Novel Luciferase Reporter System for In Vitro and Organ-Specific Monitoring of Differential Gene Expression in *Listeria monocytogenes*

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In this paper we describe construction of a luciferase-based vector, pPL2lux, and use of this vector to study gene expression in *Listeria monocytogenes*. pPL2lux is a derivative of the listerial integration vector pPL2 and harbors a synthetic luxABCDE operon encoding a fatty acid reductase complex (LuxCDE) involved in synthesis of the fatty aldehyde substrate for the bioluminescence reaction catalyzed by the LuxAB luciferase. We constructed pPL2lux derivatives in which the secA and hlyA promoters were translationally fused to luxABCDE and integrated as a single copy into the chromosome of *L. monocytogenes* EGD-e. Growth experiments revealed that hlyA was expressed predominantly in the stationary phase in LB medium buffered at pH 7.4, whereas secA expression could be detected in the exponential growth phase. Moreover, the correlation between luciferase activity and transcription levels, as determined by reverse transcriptase PCR, was confirmed using conditions known to lead to repression and activation of hemolysin expression (addition of cellobiose and activated charcoal, respectively). Furthermore, hemolysin expression could be monitored in real time during invasion of an intact monolayer of C2Bbe1 (Caco-2-derived) cells. Finally, hemolysin expression could be detected in the livers, spleens, and kidneys of mice 3 days postinfection. These experiments clearly established the effectiveness of pPL2lux as a quantitative reporter system for real-time, noninvasive evaluation of gene expression in *L. monocytogenes*.

Listeria monocytogenes is a gram-positive intracellular pathogen that is capable of withstanding a variety of hostile environmental conditions, including the many stresses encountered during the production, preparation, and storage of food. After contaminated food is consumed, *L. monocytogenes* effectively survives the extreme conditions in the human gastrointestinal tract, including the gastric acidity encountered in the stomach and the presence of bile salts in the duodenum (13). Several virulence factors that play a role during the subsequent intracellular cycle of *L. monocytogenes* have been determined, and they include hemolysin, encoded by *hlyA*, which allows escape of listeria from the phagosomal compartment and intracytoplasmic growth (15, 44). Notably, *L. monocytogenes* is the causative agent of listeriosis, which is estimated to account for up to 35% of all deaths caused by known bacterial food-borne pathogens in the United States (24), which justifies the increased effort that is put into analysis of listerial gene expression signals and the characterization of regulatory elements (4, 20, 36, 41, 47). Moreover, it has been suggested that in order to fully investigate the expression of virulence genes, the development of new tools that allow noninvasive imaging and in situ determination of quantitative temporal changes in gene expression during infection of relevant hosts will become increasingly important in listerial research in the coming years (9).

Various reporter systems have been used to identify regulatory sequences and to monitor gene expression. Historically, bacterial promoters have been studied by insertion of random or defined chromosomal DNA fragments upstream of promot-

erless reporter genes, such as genes encoding chloramphenicol acetyltransferase (1, 27, 42), alanine racemase (7, 8), and different sugar hydrolases (19, 27, 32). These reporter systems are convenient tools for semiquantitative, plate-based assessment of promoter activities. However, more accurate quantification of promoter strength usually requires relatively laborious enzymatic assays, which typically involve bacterial cell disruption and addition of a substrate to drive the enzymatic reaction, followed by measurement of the optical density (27, 32).

Another group of reporter systems is based on the emission of light. Variants of green fluorescent protein from *Aequorea victoria* have been used to monitor bacterial promoter activity during infection of eukaryotic cell lines (14, 35). Unfortunately, naturally occurring fluorescence can lead to high background levels during in vitro and in vivo measurements. Alternative strategies for both gram-negative and gram-positive bacteria have involved the luciferase-encoding luxAB genes, typically derived from *Vibrio fischeri*, *Vibrio harveyi*, or *Photobacterium luminescens* (25). Although the level of the background bioluminescence is extremely low compared to the level of endogenous background fluorescence, luxAB systems require addition of an exogenous aldehyde as a substrate in the light emission reaction (17, 28, 30, 33). This aldehyde requirement can be overcome by using the synthetic operon luxCDABE, as luxCDE encodes a fatty acid reductase complex involved in synthesis of the fatty aldehyde substrate for the bioluminescence reaction catalyzed by the luxAB-encoded luciferase (25). The luxCDABE system has been utilized in a noninvasive, real-time fashion for tagging and assessing promoter activities of several gram-negative bacteria, including *Escherichia coli* (6, 43, 45), *Salmonella enterica* serovar Typhimurium (3), *Citrobacter rodentium* (46), and *Campylobacter jejuni* (2). Moreover, after introduction of gram-positive ribosome

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TABLE 1. Strains, plasmids, and primers used in this study and their relevant characteristics

Strain, plasmid, or primer	Relevant feature(s) ^a	Source or reference
Strains		
<i>E. coli</i> DH10B	Cloning host	Invitrogen
<i>L. monocytogenes</i> strains		
EGD-e	Wild type of serotype 1/2a for which the genome sequence is available	16
EGD-e::pPL2lux	Cm ^r , contains pPL2lux integrated at tRNA ^{Arg} locus on the <i>L. monocytogenes</i> EGD-e chromosome	This study
EGD-e::pPL2lux-P _{secA}	Cm ^r , contains pPL2lux-P _{secA} integrated at tRNA ^{Arg} locus on the <i>L. monocytogenes</i> EGD-e chromosome	This study
EGD-e::pPL2lux-P _{hlyA}	Cm ^r , contains pPL2lux-P _{hlyA} integrated at tRNA ^{Arg} locus on the <i>L. monocytogenes</i> EGD-e chromosome	This study
Plasmids		
pSB2025	Ap ^r , contains synthetic <i>luxABCDE</i> operon	34
pPL2secA2-FLAG	Cm ^r , <i>L. monocytogenes</i> site-specific integration vector harboring <i>secA2</i>	23
pPL2a	Cm ^r , pPL2secA2-FLAG derivative without <i>secA2</i>	This study
pPL2SwaI ⁻	Cm ^r , pPL2a without SwaI	This study
pPL2luxSwaI ⁺	Cm ^r , pPL2SwaI harboring <i>luxABCDE</i> derived from pSB2025	This study
pPL2lux	Cm ^r , pPL2luxSwaI ⁺ with SwaI removed from <i>luxC</i> and SwaI inserted overlapping the start codon of <i>luxA</i> , allowing exact translational promoter fusions to <i>luxABCDE</i>	This study
pPL2lux-P _{secA}	Cm ^r , pPL2lux derivative containing 0.5-kb promoter region of <i>secA</i> translationally fused to <i>luxABCDE</i>	This study
pPL2lux-P _{hlyA}	Cm ^r , pPL2lux derivative containing 0.5-kb promoter region of <i>hlyA</i> translationally fused to <i>luxABCDE</i>	This study
Primers		
IM101	5'-GAATTGGCATACTGATTATATGAAGG-3'	This study
IM102	5'-GATACACCAAGGAAAGTCTACAC-3'	This study
IM103	5'-CCATCCAAGTCGACATTTAAATTTGGAAACTTTTGCTTACATAC-3'	This study
IM104	5'-GAATGATGACTCCCAAGGAAAAATAG-3'	This study
IM105	5'-CCATCCAACCTCGAGAAGTTCCGCGACAAAGGTGCAGAAAG-3'	This study
IM106	5'-CATTTAAATTCTCCTCTACTGTTTTC-3'	This study
IM107	5'-CCATCCAACCTCGAGTTTGAGCTAGTGGTTTGGTTAATG-3'	This study
IM108	5'-CATGGGTTTCACTCTCCTTCTAC-3'	This study
IM109	5'-GTAAGTTGGGGATAACTCCG-3'	This study
IM110	5'-GCGCTTTACGCCCCATAAATC-3'	This study
IM111	5'-AAAAGGACGATTTTCGGTTTGG-3'	This study
IM112	5'-CCAATGCCCCAGAAATTTCC-3'	This study
IM113	5'-CCAATTGCGCAACAACTGAAG-3'	This study
IM114	5'-TTCCACTAATGTATTTACTGCG-3'	This study
PL95	5'-ACATAATCAGTCCAAAGTAGATGC-3'	22
PL102	5'-TATCAGACCTAACCCAAACCTTCC-3'	22

^a Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance. Underlining indicates restriction sites used in subsequent cloning procedures.

binding sites and shuffling of the gene order to *luxABCDE*, the luciferase system also appeared to be functional in gram-positive bacteria (11, 34). Transposon mutagenesis strategies have been employed to integrate the *luxABCDE* system into the chromosome of several gram-positive bacteria, including *Staphylococcus aureus* (11), *L. monocytogenes* (18), and *Streptococcus pneumoniae* (12). These tagged pathogens could be monitored in intact animal models, which allowed temporal and spatial assessment of the infection processes.

Here we describe construction of a luciferase-based reporter system, pPL2lux. This vector is based on the listerial site-specific integration vector pPL2 (22) and the *luxABCDE* system (34). Derivatives of pPL2lux harboring the listerial *secA* and *hlyA* promoters translationally fused to *luxABCDE* were constructed and integrated into the *L. monocytogenes* chromosome. The strains that were constructed allowed us to assess the functionality of pPL2lux as a reporter system in three model systems used routinely in listerial research: growth in laboratory media, invasion assays with a Caco-2-derived cell line, and animal experiments using BALB/c mice. The exper-

iments demonstrated the utility and advantages of pPL2lux as a real-time, noninvasive reporter system for quantitative assessment of listerial promoters.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The strains, plasmids, and primers used in this study are listed in Table 1. *E. coli* strain DH10B (Invitrogen, Paisley, United Kingdom) was used as a cloning host during construction of the pPL2lux derivatives (see below) and was grown aerobically at 37°C in LB medium (39). *L. monocytogenes* EGD-e (16) was routinely grown aerobically at 37°C in brain heart infusion (BHI) medium (Oxoid, Basingstoke, United Kingdom). However, during the bioluminescence assays LB medium was used. When indicated below, LB medium was buffered with 100 mM 3-(N-morpholino)propane-sulfonic acid (pH 7.4). When appropriate, antibiotics were added to the media as follows: for *E. coli*, ampicillin (100 µg/ml) or chloramphenicol (15 µg/ml); and for *L. monocytogenes*, chloramphenicol (7.5 µg/ml).

DNA techniques. Plasmid DNA was isolated from *E. coli* using a QIAprep Spin Miniprep kit according to the manufacturer's instructions (QIAGEN, Crawley, United Kingdom). Genomic DNA was isolated from *L. monocytogenes* EGD-e using a Genelute bacterial genomic DNA kit (Sigma, Steinheim, Germany) as recommended by the manufacturer. Transformation of *L. monocytogenes* was performed as described previously (29). Standard procedures were

FIG. 1. Plasmid map of pPL2*lux*. The *luxABCDE* operon was derived from pSB2025 (34) with a blunt-end *Swa*I restriction site introduced overlapping the ATG start codon of *luxA*. Cloning of PCR-amplified promoter elements into the *Sal*I and *Swa*I restriction sites allowed construction of exact translational fusions of listerial promoters to *luxABCDE*.

used for DNA manipulation in *E. coli* (39). Restriction endonucleases (Roche Diagnostics, Mannheim, Germany), T4 DNA ligase (Roche), and 2× PCR mixture (Promega, Madison, WI) were used as recommended by the manufacturers. Primers were purchased from Sigma Genosys (Haverhill, United Kingdom). PCR products required for cloning were obtained with KOD hot-start high-fidelity DNA polymerase (Merck, Nottingham, United Kingdom) using either 10 ng *L. monocytogenes* genomic DNA or 50 ng plasmid DNA as the template.

Construction of pPL2*lux* and derivatives. Plasmid pPL2*secA2*-FLAG (23) was digested with PstI, and the 6.1-kb fragment containing the pPL2 backbone was self-ligated, yielding pPL2a. Except for the modified multiple cloning site, pPL2a is otherwise identical to pPL2 (22). The unique SmaI site present in the PSA integrase-encoding gene of pPL2a was removed by site-directed mutagenesis in a PCR using pPL2a as the template and primers IM101 and IM102. The resulting 0.94-kb amplicon was digested with SphI, and the 0.73-kb fragment was ligated into SphI-SmaI-digested pPL2a, yielding pPL2*SwaI*⁻. Compared to parental plasmid pPL2a, the newly constructed plasmid had one point mutation in the SmaI site (introduced by the IM102 primer during the PCR), which did not change the protein sequence of the PSA integrase. Subsequently, pSB2025 (34) was digested with SalI and PstI, and the resulting 5.6-kb fragment harboring the synthetic *luxABCDE* operon was cloned into complementary digested pPL2*SwaI*⁻, yielding pPL2*luxSwaI*⁺. Finally, the SmaI site in the *luxC* gene harbored by pPL2*luxSwaI*⁺ was removed using a site-directed mutagenesis approach essentially as described above to introduce a point mutation into the SmaI site, using pPL2*luxSwaI*⁺ as the template and primers IM103 and IM104, as well as exchange of the native and mutated fragment by cloning the SalI-digested amplicon into the SalI-SmaI-digested backbone of pPL2*luxSwaI*⁺. The resulting vector was designated pPL2*lux* (Fig. 1) and contains a unique SmaI restriction site that overlaps the start codon of *luxA* (introduced by the IM103 primer), which allows exact translational fusion of listerial promoters to the *luxABCDE* operon (see below).

To test the functionality of the luciferase reporter system, the *secA* and *hlyA* promoters were cloned into pPL2lux. Chromosomal DNA from *L. monocy-*

genes EGD-e was used as the template in PCRs using KOD polymerase (Merck) and primer pairs IM105-IM106 and IM107-IM108. The resulting 0.5-kb PCR products harbored the regions immediately upstream of *secA* and *hlyA*, respectively. Notably, the start codons of both genes were included at the ultimate 3' end of the reverse primers. The P_{*secA*} and P_{*hlyA*} amplicons were digested with XhoI and cloned into SmaI-SalI-digested pPL2*lux*, yielding pPL2*lux*-P_{*secA*} and pPL2*lux*-P_{*hlyA*}, respectively. Subsequently, pPL2*lux*, pPL2*lux*-P_{*secA*}, and pPL2*lux*-P_{*hlyA*} were transformed into *L. monocytogenes* EGD-e, and candidate integrants were checked for site-specific integration by PCR, using template DNA obtained directly from colonies by a microwave treatment (3 min, 600 W) and primers PL95 and PL102 (22). From each transformation one colony having the anticipated genotype was selected for bioluminescence analysis using a Xenogen IVIS 100 system (Xenogen, Alameda, CA).

RNA isolation. Levels of luciferase and hemolysin transcription were determined in EGD-c::PL2*luxP_{hlyA}* grown with or without 25 mM D-(+)-cellobiose (Calbiochem, San Diego, CA) or 0.2% activated charcoal (0.3 to 0.5 mm; Merck, Nottingham, United Kingdom). Full-grown LB medium cultures were diluted 50-fold in fresh medium and grown statically at 37°C. Growth was monitored by determining the optical density at 600 nm (OD₆₀₀), and at the beginning of the stationary phase (OD₆₀₀, 0.3) cells were harvested from 10 ml after addition of 40 ml of quench buffer (60% methanol, 66.7 mM HEPES [pH 6.5]; -40°C). Following quenching (31), the cells were immediately pelleted by centrifugation at 3,220 × g for 10 min, and the cell pellets were resuspended in 0.2 ml of ice-cold distilled H₂O. The cell suspensions were added to ice-cold tubes containing MagNA Lysor green beads (Roche Diagnostics), 400 µl of acidified phenol (Sigma), 100 µl of chloroform (Sigma), 30 µl of 10% sodium dodecyl sulfate (Sigma), and 30 µl of 3 M sodium acetate (pH 5.2) (Merck). The cells were disrupted with three 40-s treatments in a MagNA Lysor (Roche Diagnostics) separated by 1 min on ice. After centrifugation, 100 µl of the aqueous phase was used for RNA isolation with QIAGEN RNeasy columns (Crawley, United Kingdom). After elution, 0.5 µl of protector RNase inhibitor was added to all RNA samples (Roche Diagnostics), and 1 µg of RNA was subjected to DNase I

treatment according to the manufacturer's instructions (Ambion, Huntingdon, United Kingdom).

cDNA synthesis and reverse transcriptase PCR. cDNA was synthesized in a 40- μ l reaction mixture containing 0.2 nmol of random hexamer primer, 10 mM dithiothreitol, 1 $\times$ Expand reverse transcriptase (RT) buffer, 50 U Expand reverse transcriptase (Roche Diagnostics, Mannheim, Germany), each deoxynucleoside triphosphate at a concentration of 0.25 mM, and 0.7 μ g DNase I-treated RNA. Reverse transcription was initiated by 10 min at 30°C, followed by 45 min at 42°C and inactivation of the reverse transcriptase by incubation at 95°C for 2 min. Subsequently, 1 μ l of the cDNA synthesized was used in PCR mixtures containing 1 $\times$ PCR mixture (Promega) and 1 pmol of the 16S RNA (IM109-IM110), *luxA* (IM111-IM112), or *hlyA* (IM113-IM114) primer pair (Table 1).

Cell invasion assay. C2Bbe1 cells (CRC-2102; American Type Culture Collection), a clone of the Caco-2 human adenocarcinoma cell line, were used for invasion assays. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/liter Glutamax (Gibco Laboratories, Grand Island, NY), 10% fetal bovine serum (Gibco), 1% (vol/vol) nonessential amino acids (Gibco), 1% (vol/vol) penicillin-streptomycin (Gibco), and 0.01 mg/ml human transferrin (Calbiochem) at 37°C in a 5% CO₂ atmosphere. For cell invasion assays, C2Bbe1 cells were trypsinized (Gibco), harvested by centrifugation at 400 $\times$ g for 8 min, and resuspended in 1 ml antibiotic-free DMEM containing 10% fetal bovine serum. Cells were seeded onto 24-well flat-bottom tissue culture plates (Sarstedt, Leicester, United Kingdom) at a concentration of 3 $\times$ 10⁵ cells per well. The plates were incubated for 72 h at 37°C in a 5% CO₂ atmosphere until confluence was reached. An overnight BHI medium culture of EGD-e::pPL2lux-P_{hlyA} or EGD-e::pPL2lux was diluted 1:20 in fresh BHI medium and incubated until the OD₆₀₀ was 1.0 (1 $\times$ 10⁹ CFU/ml). Subsequently, bacterial cells were washed twice in DMEM and added at a multiplicity of infection of 100:1, and the plates were incubated at 37°C in a 5% CO₂ atmosphere. At several times gentamicin was added to a final concentration of 100 μ g/ml, and the cells were incubated for 30 min (21). Subsequently, the cells were washed three times with phosphate-buffered saline (PBS), and the levels of bioluminescence were determined using a Xenogen IVIS 100 system (Xenogen, Alameda, CA). After this monolayers were lysed with 1 ml of ice-cold distilled H₂O, and serial dilutions were plated on BHI agar to assess the number of viable intracellular bacteria.

Animal experiments. Overnight cultures of either *L. monocytogenes* EGD-e::pPL2lux-P_{hlyA} or *L. monocytogenes* EGD-e::pPL2lux were pelleted by centrifugation (7,000 $\times$ g for 5 min), washed with PBS, and used to inoculate 8- to 12-week-old BALB/c mice intraperitoneally with 2 $\times$ 10⁶ CFU in 200 μ l of PBS. Three days after infection, the mice were sacrificed by cervical dislocation, and individual organs were examined for bioluminescence using a Xenogen IVIS 100 system. Organs were then homogenized in PBS, and serial dilutions were plated onto BHI agar, which was followed by overnight incubation at 37°C. The resulting colonies were used to calculate the number of bacterial cells per organ.

RESULTS

Functionality of the luciferase reporter system (pPL2lux).

The main goal of the work described here was construction and functional analysis of pPL2lux (Fig. 1). This vector is a derivative of pPL2 that contains a PSA listeriophage integrase gene and attachment site which directs site-specific, single-copy integration into the tRNA^{Arg} locus on the *L. monocytogenes* chromosome (22). In addition, pPL2lux harbors *luxABCDE*, a synthetic operon that contains optimized gram-positive translational initiation sequences upstream of *luxA*, *luxC*, and *luxE* (34). The *luxABCDE* operon is divergently oriented with respect to flanking genes on pPL2lux to minimize any potential read-through from upstream promoters. Importantly, the use of the SalI and SmaI restriction sites for orientation-specific cloning of PCR-amplified promoters into pPL2lux allowed exact translational fusion to the *luxABCDE* operon. Moreover, the fact that the start codon of *luxA* was not present in pPL2lux enabled inclusion of the start codon of the gene under investigation at the 3' end of the primer used during amplification

of the promoter region, thereby allowing direct identification of positive clones by their bioluminescent phenotype in *E. coli*.

To demonstrate the functionality of the luciferase reporter system in *L. monocytogenes*, the expression profiles of *hlyA* and *secA* were assessed. The latter gene encodes SecA, which is essential in *L. monocytogenes* as it is required for the initial binding of preproteins to the inner membrane and their subsequent translocation across this structure (23). Hence, this gene should be expressed in all growth phases and was used primarily as a positive control. *hlyA*, encoding hemolysin, plays an important role during listerial escape from the phagosomal compartment and intracytoplasmic growth (15, 44). The original vector, pPL2lux, and its derivatives, pPL2lux-P_{secA} and pPL2lux-P_{hlyA}, were constructed and integrated into the chromosome of *L. monocytogenes* EGD-e as described in Materials and Methods.

The stability of the integrated pPL2lux derivatives in the absence of antibiotic selection pressure was evaluated. Cultures of *L. monocytogenes* EGD-e::pPL2lux, EGD-e::pPL2lux-P_{secA}, and EGD-e::pPL2lux-P_{hlyA} were grown for 50 generations in BHI medium without antibiotic selection, dilutions of the cultures were plated, and the presence of the integrated plasmids in the resulting colonies was assessed by scoring for the chloramphenicol resistance encoded by the pPL2 backbone. All 100 colonies tested for each of the three strains were chloramphenicol resistant after 50 generations of growth (data not shown), indicating that the integrated plasmids were stably maintained without antibiotic selection pressure. This high level of stability allowed use of the luciferase reporter system in experiments in which antibiotic selection pressure could not be maintained, such as cell invasion assays and animal experiments (see below).

Overnight cultures of the constructed strains were diluted 1:50 in LB medium buffered at pH 7.4, and growth and bioluminescence were monitored over time (Fig. 2). Notably, relatively low levels of hemolysin-coupled bioluminescence were observed in our initial experiments using the luciferase reporter system and unbuffered BHI and LB media. In contrast, higher levels of bioluminescence were observed in BHI and LB media buffered at pH 7.4; the levels of expression in LB medium were the highest, while the *secA* transcription profiles appeared to be similar in the unbuffered and buffered LB and BHI media (data not shown). The growth rates of EGD-e::pPL2lux-P_{secA} and EGD-e::pPL2lux-P_{hlyA} in the different media were not affected compared to the growth rate of EGD-e::pPL2lux (data not shown). Apparently, luciferase expression at the levels reached during the experiments described here does not influence the growth rate of *L. monocytogenes*. No bioluminescence was detected at any time for the negative control strain EGD-e::pPL2lux, indicating that there was no background signal (data not shown). For strain EGD-e::pPL2lux-P_{secA}, bioluminescence was detected throughout the growth curve, as expected for this essential system (Fig. 2). However, bioluminescence declined during the late logarithmic phase of growth and decreased considerably after the strain entered the stationary phase, until it was eventually undetectable. In contrast, bioluminescence was not present in EGD-e::pPL2lux-P_{hlyA} in the early logarithmic growth phase and was detected only from the mid-logarithmic growth phase onward (Fig. 2). Higher levels of expression were observed at the end of the logarithmic phase

FIG. 2. Hemolysin expression profiles during growth at 37°C in LB medium buffered at pH 7.4. The triangles and diamonds indicate the average growth of quadruplicate cultures and the gray and black bars indicate the average luciferase expression profiles for EGD-e::pPL2lux-P_{secA} and EGD-e::pPL2lux-P_{hlyA}, respectively. BLC, bioluminescence counts; OD600nm, optical density at 600 nm. The data are representative of the data from three independent experiments. At times 1, 2, and 3, samples were taken from the cultures for RNA isolation. Subsequently, luciferase transcript levels were assessed by RT-PCR analysis (inset).

and upon entry into the stationary phase. Although the hemolysin levels fluctuated during the stationary phase, equally high levels of expression were detected after 24 h of growth compared to the levels at the beginning of the stationary phase (Fig. 2). The bioluminescence profiles for EGD-e::pPL2lux-P_{secA} and EGD-e::pPL2lux-P_{hlyA} differed significantly throughout the different growth phases, suggesting that differential expression can be monitored using the luciferase reporter system.

To investigate whether the levels of bioluminescence observed with the EGD-e::pPL2lux-P_{secA} and EGD-e::pPL2lux-P_{hlyA} stationary-phase cultures resulted from active transcription or accumulation of luciferase, an RT-PCR analysis was performed. RNA was isolated from the cultures during the log phase, at the beginning of the stationary phase, and after a prolonged time in the stationary phase (times 1, 2, and 3 in Fig. 2). After cDNA synthesis, control PCRs were performed with 16S RNA primers (Table 1), which confirmed that the cDNA concentrations were comparable in all samples (Fig. 2, inset). Moreover, the amplicons obtained using the *luxA* primers (Table 1) demonstrated that there was active luciferase transcription in EGD-e::pPL2lux-P_{secA} and EGD-e::pPL2lux-P_{hlyA} during the log phase and at the beginning of the stationary phase. In contrast, the intensity of the amplicon obtained from cDNA derived from the EGD-e::pPL2lux-P_{secA} culture after a prolonged time in the stationary phase was extremely low compared to the intensity of the amplicon obtained from cDNA derived from the EGD-e::pPL2lux-P_{hlyA} culture. These experiments demonstrated that the levels of bioluminescence accurately reflected the levels of luciferase transcription during all growth phases and that luciferase accumulation did not make a major contribution to the bioluminescence signals obtained.

Previous studies have demonstrated that *hlyA* transcription is induced in the presence of activated charcoal and is repressed by cellobiose (10, 26, 37). To further investigate

the potential of pPL2lux as a quantitative reporter system, EGD-e::pPL2lux-P_{hlyA} was grown in media with and without added cellobiose or activated charcoal. At the beginning of stationary phase the levels of bioluminescence were determined, and the results revealed that addition of cellobiose and charcoal to the medium resulted in a 16-fold decrease and a 28-fold increase, respectively, in the levels of bioluminescence compared to growth in the control medium (Fig. 3A). Despite the fact that the levels of bioluminescence in LB medium containing activated charcoal appeared to be 448-fold higher than the levels in LB medium containing cellobiose, both levels could be conveniently measured, indicating the suitability of the luciferase reporter system for assessing levels of hemolysin expression in a wide dynamic range. Simultaneously, RNA was isolated from the cultures, and after cDNA synthesis the correlation between bioluminescence and *luxA* or *hlyA* levels was evaluated by PCR analysis. Control PCRs with cDNA derived from the cultures were performed with 16S RNA primers (Table 1), and the results confirmed that the cDNA concentrations were comparable in all samples (Fig. 3B). The relatively high intensity of the amplicon obtained using the *luxA* primers (Table 1) and cDNA derived from the culture to which activated charcoal had been added indicates that the levels of *luxA* mRNA were significantly higher than the levels in the control culture. Moreover, the intensity of the amplicon from the culture grown in LB medium containing cellobiose was significantly lower, indicating that the levels of *luxA* mRNA were decreased in the presence of cellobiose. These experiments revealed that the levels of bioluminescence obtained using the pPL2lux reporter system strongly correlated with the levels of *luxA* mRNA in the same cultures. Using the same cDNA samples with primers specific for *hlyA* (Table 1), very similar relative expression profiles were obtained, namely, decreased and increased levels of *hlyA* mRNA in the cultures containing

FIG. 3. Correlation between bioluminescence and mRNA levels. EGD::pPL2*lux*-P_{*hlyA*} was grown in LB medium, LB medium containing 0.2% activated charcoal (LBAC), or LB medium containing 25 mM cellobiose (LBcell). Upon entry into the stationary phase, 1-ml portions of the cultures were imaged using the IVIS 100 system (A), and cDNA was synthesized from RNA extracted from 10-ml portions of the cultures, which was followed by PCR analysis to assess levels of 16S RNA, *luxA*, and *hlyA* transcription (B). The images in panel A were obtained using an IVIS 100 system with 5 min of exposure and a binning value of 8. The color bar indicates the bioluminescence signal intensity (in photons s⁻¹ cm⁻²). Fold induction indicates the levels of bioluminescence relative to the levels observed in LB medium.

cellobiose and charcoal, respectively (Fig. 3B). From these data, we concluded that at least in this instance, measuring bioluminescence provides an accurate depiction of transcription of the single-copy, chromosomally located *luxABCDE* operon, which in turn correctly reflects the transcription profile of the native promoter in its normal chromosomal locus. Taken together, the experiments described above demonstrate that pPL2*lux* can be used effectively as a quantitative reporter system for real-time, noninvasive detection of promoter activities during in vitro growth of *L. monocytogenes*.

Hemolysin expression during cell invasion. We also investigated the possibility of using pPL2*lux* to monitor hemolysin expression in real time during a cell invasion assay. EGD-e::pPL2*lux* and pPL2*lux*-P_{*hlyA*} were used to infect C2Bbe1 cells, and the levels of intracellular bioluminescence were monitored and the numbers of intracellular bacterial cells were determined (Fig. 4). Notably, EGD-e::pPL2*lux* and EGD-e::pPL2*lux*-P_{*hlyA*} grown in BHI medium were used to infect the C2Bbe1 cells, as these cells exhibited no detectable bioluminescence (see above). Moreover, no bioluminescence was detected at any time in the wells containing C2Bbe1 cells and control strain EGD-e::pPL2*lux*, which confirmed the absence of any detectable background bioluminescence (data not shown). Furthermore, no bioluminescence was detected in the gentamicin-treated washes (see Materials and Methods). Similarly, no bioluminescence was detected immediately after infection with EGD-e::pPL2*lux*-P_{*hlyA*}, whereas 30 min after infection relatively low absolute levels of intracellular bioluminescence were detected and the absolute levels increased as time progressed. The bacterial counts for EGD-e::pPL2*lux*-P_{*hlyA*} increased concomitant with the levels of bioluminescence, indicating that relative hemolysin expression was relatively constant during the course of the experiment (0.60 to 1.30 photons s⁻¹ CFU⁻¹). Moreover, the numbers of intracellular bacterial cells demonstrated that at each time similar numbers of bacteria were recovered from wells infected with EGD-e::pPL2*lux* and EGD-e::pPL2*lux*-P_{*hlyA*}, indicating that luciferase expression does not influence the invasive potential of *L. monocytogenes* (data not

FIG. 4. Hemolysin expression during cell invasion. EGD::pPL2*lux*-P_{*hlyA*} was used to infect monolayers of C2Bbe1 cells, and bioluminescence was monitored 0, 30, 60, and 120 min postinfection. The images were obtained using an IVIS 100 system with 5 min of exposure and a binning value of 8. BLC, bioluminescence counts (in photons s⁻¹). The color bar indicates bioluminescence signal intensity (in photons s⁻¹ cm⁻²). ND, not detectable (bioluminescence counts, <1 × 10⁴ photons s⁻¹). The values for bioluminescence counts and CFU are the means ± standard deviations from triplicate wells. The data are representative of the data from three independent experiments.

FIG. 5. In vivo analysis of *L. monocytogenes* hemolysin expression and organ-specific colonization in BALB/c mice. Two mice were inoculated with 2×10^6 bacteria via the intraperitoneal route using either EGD-e::pPL2lux (Control) or EGD-e::pPL2lux-*P_{hlyA}* (*hlyA*). Three days postinfection, the mice were euthanized, and bioluminescent imaging was performed for 5 min at a binning value of 4 using an IVIS 100 system. BLC, bioluminescence counts (in photons s^{-1}). The color bar indicates the bioluminescence signal intensity (in photons $s^{-1} cm^{-2}$). Subsequently, the numbers of bacterial CFU were determined for the individual organs. Results for the left and right kidneys were combined to obtain total BLC and CFU values. ND, not detectable (bioluminescence counts, $<1 \times 10^4$ photons s^{-1}). The data are representative of the data from three independent experiments.

shown). Overall, these experiments established the effectiveness of pPL2lux as a real-time reporter system to study listerial gene expression in an intact monolayer of C2Bbe1 cells.

Hemolysin expression in the organs of mice. Mice are used as an in vivo model for *L. monocytogenes* infections. Therefore, the suitability of the pPL2lux reporter system to monitor listerial gene expression in the organs of mice following infection was investigated. Two groups of two mice were infected with control strain EGD-e::pPL2lux or EGD-e::pPL2lux-*P_{hlyA}*. Three days postinfection all mice had developed overt symptoms of listeriosis, including piloerection and reduced activity (data not shown). The mice were sacrificed, and the levels of bioluminescence in the intact organs were determined and correlated with the numbers of bacterial cells (Fig. 5). Notably, the numbers of organ-specific CFU recovered from the mice infected with EGD-e::pPL2lux and EGD-e::pPL2lux-*P_{hlyA}* were very similar, suggesting that luciferase expression does not influence the persistence of *L. monocytogenes*. No bioluminescence was detected in any of the organs of the two mice in the control group (Fig. 5). In contrast, the livers, spleens, and kidneys obtained from the two mice infected with EGD-e::pPL2lux-*P_{hlyA}* displayed relatively high levels of bioluminescence. Furthermore, lower levels of bioluminescence were detected in the hearts and lungs of both mice infected with EGD-e::pPL2lux-*P_{hlyA}* (data not shown). These lower lev-

els of bioluminescence most likely reflected the fact that lower numbers of bacteria were recovered from these organs. Nevertheless, the data clearly indicate that hemolysin was expressed in all tested organs of mice 3 days postinfection and demonstrate that the pPL2lux reporter system can be used to monitor listerial gene expression in intact murine organs.

DISCUSSION

Here we describe construction of a luciferase-based reporter system, pPL2lux, and successful use of this system to quantitatively study gene expression in *L. monocytogenes* in three model systems (growth in broth, cellular invasion, and murine infection). In all three model systems the luciferase-expressing *L. monocytogenes* strains displayed characteristics similar to those of control strains that did not express luciferase, as judged by the growth rates and invasive potential. Naturally occurring background levels of bioluminescence have been reported to be extremely low (48), and no background bioluminescence was observed during any of the experiments described here. This feature results in higher sensitivity for luciferase-based reporter systems than for many other reporter systems, including fluorescence-based systems, for which naturally occurring background levels are relatively high (48). One potential limitation of the system described here is the fact that

the luciferase reaction requires oxygen; therefore, it remains to be seen if the system is functional in model systems with low oxygen pressures (for instance, anaerobic growth in broth or the colon). The pPL2lux vector is a site-specific integration vector that was designed for construction of exact translational fusions of promoters to the luciferase-encoding genes, mimicking transcription and translation initiation as it occurs at the native chromosomal location of the promoters under investigation. Therefore, it is not surprising that the levels of bioluminescence and *luxA* transcription closely resembled the actual levels of *hlyA* transcription found at the native chromosomal location. Moreover, the pPL2lux system allows direct, quantitative determination of luciferase activity and the corresponding promoter strength, intrinsically providing the ability to monitor promoter strength in a real-time fashion. This characteristic provides a major advantage compared with many of the reporter systems available to date for gram-positive bacteria, which typically require disruption of bacterial cells and/or addition of a substrate, followed by spectrophotometric measurement to quantitatively determine enzyme and corresponding promoter activities (1, 7, 28, 30, 32).

A previous study in which β -glucuronidase was used as a reporter system in *L. monocytogenes* revealed that hemolysin expression could be conveniently detected in LB broth buffered at pH 7.4 1 h into the stationary phase (5). Similar conditions were used here for the luciferase reporter system, and our results corroborated the previous findings. In addition, our experiments revealed that hemolysin expression is initiated during the mid-log phase in LB broth buffered at pH 7.4 and is still detectable after prolonged incubation in the stationary phase. Previously, the levels of bioluminescence from *luxABCDE* systems have been reported to decrease in the stationary phase in several gram-positive bacteria, including *Streptococcus pneumoniae* (12), *Lactobacillus casei* (28) and *L. monocytogenes* (18). To our knowledge, the hemolysin-coupled luciferase expression profiles described here are the first examples of relatively high levels of luciferase expression in a gram-positive bacterium in the stationary phase and thus overcome a final technical hurdle for the use of luciferase reporter systems to monitor bacterial gene expression in broth.

A *luxAB*-based luciferase reporter system (28) and (quantitative) RT-PCR strategies (4, 8, 28, 38, 40) have been employed previously to monitor in situ gene expression in bacteria during residence in their hosts. Both approaches have been applied only to the murine gut and require extensive homogenization of this organ prior to analysis. In contrast, hemolysin expression profiles could be obtained for intact organs using the luciferase reporter system. Hence, the system described here has great potential for direct comparison of different bacterial promoter activities in intact organs. However, organ-specific variation in quenching of bioluminescence signals does not allow quantitative interorgan comparison for each specific promoter element. Such an analysis would require homogenization of the organs prior to measurement of bioluminescence. Although the quenching differences mentioned above prevent direct correlation of the in vitro and in vivo *hlyA* promoter strengths observed in our experiments, it is remarkable that the highest relative levels of luciferase found per CFU in vitro are more than 100-fold lower than the levels found in C2 cells and the organs of mice (data not shown). Therefore, our findings

further enhance previous experimental suggestions that hemolysin expression might be dramatically elevated in vivo (37).

Overall, the luciferase reporter system described here is a valuable addition to the genetic toolbox available for *L. monocytogenes*, as it can be employed for in situ real-time investigation of listerial promoter activities after infection of mice. Currently, the luciferase reporter system is being exploited in our laboratory to obtain and compare quantitative, organ-specific expression profiles for the majority of *L. monocytogenes* virulence genes and regulators, including *cwhA*, *prfA*, *ycyF*, *inlA*, and *actA*. In parallel, the expression profiles of several household genes, such as *atpI* and *ftsA*, are being obtained, which allows normalization of the expression profiles found for the virulence genes (I. R. Monk, P. A. Bron, P. G. Casey, C. G. M. Gahan, and C. Hill, unpublished data). The wealth of in situ information about spatial and temporal gene expression that will become available from this type of animal experiment is expected to contribute significantly to our understanding of the molecular mechanisms involved in the in situ behavior of *L. monocytogenes* during infection.

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