Increased Intracellular Hydrogen Peroxide Observed in *Escherichia coli* in the Presence of Two Classes of Antibiotics

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The modes of action of antibiotics on their target cells are well established. It has been shown previously that bactericidal classes of antibiotics stimulate hydroxyl radical production in cells as a result of the oxidative damage cellular pathway, in addition to their specific drug-target interactions; however, the production of reactive oxygen species (ROS) as a general cell death mechanism is a point of contention. This study aimed to investigate the effects of norfloxacin and tobramycin, two classes of bactericidal antibiotics, on growth rates and intracellular H₂O₂ production of *Escherichia coli*. Three *E. coli* strains, BW25113 (the wild type), JW3914-1, and JW1721-1 (ΔkatG and ΔkatE mutants respectively), were exposed to H₂O₂. Both mutants had a depressed growth rate compared to the wild type, although growth was further reduced in ΔkatG, which is consistent with the role of KatG as the main catalase responsible for scavenging H₂O₂ during log phase growth. When exposed to sub-inhibitory levels of tobramycin and norfloxacin, results indicated that there was no difference between the growth of BW25113 or ΔkatG. A reporter system consisting of BW25113 transformed with a plasmid containing *katG::luxCDABE* was used to establish if norfloxacin and tobramycin indirectly induced the production of H₂O₂. Luminescence was greater in the presence of tobramycin compared to norfloxacin, although both antibiotics did produce a signal. This experiment therefore supports the hypothesis that bactericidal antibiotics elicit the production of ROS, as indicated by the results of our lux reporter system.

Our current understanding of antibiotic-mediated cell death is centered on specific drug-target interactions and their direct effects. Bactericidal antibiotics are separated into three broad classes based on their mechanism of action. Quinolones inhibit DNA replication and repair, causing double-stranded DNA breaks; aminoglycosides cause protein mistranslation resulting in an altered cell membrane structure and increased permeability to the drug; and, β-lactams inhibit cell-wall turnover compromising the structural integrity of the cell (4, 10). Recent work has shown that despite the specific mode of action utilized by each of these drugs, antibiotics may work to induce cell death in a more complex fashion than just primary drug-target interaction. It has been proposed that in addition to their primary targets, bactericidal antibiotics work via a common killing mechanism that induces formation of hydroxyl radicals (5, 11, 12, 15).

The hydroxyl radicals produced are the end products of an oxidative damage cellular death pathway involving the reduction of hydrogen peroxide, and alterations in central metabolism and iron metabolism (5, 10). Cells growing under aerobic conditions are exposed to hydrogen peroxide (H₂O₂), which is a byproduct of aerobic respiration (7). In the presence of increased concentrations of H₂O₂, bacteria will upregulate catalase enzymes as a protective measure (9). *Escherichia coli* carries two distinct catalase genes, *katG* and *katE*, which are activated under different conditions (6) although they both convert H₂O₂ into water and oxygen (7). The catalase encoded by *katG* is part of the OxyR regulon and is induced in the presence of H₂O₂ (2). In contrast, *katE* which is fully induced under aerobic conditions, is not further induced in the presence of H₂O₂, and is not a part of the OxyR regulon (3, 17). While aerobic cells are able to eliminate the toxicity presented by H₂O₂ with catalase enzymes, there is no enzyme to detoxify the hydroxyl radical (7).

A lux reporter system, *luxCDABE* fused to a *katG* promoter, was used to detect the level of peroxides present in the cellular environment (2, 8). The presence of peroxides oxidizes the OxyR transcription factor, which induces the *katG* promoter and thus expresses the downstream luciferase protein, which fluoresces. The amount of luminescence produced is proportional to the amount of peroxides present, and therefore allows for a quantitative measurement of peroxide levels within the cell. In this study we investigated the basic premise of
Intracellular hydroxyl radical production by bactericidal antibiotics. Two broad-spectrum antibiotics, tobramycin (an aminoglycoside) and norfloxacin (a quinolone), were examined to determine whether any production of ROS was limited to either class of antibiotics, or constituted a general mechanism of killing as previously suggested (11, 20). We found that exposure to both tobramycin and norfloxacin increased the level of peroxides within the cell, supporting the hypothesis that bactericidal antibiotics generate ROS as a general killing mechanism.

MATERIALS AND METHODS:

Bacterial strains and growth conditions. E. coli strains BW25113 (the parent strain), JW1721-1 (referred to subsequently as ΔkatE), and JW3914-1 (referred to subsequently as ΔkatG) (Table 1) were obtained from the University of British Columbia’s (UBC) bacterial strain collection, originally from the Keio collection (1). Strains BLST11W-1 and BLST11W-2 were constructed by isolating the pKatGlux2 plasmid from the DPD2511 strain (2) using the Fermentas GeneJet plasmid isolation kit (lot 00024023) and transforming the plasmid into BW25113 and JW1721-1. Cells were chilled on ice and made chemically competent with 0.1 M calcium chloride. 200 ng of pKatGlux2 was added and cells incubated on ice for 30 min, prior to a 45 sec heat shock at 42°C. Transformants were recovered in Luria broth for 60 min at 37°C, and plated on Luria agar (1% tryptone, 0.5% yeast extract (Bacto), 0.5% NaCl (Fisher), 1.5% agar (Invitrogen)) containing 100 µg/ml ampicillin (Sigma) as a selection marker. All strains were carried out at 37°C on a shaking platform in M9 salts minimal media, consisting of 0.05% NaCl (Fisher), 0.7% NaHPO₄ (ACS), 0.3% KH₂PO₄ (EM Science), 0.1% NH₄Cl (BDH Chemicals), 0.02% MgSO₄⋅7H₂O (Gibco), 0.2% glycerol (Sigma Aldrich).

Luminescence testing of transformed strains. Following transformation of BW25113, ΔkatE, and ΔkatG with pKatGlux2, transformed strains were challenged with H₂O₂, and production of luminescence was observed in order to confirm successful transformation. Luminescence was monitored using Turner Biosystems Luminometer TD-20/20, over a period of 1 hr.

Minimum inhibitory concentration (MIC) assay. Overnight cultures of BW25113, ΔkatE, and ΔkatG were added in duplicate to a 96-well microtitre plate containing tobramycin (1, 0.5, 0.25, 0.125, 0.063, and 0.032 µg/ml), norfloxacin (0.25, 0.125, 0.063, 0.032, 0.016, 0.008 µg/ml), and a positive control containing no antibiotic. Sterile M9 salts minimal media was added to empty wells as a negative control. The plate was incubated overnight at 37°C, and the MIC was established as the lowest concentration of antibiotic at which there was no visible growth.

Cell growth assay with H₂O₂. Overnight cultures of BLST11W-1, BLST11W-2, and ΔkatG were diluted 1/2 with M9 salts minimal media and incubated for 30 min. After pre-incubation, cultures from each strain were added in triplicate to a 96-well microtitre plate containing tobramycin (0.3, 0.6, and 1.2 µg/ml) norfloxacin (0.055, 0.11, and 0.22 µg/ml), H₂O₂ as a positive control (15, 30, and 45 µg/L), and a negative control containing no antibiotic. The plate was incubated over the course of 120 minutes and the optical density was read at 620 nm using the Tecan Spectrafluor-Plus Reader. The initial reading of the plate took 4 min. This lag in time was compensated by normalizing the readings of the H₂O₂ treatments to the optical density of the control condition for each strain. This experiment was supposed to supplement data from an additional lux reporter assay using transformed strains, different from the MIC assay. The ΔkatG strain was not successfully transformed with the pKatGlux2 plasmid.

Cell growth assay with antibiotics. Overnight cultures of BW25113 and ΔkatG were diluted in additional M9 salts minimal media to an OD₅₉₀ of 0.15 and incubated for 30 min. After pre-incubation, cultures from each strain were divided into three equal portions, and treated with tobramycin (0.3 µg/ml), norfloxacin (0.055 µg/ml), or left untreated as the negative control. Samples were taken over the course of 120 min, and optical density was measured in a Biochrome Ultrospec 2000 UV/Visible Spectrophotometer at 500 nm.

lux reporter assay monitoring katG expression. An overnight culture of BLST11W-1 was diluted 1/2 with additional media, and was incubated for 30 minutes. The culture was divided into equal sized portions and treated as follows: tobramycin (0.3, 0.6, and 1.2 µg/ml), norfloxacin (0.055, 0.11, and 0.22 µg/ml), H₂O₂ as a positive control (30 µg/ml), or a negative control containing no antibiotic. Flasks were left stationary and at room temperature, and luminescence was measured with a Turner Biosystems Luminometer TD-20/20 over a 75 min time-course. The last datum point for the positive control (Fig. 3) was calculated from a 1-in-5 diluted sample, since the upper limit of luminescence reading was 10000 RLU. The lux reporter assay was performed with only one strain, BLST11W-1, due to time limitations and inconsistent luminescent readings of BLST11W-2.

RESULTS

MIC Assay. Of the antibiotic concentrations tested, all three strains (BW25113, ΔkatE, ΔkatG) had the same MIC of 0.5 µg/ml for tobramycin and 0.063

<table>
<thead>
<tr>
<th>Strain Abbrev.</th>
<th>Strain Characteristics</th>
<th>Reference Collection</th>
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<tbody>
<tr>
<td>BW25113</td>
<td>Parent strain</td>
<td>UBC MICB 421</td>
</tr>
<tr>
<td>JW1721-1</td>
<td>ΔkatE</td>
<td>UBC MICB 421 collection</td>
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<tr>
<td>JW3914-1</td>
<td>ΔkatG</td>
<td>UBC MICB 421 collection</td>
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<tr>
<td>DPD2511</td>
<td>RFM443 parent strain containing pKatGlux2</td>
<td>UBC MICB 421</td>
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<tr>
<td>BLST11W-1</td>
<td>BW25113 parent strain containing pKatGlux2</td>
<td>UBC MICB 421</td>
</tr>
<tr>
<td>BLST11W-2</td>
<td>JW1721-1 strain containing pKatGlux2</td>
<td>UBC MICB 421</td>
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TABLE 1. Escherichia coli K12 strains used in this study
showed slightly heightened RLU when challenged with heightened RLU to transformed mutants in growth assay. Treatment with tobramycin or norfloxacin, hence results can be applied to transformation with a well format. Data were normalized to the control turbidity within each strain.

FIG. 1. Differential growth in response to increasing H$_2$O$_2$ concentrations in (A) BLST11W-1, (B) BLST11W-2, and (C) $\Delta$katG. Each datum point represents mean of replicate well readings (n=3), error bars represent standard deviation, and trend lines are linear regression. Data were normalized to the control turbidity within each strain.

$\mu$g/ml for norfloxacin. MIC assay was conducted to estimate the ranges of antibiotic concentration suitable for the growth assay and was done prior to transformation with lux plasmid. The rationale was that the lux plasmid should not confer cross-resistance to tobramycin or norfloxacin, hence results can be applied to transformed mutants in growth assay.

Transformation and Bacterial Strains. Luminescence testing of BW25113, $\Delta$katE, $\Delta$katG transformed with pKatGlux2 showed significantly heightened RLU only in BW25113, while $\Delta$katE showed slightly heightened RLU when challenged with 30 mg/L H$_2$O$_2$, and no increased luminescence was observed with $\Delta$katG (preliminary data not shown). $\Delta$katG therefore was assumed not to be a true transformant, and due to time constraints, re-transformation of $\Delta$katG was not attempted.

Cell growth assay with H$_2$O$_2$. Transformed strains (BLST11W-1 and BLST11W-2) and $\Delta$katG were used in this assay to examine whether differential growth would be observed in BLST11W-1 in comparison to the knockout strains. This was a complementary set of data for lux reporter assay in a 96-well format, hence transformed strains were used and $\Delta$katG was also included due to extra well availability. Each strain had a different cell concentration after recovery to mid-log phase from overnight culture, especially BLST11W-2, which was significantly less turbid than BLST11W-2 and $\Delta$katG by visual inspection. All samples from each strain had the same initial cell concentration.

When challenged with different concentrations of H$_2$O$_2$, a dose-dependent effect on growth was observed in BLST11W-1 and BLST11W-2, where higher concentrations led to more depressed growth curves (Fig. 1A and 1B). However, growth of BLST11W-2 halted upon challenge by 30 and 45 mg/L H$_2$O$_2$, indicating that the loss of katE may affect ROS resistance at higher H$_2$O$_2$ concentrations. For $\Delta$katG, no growth was observed upon addition of any H$_2$O$_2$ (Fig. 1C). It is also important to note that the growth rate of $\Delta$katG was better than BLST11W-1 and BLST11W-2, which may indicate that the presence of pKatGlux2 depressed the growth rate.

Cell growth assay with antibiotics. Untransformed strains were used to avoid the potential effect of pKatGlux2 plasmid on growth. Only BW25113 and $\Delta$katG strains were used due to the limited number of samples that could be processed. BW25113 and $\Delta$katG appeared to grow similarly in control and antibiotic treatments within the time frame (120 min) of the assay (Fig. 2). No significant differences were observed in the growth rates as measured by turbidity. Fig. 2A showing growth under control conditions, and Fig. 2B showing growth in the presence of norfloxacin, looked similar in that the growth of BW25113 and $\Delta$katG seemed to converge with time. However, in Fig. 2C showing growth in the presence of tobramycin, the growth of BW25113 and $\Delta$katG did not converge, but rather, grew at similar rates (parallel growth trend lines). Although growth was also monitored by viable plate counts, the counts obtained were out of a statistically reliable range. These inconsistent and highly variable data were not presented. Note that the datum point taken at 0 min for the BW25113 in Fig. 2A control condition was an
gave readings above the negative control, indicating that H$_2$O$_2$ was produced intracellularly. Tobramycin appeared to cause a higher production of H$_2$O$_2$ than norfloxacin (Fig 3). A closer look at the norfloxacin treated samples (Fig. 3C), showed that maximum induced luminescence was observed between 45 to 60 min, after which the signal stabilized. The luminescence induction in norfloxacin treatments was approximately 2-fold greater than the negative control and no dose-dependent induction of luminescence was observed. In tobramycin treated samples (Fig. 3D), maximum induced luminescence was observed between 30 to 45 min and was approximately 5.5-fold greater than the negative control. Interestingly, the highest tobramycin concentration tested (1.2 µg/ml) had lower luminescence induction, at approximately 40% of luminescence than lower tobramycin concentrations. This high concentration is approximately 2-fold the MIC concentration for tobramycin; the lower luminescence induction may be due to rapid inhibition of protein synthesis and growth.

**DISCUSSION**

Previous studies that looked at the generation of ROS by bactericidal antibiotics performed their experiments using antibiotics of three different classes: quinolones, aminoglycosides, and β-lactams (11, 20). In this study, norfloxacin, a first generation fluoroquinolone, was selected as a representative of the quinolone group of bactericidal antibiotics. Norfloxacin binds to DNA gyrase and topoisomerase IV, and traps them on the DNA at the cleavage stage, which arrests DNA replication and introduces dsDNA breaks (10). Many bacterial strains used in this study carried kanamycin resistance; therefore tobramycin was selected as a representative aminoglycoside antibiotic as there was no risk of cross-resistance (19). Tobramycin works specifically by binding to both the 30S and 50S subunits of the bacterial ribosome, which prevents protein synthesis and eventually leads to cell death (13).

In the H$_2$O$_2$ exposure growth assay, the dose-dependent effect of H$_2$O$_2$ on growth suppression of BLST11W-1 and ΔkatE (Fig. 1A and 1B), may reflect the increasing degree of ROS damage after induction and possible saturation of KatG by increasing H$_2$O$_2$ concentrations, highlighting the importance of KatG at higher concentrations of H$_2$O$_2$ (20). The similarity of three strains in the control condition (Fig. 1 gray trend lines) reflects some functional redundancy in ROS scavenging enzymes at endogenous H$_2$O$_2$ levels produced from aerobic metabolism (20). While the

outlier. The cuvette used was chemically stained and thus semi-opaque. A new cuvette was used for the reminder of the assay.

**lux reporter assay monitoring katG expression.**

In the katG promoter-controlled lux reporter assay, the controls displayed luminescence readings as expected: the positive H$_2$O$_2$ control induced luminescence 21-fold in comparison to the base signal observed in the negative control at 75 min (Fig. 3). In Fig. 3A and 3B, both the norfloxacin and tobramycin treated samples
importance of KatG is highlighted in BLST11W-1 and ΔkatE under control conditions, comparable growth observed in ΔkatG may be due to the presence of other H₂O₂ scavenging enzymes such as alkyl hydroperoxide reductase (Ahp) (encoded by ahpCF). (18). Ahp is also controlled by the OxyR regulon and has been suggested to act as the primary scavenger in the presence of low endogenous H₂O₂ due to higher affinity and lower Km (18). Comparable growth observed in ΔkatG in the control condition might be due to functional Ahp (20). The detrimental effect on growth observed in ΔkatG when exogenous H₂O₂ was added, regardless of the three concentrations tested, could have been due to saturation of Ahp by H₂O₂ concentrations beyond the enzyme’s Km in addition to the absence of KatG, leading to lethal ROS damage (18).

As expected, the lux reporter in the positive control (exposure to 30 mg/L of exogenous H₂O₂) showed a robust luminescence signal (Fig. 3A and 3B). Whereas the signal observed in the negative control was weak due to stimulation only from endogenous sources of H₂O₂ produced by aerobically growing cells. Both norfloxacin and tobramycin treated BLST11W-1 cells showed a luminescence signal greater than the negative control (Fig. 3C and 3D). This provides strong evidence that the katG promoter is being induced in the presence of antibiotics, in which increased endogenous levels of H₂O₂ drives the expression of luciferase.

Although signal was induced by both antibiotic treatments, norfloxacin gave a weaker signal in general, approximately a third of that produced by tobramycin (0.3 μg/ml and 0.6 μg/ml). This may be explained by the specific mechanism by which each antibiotic interacts with its targets and the limitation of the lux reporter assay. The lux reporter assay has been frequently utilized to track gene expression due to its low background in non-luminescent bacteria, high sensitivity and relatively large range of linearity between lux induction and luminescence (14). However, the dependence of the lux reporter assay on successful luciferase transcription, translation and expression may interfere with the quantification of katG promoter induction in this experiment. Norfloxacin inhibits DNA gyrase and topoisomerase IV, which are necessary for stabilizing and relieving topological stress produced by the unwinding of supercoiled DNA during replication or transcription (4). Chromosomal instability and its subsequent effects on replication and...
transcription in norfloxacin treatment may be too detrimental to the cell to produce a signal comparable to that seen in tobramycin treated samples even if similar levels of \( \text{H}_2\text{O}_2 \) were induced. This is consistent with the observation that the highest concentration of tobramycin (1.2 \( \mu \text{g/mL} \)) produced a weaker luminescence of approximately 40% of the signal in lower tobramycin concentrations (Fig. 3D). At lower concentrations of the aminoglycoside tested, a relatively strong signal approximately 5.5-fold that of the negative control was observed. However, the highest concentration of tobramycin produced only a weak signal in similar ranges as that seen for norfloxacin treated cells. Tobramycin is known to prevent the formation of the 70S bacterial ribosome, and thus protein synthesis (13). At higher concentrations of tobramycin, it is possible that translation is so inhibited that the luciferase enzyme cannot be synthesized resulting in weaker luminescence even though the same or greater levels of \( \text{H}_2\text{O}_2 \) are induced. Therefore, even though the \( \text{katG-lux} \) reporter assay provided strong evidence that increased levels of \( \text{H}_2\text{O}_2 \) were produced in antibiotic-treated cells, this assay was not suitable for accurate quantification of \( \text{H}_2\text{O}_2 \) induction in this experiment. Due to a lack of time, BLST11W-2 was not tested in the \( \text{lux} \) reporter assay.

The cell growth assay performed using the BW25113 and \( \Delta\text{katG} \) strains showed no differences in growth rates in the three conditions tested: control, norfloxacin and tobramycin. It would be expected that if these antibiotics were generating ROS, such as \( \text{H}_2\text{O}_2 \), then the strain deficient in KatG would not be able to sufficiently detoxify the cell, resulting in a depressed growth rate. However, it is important to note that sub-inhibitory MIC concentrations of the antibiotics were used in this experiment. These concentrations may not have been high enough to elicit a detrimental phenotype such as depressed growth rate as measured by optical density. This is consistent with the positive growth still observed even when cells are treated with antibiotics (Fig. 2). The luminescence produced by cells treated with tobramycin and norfloxacin was approximately one-half to one-fifth less, respectively, than that observed when induced by 30 mg/L \( \text{H}_2\text{O}_2 \). This suggests that the low concentrations of antibiotics used may have only produced a very low concentration of \( \text{H}_2\text{O}_2 \) that was detectable by this assay, but may be too low, like the antibiotic concentrations, to contribute to cell death. A study by Wang et al. showed that cells lacking KatG had a susceptibility similar to the wildtype when treated with ampicillin and kanamycin five times greater than the MIC (20). The \( \text{H}_2\text{O}_2 \) generated by antibiotics may be below the \( K_M \) of KatG and is instead scavenged by Ahp, which is thought to be the primary scavenger of peroxides at low substrate concentrations due to higher affinity (lower \( K_M \)) than KatG (20).

Increasing the concentration of antibiotic may cause a more observable change within the time frame of the assay, as it has been shown that an increase in hydroxyl radicals is associated with lethal levels of bactericidal antibiotic treatment (11). It should also be noted that although KatG is the main scavenging enzyme of \( \text{H}_2\text{O}_2 \) (20), there may be other epistatic factors contributing to the lack of change in growth rate seen in this experiment. Also, growth was measured by turbidity, which may not be indicative for assessing changes in cellular shapes and sizes, and viability. Unfortunately, the complementary viable plate counts attained from this assay were inconsistent and out of statistical reliable counting range. Hydroxyl radicals are derived from \( \text{H}_2\text{O}_2 \), and \( \text{H}_2\text{O}_2 \) is generated mainly from superoxide (9). If ROS are being produced by the antibiotics, \( \Delta\text{katG} \) may still be able to compensate via the presence of other antioxidant enzymes, such as Ahp and catalase KatE.

Although the cell growth assay using antibiotic treatments showed no depressed growth, it was likely due to sub-inhibitory antibiotic concentrations that were insufficient to produce a detrimental effect on cells. However, the growth assay using \( \text{H}_2\text{O}_2 \) and the \( \text{lux} \) reporter assay provided convincing support that the tested antibiotics, norfloxacin and tobramycin, both induced the production of \( \text{H}_2\text{O}_2 \), suggesting the existence of a common mechanism, in addition to their specific antimicrobial action.

**FUTURE DIRECTIONS**

Detection of \( \text{H}_2\text{O}_2 \) production in bacterial culture remains a challenge due to the instability of the peroxide. Despite the \( \text{H}_2\text{O}_2 \) quantification limitations of the \( \text{katG-lux} \) reporter assay as suggested in the discussion, it provides valuable insight into the potential of an antibiotic-induced ROS mechanism by qualitative comparison between the treatment signals and the control. To fully explore this potential ROS mechanism, a broader selection of antibiotics from different classes should be tested and other model organisms should be used before a conclusive statement regarding the broad and general characteristic of this mechanism among bacteria can be made. Also, the \( \text{katG-lux} \) reporter can be transformed into a \( \text{katG} \) knockout strain to increase reporter sensitivity by preventing any induced endogenous \( \text{H}_2\text{O}_2 \) to be
scavenged and detoxified prior to inducing OxyR. The increased reporter sensitivity may lead to better resolution of weaker induced luminescence. A more direct method in monitoring H$_2$O$_2$ generation is by measuring levels of OxyR, which is directly induced by H$_2$O$_2$ (16). This can be done using real time quantitative polymerase chain reaction (RT-qPCR), in which oxyR mRNA transcripts are reverse transcribed into complementary DNA, quantified via PCR amplification and incorporation of double-stranded DNA-binding dyes. Use of RT-qPCR would bypass the need for translation in the lux-reporter assay, which would be especially useful when a protein translation inhibitor is used. Also, the induction of oxyR by endogenous H$_2$O$_2$ will be more rapid than katG-lux.

**ACKNOWLEDGEMENTS**

We would like to extend our sincere thanks Dr. William Ramey and Matt Mayer for their continued help and guidance throughout the course of this study, to the Hancock Lab for providing resources and equipment, and to Dr. Shimshon Belkin of The Hebrew University in Jerusalem for providing the pKatGLuxP plasmid. Finally, we thank the Department of Microbiology and Immunology, University of British Columbia for the financial support for this project.

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