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Use of Luminescent *Leptospira interrogans* for Enumeration in Biological Assays▽

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Rapid and reliable *in vitro* methods for the detection of pathogenic leptospires, such as *Leptospira interrogans*, are lacking. The present study investigated the use of luminescence to replace the existing enumeration techniques. Transposon Tn*SC189* was modified to incorporate the *luxCDABE* cassette from *Photorhabdus luminescens* and was used to construct luminescent *Leptospira* spp. There was a linear relationship between luminescence and cell number, with the theoretical detection limit being less than 10^4 leptospires. A comparison of enumeration by a standard method (counting by dark-field microscopy) and enumeration by luminescence was conducted with luminescent *L. interrogans*. There was a good correlation between the two methods of enumeration ($R^2 = 0.766$), although variation in the luminescence early and late in growth phase reduced the degree of correlation. To demonstrate the utility of luminescence as a viability and cell number reporter, *in vitro* assays, including MIC determination, an extracellular matrix binding experiment, and a complement killing experiment, were conducted. In each case, the results obtained by luminescence matched those obtained by traditional means with high correlations (binding assay $R^2 = 0.916$, complement killing assay $R^2 = 0.988$). A strain expressing the *luxCDABE* transposon retained virulence in the hamster model of infection. Despite some variation in luminescence as a result of the growth phase or the particular assay conditions, enumeration by luminescence was found to be a quick, reliable, and highly sensitive method for the *in vitro* detection of leptospires that has the potential to replace more time-consuming methods of enumeration.

The spirochete *Leptospira interrogans* causes widespread zoonosis, contracted through contact with contaminated water, soil, or animal urine. Disease syndromes can range from no symptoms and a mild influenza-like illness through to multiple-organ failure and death (3).

Despite intensive research into the pathogenic mechanisms of *L. interrogans*, progress has been limited due to the practicalities of working with these bacteria. One significant technical difficulty is the quantitative detection of leptospires *in vitro*. Conventional bacterial enumeration methods are unsuitable due to the unusual features of *Leptospira* spp. A very slow rate of growth (*Leptospira* spp. take up to 4 weeks to form colonies on plates [1]) makes growth-dependent outputs such as CFU counts and minimal dilution slow and prone to overgrowth by contaminants. The unusual morphology of leptospires makes identification by light microscopy difficult, while a low maximum culture density makes estimation of the concentration by measurement of the optical density (OD) of limited use. Quantitative real-time PCR may be used to estimate the leptospiral cell number through the detection of chromosomal DNA (10), but this technique is time-consuming and expensive and does not distinguish live cells from dead cells. The current standard for the enumeration of leptospires involves counting by use of a Petroff-Hausser chamber under dark-field illumination (3). This process requires a trained operator, is time-consuming, and puts the operator at risk of eye strain. Furthermore, this technique is difficult in the presence of other diffracting material that may be present in the output of an experimental assay (e.g., cell debris) and is compromised by the autoagglutination of leptospires and the high degree of motility of some strains. New methods of enumeration of spirochetes are required, such as the recently described flow cytometry method for the enumeration of *Treponema denticola*, an oral spirochete that shares many of the difficult features found in *Leptospira* (16).

To further complicate the study of *L. interrogans*, this pathogen has no replicating plasmids and genetic manipulation through mutagenesis is very difficult. Recently, an *L. interrogans* mutagenesis method was developed with a mariner transposon, Tn*SC189* (1, 12). This is currently the only efficient means for constructing defined leptospiral mutants. This method has been applied for widespread random mutagenesis of the bacterial chromosome (13), facilitating the identification of two virulence factors (14, 17) and the unexpected finding that the major outer membrane protein (LipL32) is not required for pathogenesis (15).

Luminescent bacteria have previously been used for *in vitro* enumeration and viability reporting and for monitoring the progress of infection *in vivo* (8, 9). In the study described here, in the absence of replicating plasmids for *L. interrogans*, transposon Tn*SC189* was modified to incorporate the *luxCDABE* cassette from *Photorhabdus luminescens* (20). This construct was used to stably integrate *luxCDABE* into the chromosome, producing luminescent *Leptospira interrogans* isolates. Luminescence was found to be a highly efficient means for determining bacterial numbers and population viability.

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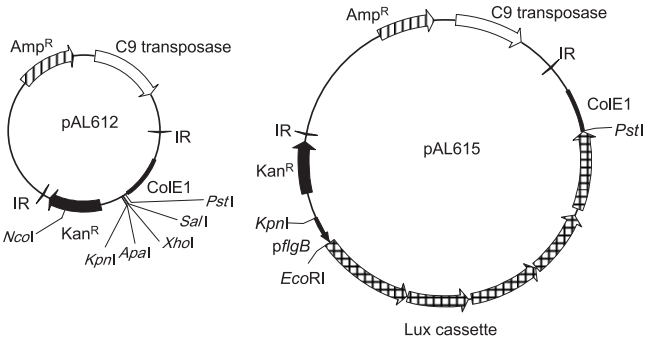

FIG. 1. Features of the plasmid constructs used in this study. Kan^r, kanamycin resistance gene; Amp^r, ampicillin resistance gene; IR, transposon inverted repeat. The relevant endonuclease restriction sites are indicated.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *L. interrogans* serovar Manilae strain L495 was obtained from N. Koizumi, National Institute of Infectious Diseases, Tokyo, Japan. The bacteria were grown in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Becton Dickinson, NJ) at 30°C without aeration. The plates were made by solidification of the medium with 1.5% agar. Kanamycin (25 μg/ml) was added where appropriate. Growth curves were made by diluting stationary-phase leptospires into fresh medium to a concentration of 2×10^7 cells/ml. Counts were performed with a Petroff-Hausser chamber.

Construction of plasmids. The features of the key plasmids used in this study are shown in Fig. 1. During the manipulation procedures, plasmid pSC189 (2) and its derivatives were maintained with ampicillin. To make the transposon easier to propagate in *Escherichia coli* host cells, plasmid pSC189 was modified to remove the *pir*-dependent *oriR6k* and incorporate the ColE1 origin of replication from pBluescript II (13) to make pAL612.

To make a transposon construct containing *luxCDABE* driven by the *flgB* promoter from *Borrelia burgdorferi*, the promoter was amplified from a plasmid construct (kindly provided by Mathieu Picardeau, Pasteur Institute) with primers TTAGGTACCATAATACCCGAGCTTCAAG and TTTCCATGGGCCTAAT TGAGAGAAGTTTC. The final plasmid was constructed in a three-way ligation with the following components: the promoter PCR product digested with KpnI-EcoRI, the *luxCDABE* cassette excised from plasmid pSB406 (20) with EcoRI and PstI, and pAL612 digested with PstI-KpnI.

Transposon mutagenesis. Transposon mutagenesis was conducted as described previously (1, 13). In brief, the leptospires were grown to late log phase and made electrocompetent by repeated washing in ultrapure water. Plasmids containing the Tn*SC189* derivatives were introduced into cells by electroporation, and transformants were selected on EMJH medium plates containing kanamycin. The location of transposon insertion was determined by direct sequencing of the genomic DNA (12).

Measurement of luminescence. Luminescence was measured with an Infinite 200 plate reader (1,000-ms integration, no signal attenuation; Tecan, Switzerland) in a 96-well opaque microtiter tray (Dynatech Laboratories), with the output measured in relative luminescence units (rlu). For the serial dilution assay, serial dilutions of triplicate cultures were measured. The average reading for the medium-only controls was subtracted from the sample readings. Negative values were then adjusted to 1 (the limit of detection) to permit graphing on a log axis.

Antibiotic MIC assay. MICs were determined by serial 2-fold dilution of antibiotics in EMJH medium containing bacteria at 2×10^6 cells/ml in triplicate wells. After 7 days, bacterial viability was assessed by dark-field microscopy and luminescence. The experiment was repeated three times.

Extracellular matrix-binding assay. An extracellular matrix-binding assay was conducted as described previously (7, 15). In brief, quadruplicate wells of a microtiter tray were incubated at 4°C overnight with 100 ml of a 500-μg/ml solution of the extracellular matrix-like compound Matrigel (BD Biosciences) in phosphate-buffered saline and washed, and then a known quantity of bacteria was added. After 1 h of incubation, the wells were aspirated and washed. Adherent bacteria were by removed by digestion with trypsin (200 μg/ml) at 37°C for 5 min and were then enumerated by dark-field microscopy and measurement in a luminometer. The experiment was repeated three times.

Serum killing assay. Luminescent *L. biflexa* serovar Patoc (10^8 cells/ml) was mixed with pooled, normal human serum or serum inactivated by heat (56°C for 30 min) in triplicate wells. The tray was incubated at 37°C, and luminescence and viability were measured at 30-min intervals. Viability was assessed by counting at least 100 cells, noting the number of cells that were alive (motile) and dead (nonmotile or damaged). The percent survival was calculated by expressing the reading (either the counts or the luminescence) for the serum-incubated sample as a percentage of the reading for the heat-inactivated serum sample. The experiment was repeated three times.

Virulence trial in hamsters. Four- to 6-week-old hamsters in groups of 10 were injected intraperitoneally with 10^3 leptospires in 100 μl of EMJH medium. Control animals received 100 μl EMJH medium. The hamsters were monitored for 21 days, and moribund animals were killed in accordance with animal ethics requirements.

RESULTS AND DISCUSSION

Construction of transposon cassettes for luminescence. The *luxCDABE* cassette from *Photorhabdus luminescens* was chosen to construct luminescent leptospires (20). Use of this cassette has advantages over the use of other systems, such as those that use the *Vibrio*-derived *lux* gene and firefly luciferase, because it does not require addition of an exogenous substrate and is active over a wider temperature range (21). Plasmid vectors do not replicate in *L. interrogans*; hence, transposon Tn*SC189* was modified to carry the *luxCDABE* genes for luminescence and was used to introduce the cassette into the *L. interrogans* chromosome. This method has previously been used to successfully complement an *L. interrogans* transposon mutant (17). A transposon encoding luminescent genes has previously been used in *Streptococcus pneumoniae* to circumvent plasmid stability issues (4). Luminescence is a superior viability reporter to fluorescence because it requires energy production, so it indicates the health of the cells. In contrast, metabolically inactive cells can still be detected by fluorescent markers (18).

The key plasmids used in this study are illustrated in Fig. 1. Plasmid pAL612 contains transposon Tn*SC189* (2) and a ColE1 origin of replication from pBluescript II (13). Plasmid pAL615 was constructed by insertion of the *flgB* promoter from *B. burgdorferi* and the *luxCDABE* cassette from plasmid pSB406 (20). The *flgB* promoter is a strong, σ^{70}-like promoter from a flagellar biogenesis cluster of *B. burgdorferi* and is active in a number of different bacteria, including *L. interrogans* (1, 5).

Transformation with *luxCDABE* constructs produces luminescent leptospires. Transposon mutagenesis was conducted as described previously (1, 13) with *L. interrogans* serovar Manilae or *L. biflexa* serovar Patoc. Transformation of both the saprophytic species *L. biflexa* and the pathogenic species *L. interrogans* serovar Manilae produced luminescent leptospires (Fig. 2). The transposon was found to have inserted in various locations, determined by sequencing directly from genomic DNA (12). Bacteria transformed with the transposon not carrying the *luxCDABE* cassette showed no detectable luminescence (Fig. 2). Likewise, transformation with a Tn*SC189*::*luxCDABE* construct lacking a promoter also failed to produce luminescent leptospires (data not shown). Strain M1307, which had an intergenic transposon insertion between the LA1036 gene (which encodes a predicted histidine kinase) and the LA1039 gene (which encodes a hypothetical protein), was chosen for further analysis.

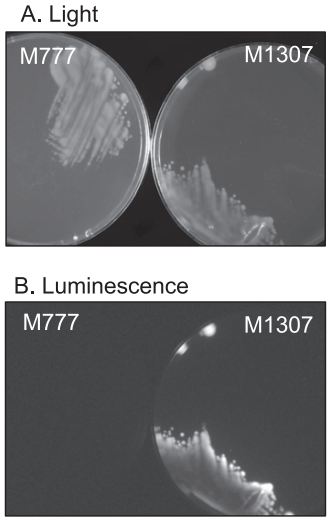

FIG. 2. Colonies of luminescent *L. interrogans* serovar Manilae strain M1307 (transformed with the *luxCDABE* transposon construct pAL615) and control strain M777 (transformed with control transposon pAL612 lacking the *luxCDABE* cassette). The strains were visualized under illumination (A) and without illumination for the detection luminescence (B). The image was taken with LAS3000 film (Fujifilm, Japan).

There is a linear relationship between luminescence and leptospiral cell number. A log-phase culture of strain M1307 was serially diluted in triplicate, and the luminescence was measured. The luminescence measurement demonstrated a linear relationship between luminescence and cell number (Fig. 3). Background noise (determined by taking measurements for the L495 wild-type control) remained under 5 rlu or 8 rlu (when the standard deviations are considered). By using the data points for M1307 above 8 rlu, there was a high degree of correlation between cell number and luminescence (R^2 = 0.998, Pearson correlation). The level of luminescence produced by each cell (luminescence index) was calculated to be 3.0×10^{-4} rlu, although this depended upon the growth phase (see below) and the temperature (data not shown). By use of this number, the theoretical limit of detection was 3.3×10^3 cells (for a luminescence of 1 rlu), although the background noise indicated a practical minimum level of detection of 2.6×10^4 cells. This compares favorably with the level of detection of other bacterial expression constructs (6) and is a useful limit for applications in an *in vitro* assay in which bacterial numbers are relatively high (approximately 10^7 cells/ml).

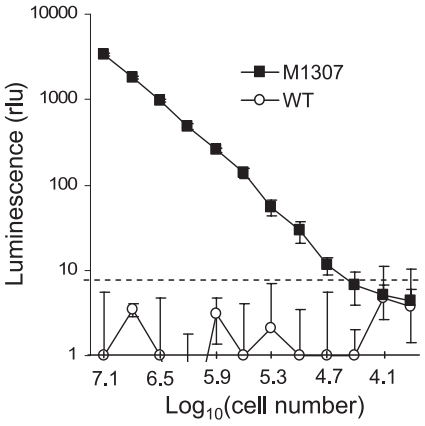

FIG. 3. Comparison of cell number and luminescence. Doubling dilution series of luminescent M1307 leptospires and the parent strain (wild type [WT]) were made and measured by luminescence. Dashed line, practical limit of detection above the background noise (approximately 2.6×10^4 cells). Error bars correspond to 1 standard deviation.

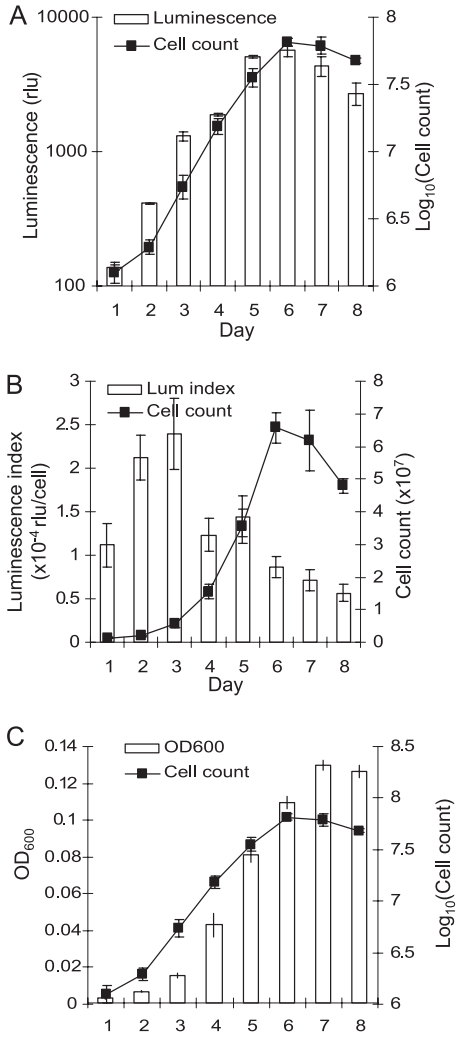

FIG. 4. Analysis of luminescence with growth phase. A growth curve was prepared to assess the value of luminescence for the determination of the cell numbers over different growth phases. Error bars represent 1 standard deviation. (A) Comparison of direct cell counts (right axis, solid line) to luminescence (left axis, histogram). (B) Variation of luminescence (Lum) index (left axis, histogram) with growth phase (right axis, solid line). The luminescence index was determined by dividing the luminescence by the number of leptospires present. (C) Comparison of direct cell counts (right axis, solid line) to the OD_{600} (left axis, histogram).

A growth curve was made to determine whether the luminescence changed with the bacterial growth phase (Fig. 4). Stationary-phase strain M1307 was diluted in triplicate into fresh medium to a concentration of 2×10^7 cells/ml. The counts were determined with a Petroff-Hausser chamber, while luminescence was measured as described above. There was a

strong correlation between the luminescence and the cell counts (Fig. 3A; $R^2 = 0.766$, Pearson correlation), although the variation in luminescence early and late in the growth phase weakened the relationship. This was further investigated by calculating the luminescence index and plotting it against the bacterial growth phase (Fig. 4B). The luminescence index was highest at early log phase (2.5×10^{-4} rlu/cell) and lowest after late log phase (5×10^{-5} rlu/cell). Nevertheless, the correlation between the cell count and luminescence remained strong over the 8-day growth period.

Variations in the luminescence index with growth phase have been observed in other luminescent bacterial systems and may relate to a slowing of the metabolism of cells and the decreased availability of reduced flavin mononucleotide in later growth phases (see reference 4 and references therein). Alternatively, this may also be due to the use of a probable σ^{70} promoter to drive *luxCDABE* expression. σ^{70} has a housekeeping role, and reduced luminescence during stationary phase may reflect a transition from σ^{70}-directed expression to expression via alternative sigma factors. If this is the case, then an investigation of different promoters may identify a promoter that makes luminescence growth independent.

The OD at 600 nm (OD_{600}) was also measured to determine its value for the enumeration of leptospires (Fig. 4C). While the OD_{600} accurately reflected the direct counts ($R^2 = 0.913$, Pearson correlation), the absorbance readings did not exceed a value of 0.1. Measurements of optical density are not suitable for application in an *in vitro* assay because the density of bacteria in a typical assay is too low to be detected and particulate matter from an assay (e.g., tissue culture cell debris) may obscure the readings. Furthermore, it has been observed that the optical density varies with cell health due to morphology changes (unpublished observations) and does not distinguish between live and dead bacteria.

Luminescence is an efficient reporter of viability and cell number for *in vitro* assays. The utility of luminescent bacteria for viability reporting and the enumeration of viable cells was tested in a number of assays, and the results were compared with those of the conventional method of counting by dark-field microscopy.

Luminescence has been used in a number of bacterial species as a viability reporter for the analysis of sensitivity to antimicrobial agents (9). An MIC experiment was conducted to demonstrate that leptospire luminescence can be used as a viability reporter. Bacteria suspended in EMJH medium were added to dilutions of chloramphenicol or EMJH medium (control). After 7 days, bacterial viability was assessed by dark-field microscopy and the measurement of luminescence (Fig. 5A). The baseline reading for inhibited bacteria was approximately 100 rlu (chloramphenicol concentration, 20 μg/ml to 1.25 μg/ml), while the readings for the control bacteria grown in EMJH medium varied from approximately 4,700 to 13,000 rlu. At a concentration of 0.625 μg/ml chloramphenicol, approximately 50% of the signal (2,041 rlu) was observed, and the cell density was confirmed by dark-field microscopy (data not shown). The chloramphenicol MIC for strains of *L. interrogans* may vary from 0.78 to 6.5 μg/ml (11); hence, an MIC of 0.625 μg/ml is at the lower end of the range of antibiotic sensitivity. This finding thus demonstrated the utility of luminescence as a leptospire viability marker and for the determination of MICs.

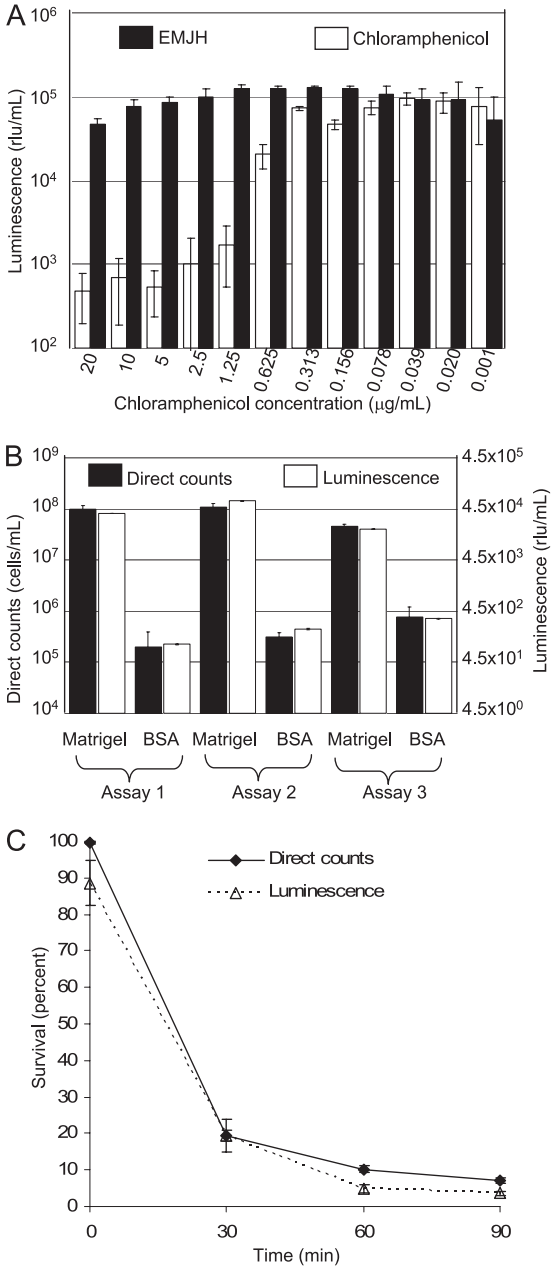

FIG. 5. Evaluation of luminescence for viability reporting and enumeration in different *in vitro* assays. Error bars correspond to 1 standard deviation. (A) MIC assay with chloramphenicol. Doubling dilutions of chloramphenicol (20 μg/ml) or EMJH medium (control) were made in an EMJH medium diluent containing strain M1307 at 10^6 cells/ml. Measurements were made after 1 week of growth. The baseline level of inhibited bacteria was approximately 100 rlu. (B) Extracellular matrix binding assay. Bacteria were added to wells coated with the extracellular matrix-like compound Matrigel or BSA (control), removed, and enumerated. The output for direct counts (solid histograms, left axis) is compared to that for luminescence (open histograms, right axis) for Matrigel- or BSA-coated wells. Comparisons are presented for three independent assays. The luminescence index for this assay was calculated to be 4.5×10^{-4} rlu/cell. (C) Complement sensitivity assay. Luminescent *L. biflexa* serovar Patoc was mixed with 5% human serum or 5% heat-inactivated human serum. The percent survival (survival rate in 5% serum/survival rate in 5% heat-inactivated serum) was assayed by the use of either direct counts (diamonds, solid line) or luminescence (triangles, broken line).

An extracellular matrix-binding assay was performed to demonstrate that luminescence could report the number of bacteria present in an assay readout. *L. interrogans* serovar Manilae adheres to the extracellular matrix-like compound Matrigel coated to the wells of microtiter trays (7). A binding assay was performed three times, as described previously (7, 15) (Fig. 5B). When the bacteria were enumerated by direct counts, an average of 4.7% of the bacteria bound to Matrigel-coated wells, while only 0.028% of the bacteria bound to the bovine serum albumin (BSA)-coated control wells, similar to previous findings (7, 15). Comparison of the different assay readouts showed that the luminescence readings closely resembled the direct microscopic counts ($R^2 = 0.916$, Pearson correlation). This result clearly demonstrates that luminescence is a useful tool for the enumeration of cells in adhesion assays, even when low numbers of cells are recovered. The luminescence index for this assay was calculated (from the counts and luminescence data presented in Fig. 5B) and was found to be 4.5×10^{-4} rlu/cell, which is within the range of luminescence indices identified in the growth curve.

The value of luminescence for detection of the survival of leptospires in a killing assay was tested in a complement resistance experiment. *Leptospira interrogans* serovars are resistant to the bactericidal activity of serum complement, while *L. biflexa* strains are highly susceptible (19). Luminescent *L. biflexa* serovar Patoc cells (10^8 cells/ml) were mixed with pooled, normal human serum at a final concentration of 5%. The bacteria were incubated at 37°C and luminescence was measured at 30-min intervals, while viability was also assessed by microscopy. The counts were compared to those for the controls (incubated in heat-inactivated serum) to calculate the percent survival. There was a very strong correlation between the alternative measurements of survival ($R^2 = 0.988$). In both cases, viability dropped from approximately 100% to 20% in the first 30 min and then continued to decline to approximately 5% (Fig. 5C). The similarity in the results obtained by the different methods of enumeration demonstrated the utility of luminescence in survival assays.

Luminescent *L. interrogans* serovar Manilae retains virulence. A further application of luminescent bacteria is in animal models to track the progress of infections (8). To determine if expression of the *luxCDABE* cassette affected the *in vivo* fitness of *L. interrogans*, luminescent serovar Manilae strain M1300 (with a transposon insertion in the LA2117 gene, a predicted anti-sigma factor antagonist) was injected intraperitoneally into hamsters along with the parent strain ($n = 10$ hamsters per group, dose of 10^3 leptospires), as described previously (14). Control hamsters injected with EMJH medium failed to show any signs of disease. Nine hamsters infected with M1300 succumbed to infection between days 6 and 9, and so there was one survivor. For the parent strain control, nine hamsters died between days 6 and 8 and the remainder died on day 11. There was no significant difference between the groups ($P > 0.05$, Fisher's exact test). The bacteria recovered from the M1300-infected hamsters retained the luminescence. This illustrated that expression of the *luxCDABE* cassette does not significantly affect the *in vivo* fitness of *L. interrogans* in the hamster model of infection and that the infecting leptospires retain the *lux* cassette.

A number of caveats need to be observed with this system. Transposon mutagenesis of *L. interrogans* is difficult, although once a luminescent strain is constructed, it can be stored frozen and used indefinitely. While the system functions well over a wide temperature range, comparisons between readings are valid only if they are taken at the same temperature. Since transposon mutagenesis is random, the location of the insertion should be determined to ensure that the mutation does not affect the phenotype being examined. Alternatively, a number of different transposon mutants may be screened simultaneously to minimize the possibility that the mutations will affect the phenotype.

In conclusion, the use of luminescence for the detection of *Leptospira* numbers and the reporting of viability has many advantages over the use of conventional detection methods: luminescence readings are rapid (readings take less than 1 min to complete) and sensitive, they discriminate live from dead cells, and they are not affected by diffracting material that may be present in the output of *in vitro* assays. The use of luminescent leptospires may complement or replace currently used methods of leptospire enumeration. Despite some variations in luminescence according to the assay conditions or the growth phase, the method has many advantages over alternative enumeration methods and remains valid for comparisons within a given assay. Future work may use luminescent bacteria to image infections in live animals. Large numbers of mutants have now been generated in *L. interrogans* (13), and the *lux* reporter construct will be useful as a tool for the high-throughput investigation of mutant phenotypes in various *in vitro* assays.

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