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Alternative Luciferase for Monitoring Bacterial Cells under Adverse Conditions

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The availability of cloned luciferase genes from fireflies (*luc*) and from bacteria (*luxAB*) has led to the widespread use of bioluminescence as a reporter to measure cell viability and gene expression. The most commonly occurring bioluminescence system in nature is the deep-sea imidazolopyrazine bioluminescence system. Coelenterazine is an imidazolopyrazine derivative which, when oxidized by an appropriate luciferase enzyme, produces carbon dioxide, coelenteramide, and light. The luciferase from the marine copepod *Gaussia princeps* (*Gluc*) has recently been cloned. We expressed the *Gluc* gene in *Mycobacterium smegmatis* using a shuttle vector and compared its performance with that of an existing *luxAB* reporter. In contrast to *luxAB*, the *Gluc* luciferase retained its luminescence output in the stationary phase of growth and exhibited enhanced stability during exposure to low pH, hydrogen peroxide, and high temperature. The work presented here demonstrated the utility of the copepod luciferase bioluminescent reporter as an alternative to bacterial luciferase, particularly for monitoring responses to environmental stress stimuli.

Bioluminescence is widely distributed in nature, occurring in a remarkably diverse set of organisms, including bacteria, dinoflagellates, fungi, fish, insects, shrimp, and squid (31, 39, 40, 50). Bioluminescence arises from oxidation of a substrate (a luciferin) by an enzyme (a luciferase), usually in the presence of molecular oxygen. Luciferin and luciferase are generic terms as none of the major classes exhibit sequence homology. While phylogenetic analyses suggest that bioluminescence has had more than 30 independent origins, there are five basic luciferin-luciferase systems. The most widely studied bioluminescence systems are those belonging to luminous beetles in the family Lampyridae, more commonly known as fireflies (such as *Photinus pyralis*) (18, 72), and the luminous bacteria (*Vibrio* sp., *Photobacterium* sp., and *Photorhabdus luminescens*) (2, 3, 14, 22, 23, 26, 64). The firefly luminescence reaction is catalyzed by a monomeric ca. 62-kDa luciferase encoded by a single gene (*luc*) and involves the oxidation of a benzothiazoylthiazole luciferin and ATP, resulting in the production of oxyluciferin, AMP, CO_2, and light (18, 47, 51, 72). In contrast, the bacterial (*lux*) luminescence reaction involves the oxidation of a long-chain aldehyde (RCHO) and reduced flavin mononucleotide, resulting in the production of oxidized flavin mononucleotide and a long-chain fatty acid (RCOOH), along with the emission of blue-green light at 490 nm (10, 32, 73). The reaction is catalyzed by bacterial luciferase, a heterodimeric 77-kDa enzyme composed of an alpha subunit and a beta subunit encoded by the *luxA* and *luxB* genes, respectively (22, 25). Bioluminescence is an excellent reporter system (recently reviewed in reference 56), a sensitive marker for microbial detection (13, 15, 27, 28, 49, 57, 58, 70), a real-time, noninvasive reporter for measuring gene expression (11, 12, 19, 33, 36, 48, 51, 53, 54, 55, 69), and a way to measure intracellular biochemical function (cell viability) (1, 4, 5, 16, 17, 27, 28, 29, 30, 34).

The rapid growth of applications of bioluminescence has stimulated research into investigation and exploitation of new bioluminescent systems (44). The most commonly occurring natural bioluminescence system is the deep-sea imidazolopyrazine bioluminescence system that has been found in seven phyla and approximately 90 genera, including copepods, ostracods, cephalopods, and amphipods (66). Coelenterazine is an imidazolopyrazine derivative that acts as the luciferin which, when oxidized by the appropriate luciferase, produces carbon dioxide, coelenteramide, and light (59, 60). One of the most widely studied coelenterazine-catalyzing luciferases is Ruc produced by *Renilla reniformans*, a sea pansy that displays bioluminescence upon mechanical stimulation. Ruc was first purified and characterized by Matthews et al. (45), and the cDNA was later isolated and expressed in *Escherichia coli* (42), transgenic plant tissues (46), and mammalian cells (41) and is now commercially available as an assay system (Promega Corporation).

Gaussia princeps is a bioluminescent marine copepod with a 10-mm-long body that lives at depths between 350 and 1,000 m. It emits bioluminescence as a secretion from 30 glands located in the antennas, cephalothorax, thorax, and abdomen in response to mechanical, electrical, or light stimuli (6–8, 38). The release of a luminous bolus from *G. princeps* is accompanied by rapid swimming that propels the copepod away from the bolus. In this manner, bioluminescence most likely serves as a defense mechanism that startles and blinds dark-adapted predators, providing a glowing decoy to hold the predator's attention while the copepod escapes. The luciferase (*Gluc*) gene from *G. princeps* has recently been cloned and shown to oxidize coelenterazine to produce light (9). The *Gluc* luciferase was subse-

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TABLE 1. Plasmids used in this study

Plasmid	Description	Reference or source
pTKmx	Mycobacterium-*E. coli* shuttle vector encoding a promoterless *xylE* reporter gene, Km^r	37
pSMT1	Mycobacterium-*E. coli* shuttle vector encoding the *luxAB* cassette from *V. harveyi*, Hyg^r	62
pSKLx	Mycobacterium-*E. coli* shuttle vector encoding the promoterless *luxAB* (*V. harveyi*) cassette, Km^r	This study
pSHKLx	Mycobacterium-*E. coli* shuttle vector encoding the promoterless *luxAB* (*V. harveyi*) cassette, Km^r Hyg^r	This study
pSHKLx1	Mycobacterium-*E. coli* shuttle vector encoding a promoted *luxAB* (*V. harveyi*) cassette, Km^r Hyg^r	This study
pUC19Gluc	Cloning vector containing the 540-bp luciferase gene (*Gluc*) from *G. princeps*	Nanolight Technology, Prolume Ltd.
pSHKGluc1	Mycobacterium-*E. coli* shuttle vector containing the promoted *Gluc* gene from *G. princeps*, Km^r Hyg^r	This study

quently used as a bioluminescent reporter of DNA hybridization and shows promise as a detection reagent in immunoassays (68) and in mammalian cells (65).

The present study was designed to investigate the possible use of the *Gluc* luciferase as a reporter system in mycobacteria. An estimated 2 billion people are latently infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis (21). There are about 8 million new infections per year and 2 million deaths, and many of them are in patients already infected with human immunodeficiency virus. New antituberculosis drugs and better vaccines are urgently needed, and research in these areas is a high priority (63). *M. tuberculosis* research requires containment level 3 facilities, which coupled with a slow doubling time (nearly 24 h) makes studies using conventional microbiological techniques challenging. In our laboratory (35, 62, 67) and other laboratories (4, 5, 16, 17, 20, 34) extensive use has been made of the bacterial and beetle luciferases as reporter genes in mycobacteria to determine cell numbers and viability. We have shown that the *Gluc* luciferase is expressed in the fast-growing organism *Mycobacterium smegmatis* and have characterized its performance under different stress conditions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. smegmatis* mc^2 155 (61) and *E. coli* DH5α were used in this study. Liquid cultures of bacteria were grown with shaking at 200 rpm at 37°C in Luria-Bertani (LB) medium supplemented with hygromycin (50 μg ml^{-1}) or kanamycin (100 μg ml^{-1}) as appropriate.

Construction of bioluminescent reporter plasmids. The plasmids and primers used in this study are shown in Tables 1 and 2. Plasmid pTKmx is a mycobacterium-*E. coli* shuttle vector that harbors a promoterless *xylE* reporter gene downstream of a transcriptional terminator (37). The *xylE* gene was excised from the vector by digestion with the restriction enzymes KpnI and SphI. The *luxAB* genes derived from *Vibrio harveyi* were obtained by PCR from plasmid pSMT1 (62) using primers P1 and P2 and directionally cloned using the KpnI and SphI sites in pTKmx to obtain the promoterless reporter plasmid pSKLx. Primers P1 and P2 introduced XbaI and BamHI sites upstream of the *luxAB* genes and an SpeI site downstream of the *luxAB* genes. Kanamycin is not the antibiotic of choice when working with mycobacteria as spontaneous mutants frequently arise. For this reason the gene for resistance to the antibiotic hygromycin was obtained by PCR from plasmid pSMT1 (62) using primers P3 and P4 and was directionally cloned using the SphI and HindIII sites in pSKLx to obtain plasmid pSHKLx. In order to obtain high levels of light expression, the 600-bp promoter for the heat shock gene *hsp60* was obtained by PCR from *M. tuberculosis* H37Rv DNA using primers P5 and P6 and was directionally cloned using the XbaI and BamHI sites in pSHKLx to obtain the promoted construct pSHKLx1 (Fig. 1A). The *luxAB* genes were removed from pSHKLx1 as a BamHI and SphI fragment, and the vector was blunt ended using the Klenow fragment (New England Biolabs, United Kingdom) and dephosphorylated. The 540-bp *Gluc* luciferase gene from *G. princes* was excised from plasmid pUC19Gluc (Nanolight Technology, Prolume Ltd., Pinetop, AZ) using EcoRI and XbaI and was blunt ended using the Klenow fragment (Fig. 1B) before ligation into the pSHKLx1 plasmid with *luxAB* deleted to create pSHKGluc1.

Luminescence assays. Luminescence measurements were obtained in triplicate at room temperature using a tube luminometer (Berthold Autolumat LB953). Luminescence was measured immediately after addition of the substrate for 10 s using an integration time of 1 s, and the results were expressed in relative light units (RLU). For bacteria expressing *Gluc*, a 10-mmol $liter^{-1}$ stock of the substrate coelenterazine (Nanolight Technology, Prolume Ltd., Pinetop, AZ) was prepared in methanol for use at a final concentration of 10 μmol $liter^{-1}$. All coelenterazine solutions were stored at −20°C, and working solutions were kept on ice in the dark during preparation. For bacteria expressing *luxAB* a 1% stock of the aldehyde substrate (decanal) was prepared in ethanol.

Effects of growth phase on the bioluminescence responses of *M. smegmatis*. Liquid cultures of bacteria were grown with shaking at 200 rpm at 37°C in LB medium supplemented with hygromycin. Samples were analyzed during the ex-

TABLE 2. Primers used in this study[a]

Primer	Sequence
P1	.....GGTCTAGAGGATCCGGAGGAATGTTATGAAATTTGG
P2	.....GGACTAGTTTACGAGTGGTATTTGACGA
P3	.....GGGCATGCGAATTCCCGGGGATCCGGTGATTG
P4	.....CCCAAGCTTTCAGGCGCCGGGGGCGGTGTCCGGC
P5	.....GGTCTAGAGGTGACCACAACGACGCCCCCGCT
P6	.....GGGGATCCGCAATTGTCTTGGCCAT

[a] Restriction sites are underlined.

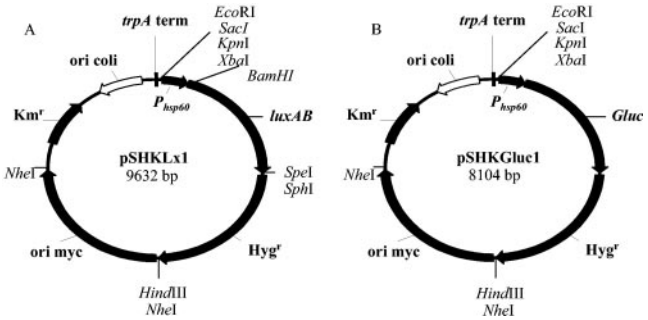

FIG. 1. Mycobacterium-*E. coli* shuttle vectors pSHKLx1 (encoding a promoted *luxAB* [*V. harveyi*] cassette) (A) and pSHKGluc1 (encoding a promoted *Gluc* luciferase gene from *G. princeps*) (B). *trpA* term, terminator sequence; Hyg^r, hygromycin resistance gene; Km^r, kanamycin resistance gene; ori myc, mycobacterial origin of replication (pAL5000); ori coli, *E. coli* origin of replication (pUC); P_{hsp60}, promoter from *M. tuberculosis hsp60* gene.

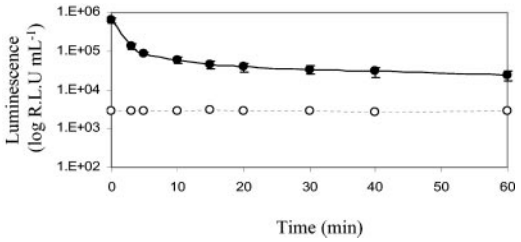

FIG. 2. *M. smegmatis* is capable of expressing the *Gluc* bioluminescence reporter gene, and the luminescence reaction that it catalyzes is a flash reaction. The luminescence is shown for a culture of *M. smegmatis* pSHKGluc1 (OD_{600}, 0.5) after addition of 10 μmol liter^{-1} coelenterazine (●). The luminescence of a comparable culture of *M. smegmatis* without the *Gluc* gene, demonstrating the background chemiluminescent signal of the coelenterazine substrate when it was incubated with bacterial cells, is also shown (○). The error bars indicate standard deviations.

ponential phase (optical density at 600 nm [OD_{600}], 0.5) and during the stationary phase (OD_{600}, 1.2 and 2.5) to determine the effects of the growth phase on bioluminescence. Assays were performed with three independent replicate cultures.

Bioluminescence responses of *M. smegmatis* to pH. Assays were performed with exponential-phase cultures (OD_{600}, 0.5). In the wells of a 24-well microtiter plate, 500-μl aliquots of bacterial cells were added to 500-μl aliquots of LB medium acidified using 2 M HCl as previously described (52). Cultures were incubated statically at 37°C and assayed for bioluminescence (as described above) and cell viability (by plating serial dilutions onto Middlebrook 7H11 agar plates supplemented with 10% oleic acid-albumin-dextrose-catalase and hygromycin as appropriate) at 0 and 1 h. Assays were performed twice with three independent replicate cultures.

Bioluminescence responses of *M. smegmatis* to hydrogen peroxide. Assays were performed with exponential-phase cultures (OD_{600}, 0.5). In the wells of a 24-well microtiter plate, 500-μl aliquots of bacterial cells were added to 500-μl aliquots of LB medium containing hydrogen peroxide that resulted in final concentrations in the range from 0 to 32 mM. Cultures were incubated statically at 37°C and were assayed for bioluminescence and cell viability at 0 and 90 min. Assays were performed twice with three independent replicate cultures.

Bioluminescence responses of *M. smegmatis* to heat shock. Assays were performed with exponential-phase cultures (OD_{600}, 0.5). Bacterial cultures were subjected to 30 min of heat shock at 45°C and 48°C and then incubated at 37°C to recover. Cultures were assayed for bioluminescence prior to heat shock and at regular intervals during recovery. Assays were performed twice with three independent replicate cultures.

RESULTS

Expression of a novel bioluminescence gene in *M. smegmatis*. The gene encoding the luciferase enzyme from *G. princeps* (*Gluc*) was cloned downstream from an *hsp60* promoter in the mycobacterial shuttle vector pSHKGluc1 (Fig. 1) and introduced into *M. smegmatis* by electroporation. The novel bioluminescence reporter gene was stably expressed in both *M. smegmatis* and *E. coli* and catalyzed a flash reaction in which luminescence decreased approximately 10-fold in the first 5 min (Fig. 2). After this initial decrease the rate of decreased slowed, and luminescence remained detectable above the background level after 60 min. The amount of luminescence was dependent on the concentration of substrate added and followed a sigmoid curve. At very low coelenterazine concentrations (below 0.5 μmol liter^{-1}) the flash reaction proceeded extremely fast and the luminescence rapidly decayed to background levels, while at high concentrations the substrate became saturating. However, at final concentrations between 0.1 and 10 μmol liter^{-1} there was a linear relationship between

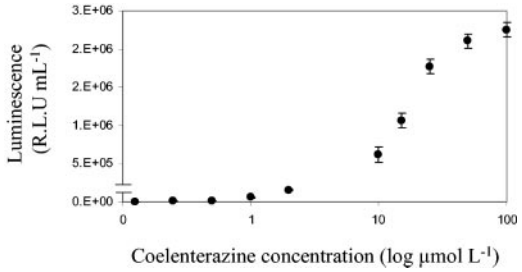

FIG. 3. Relationship between luminescence and coelenterazine concentration in *M. smegmatis* expressing *Gluc*. The results are corrected for the background. The error bars indicate standard deviations.

light output and coelenterazine concentration (Fig. 3). A final concentration of coelenterazine of 10 μmol liter^{-1} was selected for all further assays. At this concentration, coelenterazine exhibited background chemiluminescence of approximately 100 RLU ml^{-1}. The background signal was higher in the Middlebrook 7H9 medium commonly used for mycobacterial growth, and in order to minimize this, LB medium was used throughout the present study. This is in contrast to the lower background signal of the aldehyde substrate used by the bacterial luciferase, which is around 50 RLU ml^{-1} and is not affected by medium composition.

***Gluc* bioluminescence correlates with bacterial cell number throughout the exponential and stationary phases of growth in vitro.** To determine the effects of the growth phase on bioluminescence, *M. smegmatis* cultures were grown in LB medium, and samples were taken at various stages during the exponential and stationary phases of growth. While luminescence from *M. smegmatis* expressing *luxAB* correlated with cell number during the exponential phase of growth, when cells entered the

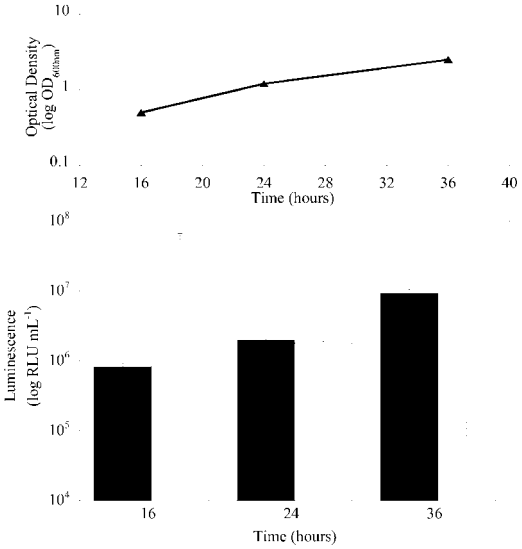

FIG. 4. *Gluc* bioluminescence correlates with bacterial cell number throughout the exponential and stationary phases of growth. The upper panel shows optical density plotted against time, and the lower panel shows a plot of luminescence versus time. Solid bars, luminescence from *M. smegmatis* expressing *Gluc*; open bars, luminescence from *M. smegmatis* expressing *luxAB*. The error bars indicate standard deviations.

TABLE 3. *Gluc* bioluminescence is not affected by exposure to low pH[a]

pH	Luminescence (RLU ml^{-1}) from *luxAB*-expressing cells	Luminescence (RLU ml^{-1}) from *Gluc*-expressing cells
6.80	3.95×10^5 ($\pm 0.77 \times 10^5$)	3.13×10^5 ($\pm 0.38 \times 10^5$)
4.75	4.22×10^5 ($\pm 0.94 \times 10^5$)	7.39×10^5 ($\pm 0.81 \times 10^5$)
3.40	4.09×10^5 ($\pm 0.10 \times 10^5$)	5.19×10^5 ($\pm 0.32 \times 10^5$)
2.75	8.07×10^4 ($\pm 3.14 \times 10^4$)	3.73×10^5 ($\pm 0.20 \times 10^5$)
1.50	3.48×10^2 ($\pm 0.18 \times 10^2$)	3.90×10^5 ($\pm 0.28 \times 10^5$)

[a] The data show luminescence (standard deviations in parentheses) measured after 1 h of exposure to LB medium acidified with HCl.

stationary phase, the luminescence decreased (Fig. 4) (62). Indeed, the RLU/CFU ratios ranged from 0.25 during the mid-exponential phase to 5.81×10^{-5} in the late stationary phase. In contrast, while the luminescence of *Gluc*-expressing cells was lower than that of *luxAB*-expressing cells during the exponential phase, it correlated with the cell number throughout both the exponential and stationary phases of growth (Fig. 4). Indeed, the RLU/CFU ratios remained constant at ca. 0.004 throughout the experiment.

***Gluc* bioluminescence is not affected by exposure to low pH.** To determine the effects of pH on bioluminescence and viability, *M. smegmatis* cultures were incubated for 1 h in LB medium acidified with HCl. With the exception of incubation at pH 1.5, which resulted in a 10-fold decrease in CFU, this treatment was found to have no effect on the viability of *M. smegmatis*, as assessed by the ability of washed cells to form colonies on Middlebrook 7H11 agar (data not shown) (52). The luminescence output from the *luxAB* reporter decreased significantly under these conditions, dropping 10-fold after 1 h of incubation at pH 2.75 and 100-fold after incubation at pH 1.5 (Table 3). In contrast, this treatment had no effect on the bioluminescence of *Gluc*-expressing cells (Table 3).

***Gluc* bioluminescence is not affected by exposure to hydrogen peroxide.** To determine the effects of reactive oxygen species on bioluminescence and viability, *M. smegmatis* cultures were incubated in LB medium containing various concentrations of hydrogen peroxide. This treatment was found to have no effect on the viability of *M. smegmatis* (data not shown). In contrast, the luminescence of *luxAB*-expressing cells was very sensitive to the presence of hydrogen peroxide; there was an almost 10-fold decrease in light output after 90 min of incubation with 0.5 mM hydrogen peroxide, and the number of RLU fell below the limit of detection when the concentration of hydrogen peroxide was greater than 8 mM (Fig. 5). Again, the sustained bioluminescence output of *Gluc*-expressing cells paralleled the viability as assessed by CFU (Fig. 5).

***Gluc* bioluminescence is not affected by heat shock.** *M. smegmatis* cultures were subjected to 30 min of heat shock at 45°C and 48°C. Samples were analyzed before the heat shock, immediately after the heat shock, and after 30 min of recovery at 37°C. This treatment was found to have no effect on the viability of *M. smegmatis* (data not shown). This was reflected by the sustained luminescence of *Gluc*-expressing cells, in contrast to the 5- and 10-fold reductions in *luxAB* luminescence after incubation at 45°C and 48°C, respectively (Fig. 6) (24, 43). The luminescence of *luxAB*-expressing cells was rapidly restored after a 30-min recovery period at 37°C (Fig. 6).

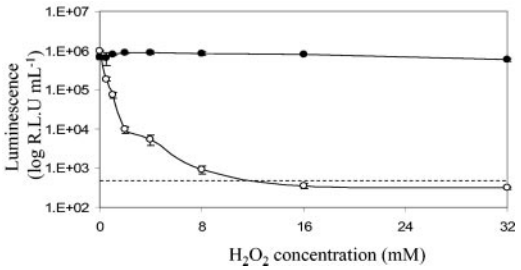

FIG. 5. *Gluc* bioluminescence is not affected by exposure to hydrogen peroxide. Symbols: ●, luminescence from *M. smegmatis* expressing *Gluc*; ○, luminescence from *M. smegmatis* expressing *luxAB*. The limit of detection of the *luxAB* reaction is indicated by the dashed line. The error bars indicate standard deviations.

DISCUSSION

We demonstrated that the copepod *Gluc* luciferase can be expressed in mycobacteria. It catalyzes a flash reaction, but, while luminescence decays rapidly after addition of the coelenterazine substrate, it is still detectable above the background level after 60 min. This compares favorably with both the *luxAB* (bacterial) and *luc* (firefly) bioluminescence systems (Wiles, unpublished data). A drawback to the *Gluc* system is that the chemiluminescent background of the coelenterazine substrate is different in different diluent buffers. While a low background signal was observed with 10 mM coelenterazine in LB medium (10^2 RLU ml^{-1}), the signal was 100-fold greater in the Middlebrook 7H9 medium commonly used for culture of the more fastidious organism *M. tuberculosis*. Alternative media (Sauton's medium, for example [71]) are required when the *Gluc* system is used with *M. tuberculosis*.

Experiments with the *luxAB* reporter system in mycobacteria have shown that there is a strong dependence on changes in the availability of bacterial cofactors under different growth conditions (62, 67). While this is advantageous in signaling a rapid response to the action of some drugs, a sharp decline in luminescence presents a limitation in studying nondividing bacteria in stationary-phase cultures. This is certainly not unique to mycobacteria; in many bacterial species harboring the *lux* operon, bioluminescence declines when cells enter the stationary phase during in vitro growth (28, 70), and this is most likely due to a decrease in metabolic activity. In contrast,

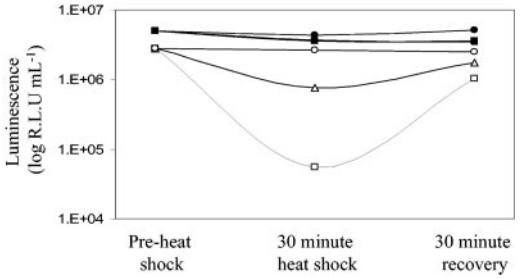

FIG. 6. *Gluc* bioluminescence is not affected by heat shock. Solid symbols, luminescence from *M. smegmatis* expressing *Gluc*; open symbols, luminescence from *M. smegmatis* expressing *luxAB*. *M. smegmatis* cultures were subjected to 30 min of heat shock at 45°C (triangles) and 48°C (squares). Control samples were incubated at 37°C (circles). The standard deviations are smaller than the symbols.

the bioluminescence of *Gluc*-expressing cells appears to be independent of cofactors that become limited during the stationary phase. Thus, the *Gluc* reporter provides a correlate of bioluminescence with bacterial number irrespective of the growth phase.

The *Gluc* system also offers advantages in experiments involving exposure to environmental stress. The ability to survive exposure to low pH and oxidative stress plays an important role in the intracellular survival and pathogenesis of *M. tuberculosis*, and the heat shock response has been used extensively as a model for studying mycobacterial gene regulation (63). However, in our hands multicopy genes under the control of the *hsp60* promoter do not respond to heat shock, presumably because the link with the heat shock regulatory circuit has been broken. Indeed, expression of the *luxAB* genes under the *hsp60* promoter results in the strongest luminescent signal when this expression is compared to the expression with other mycobacterial promoters (Wiles, unpublished results). Exposure of mycobacteria to stress conditions results in rapid dissociation between viability and the output of luminescence from the *luxAB* reporter. This dissociation is not observed with the *Gluc* reporter. Given that the two genes are cloned into and expressed in the same vector backbone under control of the same promoter, it is unlikely that this dissociation is due to increased gene expression or enzyme turnover. This difference may again reflect the relative independence of the *Gluc* reaction from bacterial metabolism or perhaps greater physiological stability of the *Gluc* luciferase enzyme. The *Gluc* system may provide a particularly appropriate reporter for studying responses associated with survival under adverse conditions, both during pathogenesis and in the environment.

Conclusions. In this paper we report the expression of a novel luciferase gene (the *Gluc* gene) from the marine copepod *G. princeps* in *M. smegmatis* that results in detectable luminescence that correlates with viable counts throughout the exponential and stationary phases of bacterial growth under normal conditions in vitro. The work presented here indicated that this luciferase should also be suitable under conditions in which the use of the more traditional bioluminescence genes is limited (for example, in studying bacterial responses to such stresses as oxidative damage and pH). We are currently constructing dual *luxAB-Gluc* systems with the aim of exploiting the relative advantages and disadvantages of the different luciferase reporters.

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