non-linear regression: second order polynomial for a) and third order polynomial for b).

The response of all strains to the nonanal substrate. The nonanal experiment was performed to determine the concentration at which nonanal is not limiting so that the luminescence is only limited by the *lux* genes. A preliminary experiment revealed that when nonanal was added to the culture directly, the luminescence readings dropped sharply after the initial reading. After nonanal was re-added to the culture before a second reading, concentrations of 30 µg/ml and below showed an increase back to the original luminescence, however, concentrations of 100 and 50 µg/ml still showed a significant decrease, compared to the first reading (data not shown). This result proposed two models in which the cultures interact with the nonanal substrate - the death model and the consumption model.

The death model suggests that the decrease in luminescence is due to death of cells from the toxicity of the nonanal. This model was tested by adding more nonanal to the culture after the luminescence has dropped. Nonanal concentrations of 100 and 50 µg/ml did not revert back to the original luminescence levels, thus, supporting the death model. Previous research by Deng in 1993 found that long carbon-chain aldehydes are toxic to bacterial proliferation (17). However, no specific concentration level of nonanal was mentioned to be toxic.

The consumption model suggests that the decrease in luminescence is due to the consumption of nonanal to produce the luminescence. Re-adding nonanal after the drop of luminescence reverted the readings back to the original reading for concentrations of 30, 20, 10, and 1 µg/ml of nonanal. This data supports the consumption model.

In the amended substrate experiment, where nonanal was added to the cuvette before measurement, the amount of luminescence increased with increase in substrate concentrations. However, this trend stops between 30 and 50 µg/ml, where the two concentrations produced similar luminescence readings. Since there was very little change in signal between 30 and 50 µg/ml of nonanal, the concentration of 30 µg/ml was chosen for future experiments.

Split *lux* reporter systems enhance luminescence response to antibiotic treatment. The strains listed in Table 1 were tested for luminescence response to treatment by tobramycin or norfloxacin. The Minimal Inhibitory Concentration (MIC) for all four strains was 2.0 µg/ml for tobramycin and 0.25 µg/ml for norfloxacin. In response to 1.0 µg/ml of tobramycin, the split *lux* systems (strains CLYV13W-2 and CLYV13W-3) produced significantly stronger luminescence throughout the experiment that was 1.5-fold and 2.8-fold higher compared to the full *lux* system (strain CLYV13W-1) at 120 minutes, respectively (Fig. 2a). Between the split *lux* systems, although strain CLYV13W-2 was predicted to have the highest signal, strain CLYV13W-3 produced a stronger response that increased over time, while strain CLYV13W-2 had a weaker response that decreased slightly over time. Similar trends were observed in response to 0.5 and 2.0µg/ml of tobramycin (data not shown).

In response to 0.25 µg/ml of norfloxacin, similar results were observed: the split *lux* systems produced greater luminescence throughout the experiment (where strain CLYV13W-3 had the highest response), while the full *lux*

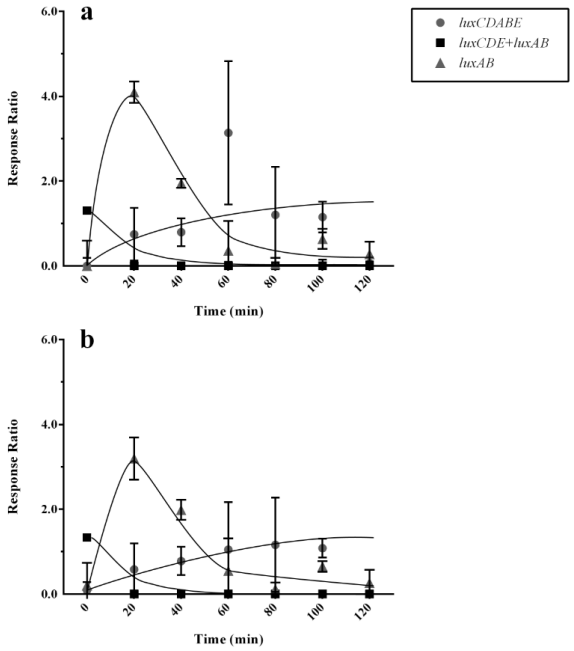

FIG. 3. Effect of antibiotic treatment on the response ratio of *E. coli* containing the full *lux* or split *lux* system. a) response ratio from 1.0 µg/ml of tobramycin; b) response ratio from 0.25 µg/ml of norfloxacin. CLYV13W-1, *katG::luxCDABE*; CLYV13W-2, *katG::luxCDE+katG::luxAB*; CLYV13W-3, *katG::luxAB*. n=2. Error bars represent compounded standard deviation. Trend lines are second order polynomial non-linear regression for a) and b).

system had a weaker response that was relatively unchanged (Fig. 2b). Similar trends were observed in response to 0.125 and 0.063 µg/ml of norfloxacin (data not shown).

For both antibiotic treatments, the wild type negative control (BW25113) produced no luminescence and background luminescence was insignificant (not shown), indicating that ROS was detected intracellularly via the expression of the *lux* genes exclusively. Overall, the expression of the split *lux* systems proved more effective in detecting ROS than the full *lux* system, suggesting that splitting the *luxCDABE* genes under their own promoters, with the addition of external substrate (nonanal), enhanced gene expression and subsequent luminescence response.

Split *katG::luxAB* system affords strongest response ratio from antibiotic treatment. Unlike the luminescence response (Fig. 2), the response ratio presented a non-uniform percent change over time. Treatment with 1.0 µg/ml of tobramycin or 0.25 µg/ml of norfloxacin produced a similar trend in response ratio that was unique to each strain (Fig. 3).

After treatment with 1.0 µg/ml of tobramycin, the split *luxAB* system (strain CLYV13W-3) showed the highest and fastest response trend, peaking at 20 minutes, before slowly decreasing over time (Fig. 3a). The maximum point of strain CLYV13W-3 at 20 minutes presented a 5.5-fold and 30-fold greater response ratio than strains CLYV13W-1 and CLYV13W-2, respectively. The split *luxCDE+luxAB* system (strain CLYV13W-2) produced a faster but lower response trend, which immediately decreased shortly after the start of the experiment. In contrast, the full *luxCDABE* system (strain CLYV13W-1) produced a slow increase and delayed response trend over time. Interestingly, strain CLYV13W-1 showed a higher response ratio compared to strain CLYV13W-2 at all time points, indicating a completely opposite response pattern than in Fig. 2a. The pattern of the response ratio was universal for the other concentrations of tobramycin tested (data not shown).

Treatment with 0.25 µg/ml of norfloxacin produced similar results: the split *luxAB* system produced the highest and fastest response ratio trend, the split *luxCDE+luxAB* system produced a faster but lower trend, and the full *luxCDABE* system produced a slow sustained response over time (Fig. 3b). Similarly, strains CLYV13W-1 and CLYV13W-3 showed greater ratios at all time points compared to strain CLYV13W-2 (Fig. 3b).

Together, response ratios from both antibiotic treatments displayed that strain CLYV13W-3 produced the highest response ratio, suggesting that the split *luxAB* system is more effective at an earlier time point than the full *luxCDABE* or split *luxCDE+luxAB* systems. However, across all time points, the full *luxCDABE* system showed a slower sustained response ratio trend than the split *luxCDE+luxAB* system, suggesting that the *lux* genes were expressed and substrate was consumed steadily throughout the full *luxCDABE* system . The non-uniformity of the data points for each strain and the inconsistency of results between the response ratio (Fig. 3) and luminescence response (Fig. 2) may be explained by the large variability in luminescence for untreated cells across strains.

***Split luxCDE+luxAB* system provides superior sensitivity for ROS detection.** To quantitatively identify the most sensitive system for ROS detection, EC_{200} and detection limit were calculated using raw luminescence data from Fig. 2. EC_{200}, the antibiotic concentration causing a twofold increase in luminescence over the untreated samples, for tobramycin and norfloxacin were lowest for strain CLYV13W-2 which contained the split *luxCDE+luxAB* system (Table 2). Like the response ratio (Fig. 3), calculation for EC_{200} was limited by the variability in luminescence of untreated samples. Results for the detection limit, the antibiotic concentration that causes a luminescence three standard deviation above the background, were less optimal; where the lowest values for detection limit for tobramycin and norfloxacin were inconclusive (Table 2). Additionally, the calculation for detection limit produced negative values, suggesting that each corresponding system may be detecting signals that are false positive, at least at low antibiotic concentrations. Additionally, the calculation for detection limit, much like for EC_{200}, was limited by the low number of concentrations tested for each antibiotic. Overall, the split *luxCDE+luxAB* system found in strain CLYV13W-2 provided the most sensitive measure of luminescence response. Interestingly for strain CLYV13W-2, EC_{200} for norfloxacin (0.18 µg/ml)

TABLE 2. EC_{200} and detection limit of tobramycin and norfloxacin using *E. coli* containing full or split *lux* systems.

Strain	Construct	Tobramycin (µg/ml)		Norfloxacin (µg/ml)	
		EC_{200}[a]	Detection[a] Limit	EC_{200}[a]	Detection[a] Limit
CLYV13W-1	*katG::luxCDABE*	2.9	-0.80	0.47	-0.01
CLYV13W-2	*katG::luxCDE+katG::luxAB*	0.1	-4.65	0.18	-0.76
CLYV13W-3	*katG::luxAB*	8.1	-5.28	0.31	-0.18

[1] values were calculated at 0 min, immediately after antibiotic treatment

was above two of the three antibiotic concentrations tested for luminescence and response ratio (0.25 µg/ml, 0.125 µg/ml, and 0.063 µg/ml), which suggest an outlier in the data points used for EC_{200} calculation or that the use 0.125 µg/ml and 0.063 µg/ml of norfloxacin were simply not ideal.

DISCUSSION

The growth experiment data was initially read incorrectly and thus, an incorrect concentration of 0.6 OD_{600} was used for the entire experiment. The actual log phase ends around an OD_{600} of 0.5, therefore, cells in their stationary phase were used. The overcrowding of the cultures could have contributed to the difference in duplicates. Overcrowding of the bacteria limits the dispersion of antibiotics and substrates in the culture (16) resulting in the bacteria being exposed unevenly to the treatments initially. Over time, however, the error due to overcrowding diminishes, as simple diffusion will eventually equilibrate the entire solution. The cells in non-log phase indicates that the cells in this culture are not as active as the cells in log phase growth. This error in choosing the wrong concentration may cause a lack of activity further in the experiment, and show a slightly smaller response to the nonanal substrate.

The split *lux* systems (strains CLYV13W-2 and CLYV13W-3) were more effective at producing a greater luminescence response compared to the full *lux* system (strain CLYV13W-1) after treatment with either tobramycin or norlfoxacin (Fig. 2a and 2b). This trend in luminescence correlates with the trend seen in the growth curve (Fig. 1) for each strain. This trend in luminescence might be because the split *lux* systems have shorter sequences to transcribe and therefore, can produce a quicker and a stronger signal (9). It was, however, unexpected that the split *luxAB* system (producing only luciferase) had a much higher signal than the split *luxCDE+luxAB* system which produces an enzyme (reductase) to provide the internal substrate in addition to luciferase. A possible explanation may relate to the fact that strain CLYV13W-3 can only use the external substrate (nonanal), whereas strain CLYV13W-2 can use both the internal substrate produced and the external substrate. As such, there might be competition between the external and the internal substrate, as seen from the reduced luminescence response of strain CLYV13W-2, where the internal substrate is preferred over the external substrate, a preference noted by Meighen (19). Therefore, the internal substrate, which needs time to be expressed is not present in non-limiting concentrations, as is nonanal. Hence, the luminescence produced by the systems expressing *luxCDE* is not as high as that produced by *luxAB* alone, suggesting that the *luxAB* system may not be burdened by any such preference towards an internal substrate. However, while the preference of the internal substrate over the external substrate was noted by Meighen (19) there have been no other studies to support this preference. It is, therefore, just as likely that strain CLYV13W-2 produces less luminescence than strain CLYV13W-3 because CLYV13W-2 has more plasmids than strain CLYV13W-3, causing the plasmids in strain CLYV13W-2 to be less stable (20).

An interesting trend was the gradual decrease in luminescence seen in strain CLYV13W-2 after treatment with either antibiotic (Fig. 2a and 2b). This might be due to the fact that the ROS being induced causes peroxidation of fatty acids (18), possibly diminishing the quantity of internal substrate preferred by systems containing *luxCDE* for producing luminescence (19). However, the decrease in luminescence is only seen in the split *luxCDE+luxAB* system and not the full *lux* system. This indicates another possible cause for the decrease in luminescence, which may be due to the preparation of the split *lux* system (9). In its preparation, perhaps some important signal that maintains the stability of either the LuxAB complex or the LuxCDE complex may have been excluded in the plasmids (19). This might explain why the luminescence of the full *lux* system increases slightly while the luminescence of the split *luxCDE+luxAB* system, reliant on the external substrate, diminishes over time.

As another measure of the effectiveness of the system, the response ratio displays the percent change of signal

detection as an adjusted luminescence ratio, wherein the signal from non-induced strains is subtracted. However, the results in Fig. 3 show no reliable trend as the response ratio was dependent on the luminescence produced by the untreated strains which produced varying amounts of luminescence (data not shown). Furthermore, as previously reported by Yagur-Kroll and Belkin, when the baseline response (in our case, the luminescence of untreated strains) varies considerably, the raw luminescence response is more representative for evaluating ROS detection than the response ratio (9). As such, the maximum peak seen for strain CLYV13W-3 at the 20 minute time point is most likely explained by the dip in luminescence seen in untreated strain CLYV13W-3 (data not shown), and is not representative of the actual ability of strain CLYV13W-3 to produce ROS-induced luminescence. The same can be said for the higher response ratio calculated for strain CLYV13W-1, which was expected to produce a lower response ratio than the split *lux* systems throughout the experiment. Therefore, although the split *luxAB* system has been shown to have the highest luminescence response and response ratio, the response ratio remains an unreliable method of detection for systems that produce a highly variable basal or non-induced signal such as ours.

The unexpectedly high and variable luminescence produced by all the untreated strains (data not shown) may be because the experiment was performed with bacterial cell cultures that were already in the stationary phase. The cells may have produced an autoinducer, known to be present in media with high cell density (19), which induces the *katG* promoter. Alternatively, the *katG* promoter may have simply been prematurely induced due to the fact that the overcrowded culture did not have enough dissolved oxygen to support the growth of all the cells.

Although the split *luxAB* system has been shown to be more effective in signal detection based on the magnitude of luminescence response, the split *luxCDE+luxAB* system is a more sensitive system for ROS detection based on its EC_{200} and the detection limit for tobramycin and norfloxacin compared to other systems. However, as the detection limit calculated for all strains produced negative values, it may be indicative of the fact that the systems are detecting false positive signals. This is, unfortunately, difficult to evaluate given our data as we only tested three different concentrations for each antibiotic and have no clear trend. Similar to the response ratio, EC_{200} is limited by the variance in luminescence from untreated strains, in addition to the low number of antibiotic concentrations tested. Based on the limitations in both calculations of EC_{200} and detection limit, it is difficult to definitively suggest which system is truly more sensitive without conducting further studies.

Our experiments determined that the split *lux* reporter systems were more sensitive to ROS than the traditional full *luxCDABE* reporter system. The split *lux* systems produced a stronger total luminescence signal with change in ROS in the presence of the antibiotics tested.

FUTURE DIRECTIONS

After assessing the role of culture phase in the responses of the *lux* reporter, it is important to understand the basis of the reduced luminescence in the systems expressing *luxCDE* (strains CLYV13W-1 and CLYV13W-2). It is therefore, important to determine if the internal substrate is preferred over the external substrate by *luxAB*. A possible experiment would be to use a system that contained the split *lux* system similar to strain CLYV13W-2 but had *luxCDE* under the control of another inducible promoter not induced by ROS. The construct should then be used in the same experimental system as that of our experiments. If the construct shows the same luminescent trend before and after inducing both *luxAB* and *luxCDE* then it does not prefer any particular substrate. However, if after induction of *luxCDE* the luminescence drops then it may indicate a preference of the system for the internal substrate. Alternatively, if a system involving Forster Resonance Energy Transfer (FRET) (21) is used it will reduce the background signal and the signal seen in cells not treated with antibiotics which was seen while using *lux* reporter systems. A possible experiment would involve creating a strain containing separate plasmids expressing interacting proteins tagged with the appropriate fluorescent tags and both under the control of a promoter induced by ROS, for example the promoter for *kat*G. Using FRET would allow for a more specific signal with reduced background as most strains do not contain fluorescing components (21).

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