Published in final edited form as:
Mol Imaging. 2008 ; 7(5): 222–233.

Stably Integrated *luxCDABE* for Assessment of *Salmonella* Invasion Kinetics

Kelly N. Flentie*, **Min Qi***, **Seth T. Gammon**, **Yasmin Razia**, **Felix Lui**, **Luciano Marpegan**, **Aashish Manglik**, **David Piwnica-Worms**, and **Jeffrey S. McKinney**
Departments of Molecular Microbiology, Pediatrics, Biology, and Developmental Biology and Molecular Imaging Center, Mallinckrodt Institute of Radiology, Washington University School of Medicine, St. Louis, MO.

Abstract

Salmonella Typhimurium is a common cause of gastroenteritis in humans and also localizes to neoplastic tumors in animals. Invasion of specific eukaryotic cells is a key mechanism of *Salmonella* interactions with host tissues. Early stages of gastrointestinal cell invasion are mediated by a *Salmonella* type III secretion system, powered by the adenosine triphosphatase *invC*. The aim of this work was to characterize the *invC* dependence of invasion kinetics into disparate eukaryotic cells traditionally used as models of gut epithelium or neoplasms. Thus, a nondestructive real-time assay was developed to report eukaryotic cell invasion kinetics using *lux* + *Salmonella* that contain chromosomally integrated *luxCDABE* genes. Bioluminescence-based invasion assays using *lux*+ *Salmonella* exhibited inoculum dose-response correlation, distinguished invasion-competent from invasion-incompetent *Salmonella*, and discriminated relative *Salmonella* invasiveness in accordance with environmental conditions that induce invasion gene expression. In standard gentamicin protection assays, bioluminescence from *lux*+ *Salmonella* correlated with recovery of colony-forming units of internalized bacteria and could be visualized by bioluminescence microscopy. Furthermore, this assay distinguished invasion-competent from invasion-incompetent bacteria independent of gentamicin treatment in real time. Bioluminescence reported *Salmonella* invasion of disparate eukaryotic cell lines, including neoplastic melanoma, colon adenocarcinoma, and glioma cell lines used in animal models of malignancy. In each case, *Salmonella* invasion of eukaryotic cells was *invC* dependent.

Eukaryotic cell invasion is used by *Salmonella* during the initial steps of pathogenesis[1] and leads to enteric symptoms and disseminated infection. *Salmonella* also localize to, and sometimes invade, cancerous tumors in mice.[2] One basic tool for dissecting the mechanisms of these bacterial–eukaryotic cell interactions is the in vitro cell invasion assay.

The standard technique to assess *Salmonella* invasion into cultured cells is the gentamicin protection assay,[3] which exploits the poor penetration of this antibiotic into eukaryotic cells.[4] Specifically, gentamicin is postulated to kill susceptible extracellular bacteria but not "protected" bacteria that have invaded. Presumably, such selective killing permits the preferential recovery of intracellular bacteria on subsequent culture of lysed cells. Gentamicin protection assays have been used to illuminate genetic and cellular mechanisms of cell invasion by *Salmonella*.[5] For example, the *invC* gene in *Salmonella* encodes an

Address reprint requests to: David Piwnica-Worms, MD, PhD, Mallinckrodt Institute of Radiology, 510 S. Kingshighway Blvd, Box 8225, Washington University, St. Louis, MO 63110; e-mail: piwnica-wormsd@mir.wustl.edu..
*These authors contributed equally to this work.

adenosine triphosphatase (ATPase) that powers a type III secretion system, triggering eukaryotic actin reorganization and *Salmonella* invasion of some eukaryotic cell lines.[6,7]

Despite their widespread use, standard gentamicin protection assays are technically and conceptually limited because they attempt to quantify the invasiveness of individual bacterial strains via direct enumeration of bacterial colony-forming units (CFUs) recovered from lysed eukaryotic cells. The lysis and CFU determination steps consume time, materials, and labor. Colonies are not necessarily correlated with bacterial numbers, so agglomerated organisms might be underenumerated. Additionally, because eukaryotic cells must be destroyed to release invaded bacteria, serial evaluations of bacterial invasion in a single temporal assay are precluded. Furthermore, by definition, current gentamicin protection, CFU-based assays of invasion require that extracellular bacteria of interest are killed by gentamicin, which is a condition not always met.

To attempt to address such limitations, we have modified current gentamicin protection assays of bacterial invasion into eukaryotic cells, including neoplastic lines, by using bioluminescence to report bacterial invasion. Contag and colleagues originally pioneered the use of bacteria expressing luciferase to monitor in vitro and in vivo pathogenesis with organisms containing plasmid-encoded luciferase.[8] We employed constitutively bioluminescent *Salmonella,* which contain chromosomally integrated *luxCDABE* genes from *Photorhabdus luminescens*,[9] and imaging systems that sensitively and specifically detect bioluminescent *Salmonella*.[10] This nondestructive assay requires neither eukaryotic cell lysis nor gentamicin. Rather, we use bioluminescence to track the invasion of *lux+ Salmonella* into various eukaryotic cells in tissue culture. These eukaryotic cells include those traditionally used for models of gastroenteritis, as well as cells previously used in whole mouse models of metastatic cancers. To determine the *invC* dependence of invasion kinetics in these different systems, we compared the invasiveness of *lux+ Salmonella* that are isogenic except for *invC*.

Methods

All experiments were performed in accordance with protocols approved by the Washington University animal studies committee.

Bacterial Strains and Eukaryotic Cell Lines

The bacterial strains and eukaryotic cell lines used in this study are listed in Table 1.

Construction of *Salmonella* Strains with Stably Integrated *luxCDABE*

Conjugative mating was performed between donor strain *Escherichia coli* S17-1 (containing the transfer plasmid pUT mini-Tn5 *lux Km2*; a gift of Michael Winson) and recipient *Salmonella enterica* serovar typhimurium strain SB300A1.11 Mating was performed as described[12] in Luria-Bertani (LB) broth, plated onto LB agar, and incubated at 37°C overnight. Mated colonies were scraped from the LB agar and onto kanamycin (50 μg/mL) MacConkey agar to discriminate *Salmonella* from *E. coli*. The isolated candidate *Salmonella* were grown at dilutions of 10^{-5}, 10^{-6}, and 10^{-7} on these agar plates for 48 hours. Replating on LB/kanamycin plates documented the kanamycin resistance of the recipients of pUT mini-Tn5 *lux Km2*. PCR confirmed the gross presence of each gene of *luxCDABE* in the new strains but not in the parent *Salmonella* SB300A1.

Identification of Site of *luxCDABE* Integration into the *Salmonella*Genome

First, the general location of *luxCDABE* integration was determined from sequences of amplicons produced using touchdown PCR[13] of the genomic DNA of our new *lux+*

Salmonella strain. Touchdown PCR used high-fidelity *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) and thermal cycling conditions (95°C for 5 minutes, then 25 cycles of 95°C for 45 seconds, annealing at variable temperature for 45 seconds [60°C in the first cycle and, at each of the 24 cycles, decreased by 0.5°C per cycle down to 47.5°C], and extension at 72°C for 2 minutes). This was followed by 25 cycles of 95°C for 45 seconds, 50°C for 45 seconds, and 72°C for 2 minutes. Primer pairs included a degenerate primer CCGAATTCCGGATNGAYKSNGGNTC (where N = A, C, G, or T; Y = C or T; K = G or T; and S = C or G), in combination with either an outward facing *luxC* or *luxE* primer (outward *luxC*: CCATCTTTGCCCTACCGTATAGAG and outward *luxE*: TGAGGATGAAATGCAGCGTA). Sequence data from the resulting amplicons suggested *luxCDABE* integration between *Salmonella* chromosomal genes *acrB* and *hha*.

The precise integration site of *luxCDABE* was then identified. PCR amplification from the genomic DNA of our new *lux+ Salmonella* strain was performed using two sets of primers. One reaction, which produced an amplicon of approximately 2.5 kb, used *luxE* (TGAGGATGAAATGCAGCGTA) and *hha* (GCCAGAACGAGGAGGCAGATAACA) primers and PCR conditions of 94°C for 3 minutes; 30 cycles of 94°C for 30 seconds, 51°C for 30 seconds, and 72°C for 3 minutes; and 72°C for 7 minutes. The second reaction produced an amplicon of approximately 3 kb, with *luxC* (ATCCAATTGGCCTCTAGCTTAGCC) and *acrB* (ACCTCAACGGATGAGTTTGG) primers, and the PCR conditions directly above. These amplicons above were sequenced by the Protein and Nucleic Acid Chemistry Laboratory at Washington University in St. Louis. Sequences were aligned with the *Salmonella enterica* serovar Typhimurium strain LT2 complete genome sequence.[14]

Growth Curves in Liquid Culture

Otherwise isogenic *Salmonella* with and without chromosomal *luxCDABE* were grown in overnight liquid cultures and then diluted 1:10 into fresh liquid medium for growth curve analysis. Growth was assessed via serial optical transmission measurements. For growth curve analyses, the growth medium was LB broth, and incubation was at 37°C in a shaker incubator at 200 to 250 rpm.

Construction of an In-Frame *invC* Deletion Mutant of *luxCDABE+ Salmonella*

An in-frame excision of *invC* nucleotides between 506 and 590 was performed using the pCVD442 suicide vector, engineered as previously described,[15] for gene allele exchange.[16] Here 5′ and 3′ segments of *invC* were amplified from wild-type *invC+ Salmonella* SB300A1[11] by PCR using the respective primer pairs 5′GGAGCGAGCTCACTGCAATATCTGGCCTACCCACA3′ with 5′GGAGCAAGCTTATCAGCATGGTCTTACCGCATCCT3′ and 5′GGAGCAAGCTTGGATATGTTGCGCGCTTCGCATAA3′ with 5′GCTATCTCGAGTTTCGCCAGGACGATATTCTCCCA3′. These four primers contain *SacI*, *HindIII*, *HindIII*, and *XhoI* sites, respectively, and nucleotides (underlined, above) of the published *Salmonella* LT2 genomic sequence for *invC*.[14] The resulting PCR products were digested with *SacI* and *HindIII* or with *HindIII* and *XhoI*, respectively, and then individually cloned into pBSIISK+. Following digestion of these two plasmids with *SacI* and *HindIII* or with *HindIII* and *XhoI*, respectively, the small fragments were cloned in tandem into *SacI* and *XhoI* digested pBSIISK+, producing pBSIISK+ (invCΔ506–590). Finally, the *SacI* delimited insert of pBSIISK+(*invC*Δ506–590) was ligated into *SacI* linearized suicide plasmid pCVD442.[16] The resulting pCVD442(*invC*Δ506–590) was transformed into *E. coli* SM10(λpir).[17] Mating was performed between the donor SM10(λpir) strain and the bioluminescent chromosomal *luxCDABE+ Salmonella* strain SB300A1FL6 on LB agar. The cells were then scraped from the LB plates, and serial

dilutions (to 10^{-7}) were made in LB. One hundred microliters of the dilutions was spread on MacConkey agar containing ampicillin (100 μg/mL) and kanamycin (50 μg/mL) to select merodiploids. Of 30 merodiploid candidates, 5 were picked and grown overnight in LB medium without salt. These cultures were then plated on 5% sucrose plates and incubated overnight at 30°C to select for *sacB* removal. Presumptive *sacB*-deficient colonies on sucrose plates were further screened on LB ampicillin (100 μg/mL) plates for a phenotype consistent with concomitant excision of the *bla* gene. One such ampicillin-susceptible clone was analyzed by PCR amplification using *invC* flanking primers. The resulting amplicon was sequenced to confirm the anticipated 84-nucleotide deletion from the 1,296 nucleotide long *invC*, between *invC* nucleotides 506 and 590. The *invC* mutant also includes a six nucleotide *HindIII* site introduced as a by-product of the subcloning steps above (ie, TGCTGATAAGCTTGGATAT, with *invC* nucleotides 506T and 590G underlined). Accordingly, the predicted InvC protein encoded by our *invC* mutation is missing intact InvC amino acids 169 to 197 and has an isoleucine-serine-leucine insert encoded by the TAA/GCT/TGG sequence created by the *HindIII* site insert. This *invC* mutant does not create a frame shift, so it should not have polar effects on adjacent genes.

Invasion Assays

Standard gentamicin protection assays were performed as described.[18] *Salmonella* grown overnight in LB broth (37°C) were diluted 1:100 or 1:10 and grown to an OD_{600} of between 0.45 and 0.7, with OD_{600} matched across samples for a given experiment. Incubations were not shaken, except where noted and as previously described.[19] These bacteria were diluted 1:10 in Dulbecco's Modified Eagle's Medium (DMEM) or to a multiplicity of infection of 100 where noted.

Diluted bacterial suspensions were added to tissue culture plates, at 500 μL to each well in 24-well plates or 100 μL to each well in 96-well plates. For 60 minutes, the bacteria were coincubated with adherent tissue culture monolayers at 60 to 100% confluence. Wells were then washed with DMEM and treated with a medium containing gentamicin at a final concentration of 100 μg/mL. The antibiotic-containing medium was replaced with phenol red-free medium after 90 minutes of treatment, and bioluminescence was measured 3.5 hours later (5 hours after the initiation of gentamicin treatment), unless otherwise noted. Gentamicin-free conditions represented use of phenol red-free DMEM lacking gentamicin after the wash step; imaging occurred 3 hours after washing.

In the CFU recovery assay, following bioluminescence imaging, bacteria were quantified by CFU recovery after immediate lysis of tissue culture cells with detergent lysis as described.[18]

Measurement of Bioluminescence

Bioluminescence measurements were performed as published.[20-22] Images were captured with a cooled charge-coupled device (CCD) camera (IVIS 100, Caliper, Hopkinton, MA). Acquisition parameters were exposure time, 30 seconds; binning, 8; no filter; f/stop, 1; field of view, 15 cm. Signals were measured as the radiance (photons/second/cm^2/sr). To calculate the bioluminescence from a given well, total photon flux (photons/second) was determined from a region of interest (ROI) positioned over the given well and an empty well, which was subtracted to correct for background machine noise using *Living Image* (Xenogen) and *Igor Pro* (WaveMetrics) software. Bioluminescence was presented as mean ± standard deviation of the mean of the total photon flux for replicate well assays.

Bioluminescence Microscopy

Henle cell monolayers cultured on glass-bottomed 35 mm dishes, with or without *luxCDABE+ Salmonella*, were treated as described for gentamicin protection invasion assays. Two to 3 hours after *Salmonella* inoculation, these plates were examined on an inverted microscope (Nikon TE 2000-S) housed in a light-tight microscope incubator (In Vivo Scientific) with the temperature maintained at 36°C. Bioluminescence was recorded with a cooled intensified CCD camera (XR/MEGA10-AW, Stanford Photonics) controlled by *Piper Imaging* software, version 1.3.6 (Agile Automation). Owing to high amplification of the signal (gain set at 400,000 lm/m^2/lx), camera noise was reduced during image acquisition by setting a minimum threshold for the signal that was kept constant for all cultures. Fifteen image sequence frames were obtained per second and integrated later in 40-minute stacks to obtain a single image. Integration, pseudo–color processing, and color merge were performed with *ImageJ* (National Institutes of Health, Bethesda, MD) and *Photoshop CS2* (Adobe) software.

Results

Chromosomal Integration of *luxCDABE*

To create a *Salmonella* strain that constitutively produced bioluminescence, a Tn5 transfer plasmid was used to engineer a strain with chromosomal integration of the *luxCDABE* operon without disrupting essential genes in this process. Independent PCR assays, followed by amplicon DNA sequencing, defined the *luxCDABE* integration site in the *Salmonella* chromosome (Figure 1). According to *Salmonella* strain LT2 complete genomic sequence annotation convention,[14] *luxCDABE* in our *Salmonella* integrated at nucleotide 528,771, 20 nucleotides 5′ to the start codon of *ybaJ*.

Despite some preference for insertion at G/C pairs,[23] Tn5 is considered to mediate near-random integration into bacterial genomes.[24] Interestingly, in bioluminescent *Salmonella* produced by another laboratory using the same suicide vector system, the transposon is reported to have integrated at *hha*.[25] Given that *hha* is immediately 3′ to *ybaJ* in the *Salmonella* genome, perhaps there is preferential integration of the Tn5 *luxCDABE* element into the *Salmonella* genome near *ybaJ* / *hha*.

Alterations in *hha* gene expression, secondary to *luxCDABE* integration, in principle could alter pathogenesis because Hha negatively regulates *hilA*,[26] and *hilA* regulates the invasive phenotype of *Salmonella*.[27] However, our new *luxCDABE+ Salmonella* does not exhibit decreased *hha* mRNA levels, assessed by reverese transcription PCR, compared with its parent (data not shown).

Fitness and Bioluminescence of *luxCDABE+ Salmonella*

The growth curves of otherwise isogenic *Salmonella* with and without *luxCDABE* were identical in LB broth (data not shown). As predicted, the *Km2* kanamycin selection marker integrated with *luxCDABE* did not bestow resistance to gentamicin at concentrations of 100 μg/mL (data not shown). Furthermore, the kanamycin resistance and bioluminescence phenotypes of our *luxCDABE+ Salmonella* were stably maintained without kanamycin selection, both in long-term in vitro cultures and in mouse infections (data not shown).

Invasion Competence of *luxCDABE+ Salmonella*

To determine the impact of integrated *luxCDABE* on *Salmonella* invasiveness, we performed parallel standard gentamicin protection assays with equal inoculations of otherwise isogenic *Salmonella*, differing only in the presence or absence of chromosomally integrated *luxCDABE*. Based on numbers of bacterial CFUs from lysed eukaryotic cells,

there was no defect in *Salmonella* invasion because of *luxCDABE* integration (data not shown).

Bioluminescence as a Reporter of Invasion by *luxCDABE+ Salmonella*

As determinates of host cell invasion, we compared *Salmonella* bioluminescence assays and CFUs from standard gentamicin invasion assays in tissue culture wells. Following measurement of bioluminescence signals from invasion assay tissue culture wells, we processed the tissue culture wells to obtain CFU data from the same assay wells that had been imaged for bioluminescence. We lysed the eukaryotic cells and used plate counts to recover and enumerate *Salmonella* CFU. There was concordance between bioluminescence output and CFU recovery in gentamicin protection assays (Figure 2). Furthermore, bioluminescence readily discriminated between invasion-competent and invasion-incompetent *luxCDABE+ Salmonella* strains. The protein encoded by *invC* is an ATPase that powers a type III secretion system, triggering eukaryotic actin reorganization and *Salmonella* invasion.[6,7] Bioluminescence distinguished between otherwise isogenic chromosomal *luxCDABE+ Salmonella* strains that had an *invC* gene that was either intact (*invC+*) or ablated by an in-frame deletion (*invC−*) (see Figure 2). Our *invC−* strain is more than 200-fold less invasive compared with isogenic *invC+ Salmonella* concordantly assessed by standard gentamicin protection assay.[6,7,18]

Salmonella invasiveness can also be modified by varying environmental conditions. For example, entry into eukaryotic cells can be significantly enhanced when invasion-competent *Salmonella* are prepared using standing rather than shaken cultures.[19] Using a bioluminescence-based gentamicin protection assay, we could discriminate these invasion phenotype differences for *invC+ Salmonella* that differ only in culture conditions prior to exposure to eukaryotic cells (Figure 3). By contrast, the otherwise isogenic *invC− Salmonella* remained minimally invasive when prepared in either standing or shaken cultures (see Figure 3).

We reproducibly observed bioluminescence enhancement with increasing *Salmonella* inoculum (Figure 4). In standard gentamicin protection assays, the multiplicity of infection often requires optimization.[3] In the bioluminescence assay, we detected wild-type *Salmonella* invasion into eukaryotic cells over a 20-fold range in multiplicity of infection (see Figure 4).

Salmonella Invasion into Diverse Eukaryotic Cells: Dependence on *invC*

Salmonella often invade eukaryotic cells postulated to be relevant to cell–cell interactions during pathologic intestinal infections (eg, Henle intestinal epithelial cells,3 HT29 colon carcinoma cells28). *Salmonella* also localize to cancerous tumors in animals,[2,29,30] including nonintestinal cells, such as melanoma, glioma, and breast and prostate neoplastic cells. *Salmonella* can be recovered from these tumors and in some cases appear by electron microscopy to have invaded the neoplastic cells.[2] Here we examined the ability of *Salmonella* to invade various cells used for models of malignancy in mice. Our wild-type bioluminescent *Salmonella* invaded not only Henle (see Figure 2) and HT29 colon cells but also eukaryotic cells of diverse origins, including colon adenocarcinoma MC38, melanoma B16F10, and even, albeit to a lesser extent, glioma C6 cells. In each case, this invasion depended on *invC* (Figure 5).

Real-Time Kinetic Measurements of Bioluminescence during Invasion Assays

The nondestructive nature of bioluminescence now permits serial assessments of the same invaded cells over time. We assessed the kinetics of *Salmonella* bioluminescence from single wells of C6 glioma cells during invasion assays. The bioluminescence from a given

well reflects several factors, including *Salmonella* cell numbers and viability. At a given time, *Salmonella* viability is influenced by the effects of gentamicin and the protection from gentamicin killing afforded by *Salmonella* invasion into eukaryotic cells. Bioluminescence versus time is shown in Figure 6. In this experiment, the distinction between bioluminescence of *invC+* versus *invC− Salmonella* was most pronounced 5 hours after the initial inoculation.

Salmonella Invasion Assay, With and Without Gentamicin Protection

Our ability to distinguish invasion-competent from invasion-incompetent *Salmonella* at time points soon after adding gentamicin (see Figure 6) raised the possibility that bioluminescence could assess invasion independent of gentamicin protection per se. Accordingly, rather than gentamicin treatment, we employed a washing step with three rounds of gentamicin-free medium to physically deplete noninvaded *Salmonella* from the assay wells. In assays in which gentamicin was not used, *invC*-dependent invasion competence of *Salmonella* could still be readily resolved (Figure 7). Although the overall assay time was shorter, the background activity was higher.

Bioluminescent *Salmonella* Visualized by Cooled CCD Microscopy

Traditionally, bioluminescent bacteria have been most extensively exploited for imaging studies of bacterial spread within whole animals, such as for *Salmonella* infections manifesting as gastroenteritis or disseminated infections[8,25] or for the targeted localization of *Salmonella* to malignant tumors.[29] By contrast, for microscopic level studies of bacterial localization[30] or gene expression,[31] fluorescent rather than bioluminescent bacteria have been most widely used. Given recent advances in cooled CCD cameras and our interest in tracking *luxCDABE Salmonella* microscopically, we attempted to visualize our *luxCDABE+ Salmonella* using a cooled CCD bioluminescence microscope. Compared to uninfected eukaryotic cell controls, tissue cultures inoculated with *luxCDABE+ Salmonella* with intact *invC* exhibited foci of bioluminescence, with maximal intensity foci clustered near or within eukaryotic cells (Figure 8). For *luxCDABE+ Salmonella* lacking *invC*, the number and intensity of these foci at the single cell level were much reduced (data not shown). The ability to use microscopy to visualize invasion by our bioluminescent *Salmonella* provides an additional advantage as bioluminescent microscopy does not require potentially cytotoxic excitation light and typically has low background signal.

Discussion

We report a new constitutively bioluminescent *Salmonella* strain (SB300A1FL6). We have subsequently deleted (in frame) specific segments of invasion competence genes in this primary *luxCDABE+ Salmonella* strain, creating a set of reagents to study the functions of specific *Salmonella* genes during bacteria–host interactions. In our *Salmonella* clones, *luxCDABE* was apparently maintained at low fitness cost. This contrasts with other *Salmonella* strains that have been engineered to be constitutively fluorescent via the presence of green fluorescent protein. For example, green fluorescent proteins can significantly inhibit *Salmonella* growth in epithelial cells and macrophages[32] and increase *Salmonella* doubling time.[33]

Plasmid-based *lux* constructs have been exploited as reporter systems for bacterial location in vitro and in vivo.[8,29] However, plasmid-based *lux* systems can suffer from instability. Using the pLITE *lux* expression plasmid in *Salmonella* infections of mice, loss rates of plasmid (and bioluminescence) exceeding 95% of bacterial colonies have been reported.[29] We observed no loss of bioluminescence of our chromosomal *luxCDABE+ Salmonella* strains after serial passages in bacterial cultures or after prolonged infections in mice, even

in the absence of kanamycin selection to maintain the *luxCDABE* / kanamycin resistance gene insert.

One motivation for constructing and characterizing these *luxCDABE+ Salmonella* strains was to use bioluminescence to assess *Salmonella* invasion kinetics into eukaryotic cells. Herein we described a robust and versatile eukaryotic cell invasion assay using chromosomal *luxCDABE+ Salmonella*. The new assay correlated well with the standard detection methodology over a broad inoculum range. The bioluminescence assay readily discriminated the invasion competencies of *invC+* and *invC− Salmonella*. Indeed, the resolution between *invC+* and *invC−* organisms was reliable across a 20-fold range of bacterial inoculated dose and multiplicities of infection. By contrast, CFU-based assays are notably nonlinear with respect to the number of inoculated bacteria.[3] Bioluminescence-based invasion assays also resolved invasion differences among *lux+ Salmonella* as regulated by environmental stimuli.[19]

Bioluminescence-based tracking of *lux+ Salmonella* during eukaryotic cell invasion permits invasion assays to be performed independent of the stringent requirements of gentamicin protection assays. For example, bioluminescence assays need not depend on the use of gentamicin to kill extracellular bacteria or eukaryotic cell lysis to report intracellular "gentamicin-protected" bacteria. This allows studies on bacteria intrinsically resistant to gentamicin or enables analysis in growth conditions that compromise gentamicin activity (eg, acidic pH or divalent cation concentrations34) while also allowing assays in which gentamicin-mediated effects on eukaryotic phenotypes are a concern.35,36

Our assay had similarities to other techniques for assessing bacterial invasion, such as direct observation of internalized bacteria following Giemsa staining[37] or direct observation of *gfp*-labeled bacteria within eukaryotic cells via fluorescent microscopy[30] or fluorescence-activated cell sorter (FACS) analysis.[31] However, *luxCDABE*-encoded bioluminescence provided potential advantages to detect intracellular bacteria. For example, in contrast to Giemsa staining of inanimate features of bacterial cell walls or to *gfp*-based fluorescence, *lux* bioluminescence only reported bacteria that were alive and biochemically active.[38] This direct detection of living bacteria removed the lag time and intermediate maneuvers imposed by experiments that rely on bacterial staining or recovery of bacterial CFUs for data. The real-time and nondestructive nature of *lux*-based tracking of *Salmonella* in eukaryotic cells also allowed serial measurements from the same well over time. Hence, it was well suited to kinetic studies of bacterial invasion and intracellular survival. From a technical perspective, bioluminescence-based detection of *lux+ Salmonella* should readily allow high-throughput experimental scaling in multiwell plate assays, with readout times within minutes.

Furthermore, the components of the experimental system described here for studying bacterial invasion into eukaryotic cells in tissue cultures can also be used to noninvasively detect and localize *luxCDABE+ Salmonella* during infections in living mice (data not shown). Thus, bioluminescence-based detection of *lux+ Salmonella* presents opportunities to more directly correlate in vitro and in vivo models of bacteria–host interactions. This can be used to detect *Salmonella* in experimental mouse models of infection and malignancy. Intriguingly, our bioluminescent *Salmonella* invade a disparate range of malignant eukaryotic cells in vitro, each in an *invC*-dependent manner. This suggests that *Salmonella* interactions with eukaryotic neoplastic cells may recapitulate features of *Salmonella* interactions with eukaryotic epithelial cells in the host intestinal tract.

Acknowledgments

Gregory Storch, Phillip I. Tarr, Virginia Miller, Kim Walker, and members of the McKinney laboratory provided helpful discussions and review of the manuscript. J.S.M. thanks Dr. Michael Winson for the kind gift of transfer

plasmid pUT mini-Tn5 *lux Km2*, Dr. Nicholas Davidson for the cell line MC38, and Joshua Aranda for technical assistance.

This work was supported by research grants from the National Institutes of Health (NIH) (Washington University Digestive Diseases Research Core Center Grant P30 DK52574, Washington University Molecular Imaging Center Grant P50 CA94056, Washington University Children's Health Research Center Grant K12 HD01487, Neuroscience Blueprint Center Core Grant P30 NS057105), the American Cancer Society, and a March of Dimes Birth Defects Foundation Basil O'Connor Scholar Research Award to J.S.M. We acknowledge student stipend support to K.N.F. from NIH Training Grant T32 GM007067 and to M.Q., F.L., and A.M. from Washington University and the Howard Hughes Medical Institute.

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Figure 1.

Foreign DNA integration site into the *Salmonella* genome, at nucleotide 528,771. Nearby loci are *acrB*, *ybaJ*, and *hha*, flanking the insert site of *luxCDABE* and *Km2* as shown. The following DNA sequence from the chromosome of our bioluminescent *Salmonella* identifies the junction between *Salmonella* genomic DNA (nonitalics, corresponding to *Salmonella* LT2 genome nucleotides 529,230 to 528,771) and foreign DNA (italics, with *luxC* coding nucleotides 1–163 in underlined italics):

AAGGCCGCGCAAGCGGCCTTTTTTACGCAAAAATCATAAAATACGCTTATTGTT
AGATTGATTATTTTTTGCCATATTAATAAAAGGTATAATCCTTACTGCGTTAAAG
GCTTTTCTTAGGAAAGTTGGCCATTTCTTAATTCAGCCATTAATTAAGAAATATT
AAGAATATTCCTGGCTATTTTCTCCTGTCAGAGTCTATTGTTTTAGCCTGAAAAG
CTAAAAAACGTTAACCCAATGATTACACAAACAATAAAACTGGTTCCTTTTTAG
GCGACCGACGATCACTGTTAAAATTCGAAAAAGTATGGCAACACGCGGCTTTCA
CGCAATTGTAATTTTTAGTAATATGACGATGAAAAGTTTTTTAGAGTAGATTATA
GTTAAATCATAAGGTGACGTGGGAAGTACCAGGTTAGTTAGTTGTATCCATCCC
GAAGGTGTTCGGTTAGTTTAAGCCCTGACTCTTATACACAAGTGCGGCCGCGTTT
AAACCCATGGACGTGTTGACAATTAATCATCGGCATAGTATATCGGCATAGTAT
AATACGACTCAGGGCCCACTAGTGGTACCCGGGGATCCTCTAGAGTCGACCTGC
AGGTCGACGGATCCGGGGAATTCAGGCTTGGAGGATACGTATGACT A A A A A
A A T T T C A T T C A T T A T T A A C G
GCCAGGTTGAAATCTTTCCCGAAGGTGATGATTTAGTGCAATCCATTAATTTTGG
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TGATTGTAATGGAAATAACGAA

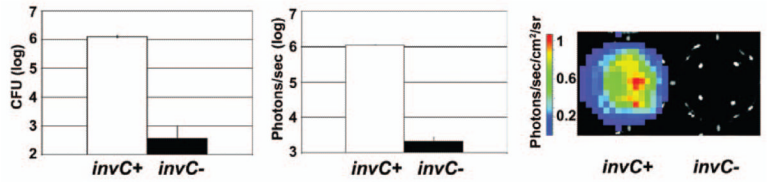

Figure 2.

Comparison of colony-forming unit (CFU) recovery and bioluminescence (photon flux, in photons/second) from gentamicin protection assays using bioluminescent *Salmonella* that vary only by *invC* gene status. Data from a representative gentamicin protection assay performed in triplicate wells are shown. Here invasion of Henle epithelial eukaryotic cells was assessed by bioluminescent *Salmonella* either with *invC* (wild type, *invC+*) or without *invC* (*invC−*). In each case, the multiplicity of infection was 100. CFUs report *Salmonella* grown from lysates, per single wells in a 24-well plate. Photon flux is in units of photons/second, also per single wells in a 24-well plate. CFU and photon flux results are shown as means (± SD). A representative pair of wells from the bioluminescence-based assay is shown with adjacent wells containing either *invC+* (*left well*) or *invC−* (*right well*) *luxCDABE+ Salmonella*; the pseudo-color scale denotes photon intensity radiance. Similar results were obtained in experiments using HT29 rather than Henle eukaryotic cells (see Figure 5 and data not shown).

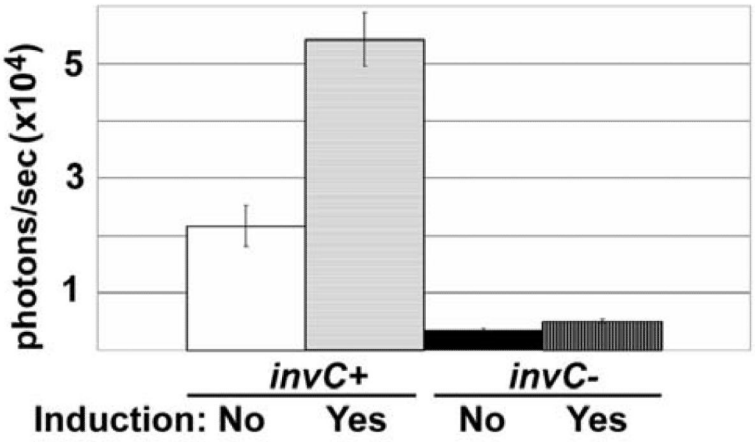

Figure 3.

Bioluminescence and gentamicin protection following differing *Salmonella* growth conditions known to induce enhanced *Salmonella* invasiveness. The bar graph compares bioluminescence data obtained from a bacterial invasion assay of HT29 eukaryotic cells by bacteria previously grown in cultures that were either shaken or standing. Standing cultures are known to induce enhanced *Salmonella* invasiveness in cell culture compared with shaken culture conditions.[19] Induction state is as indicated. Photon flux data are shown as means (± SD), in units of photons/second, and were obtained 90 minutes after adding gentamicin.

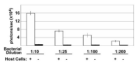

Figure 4.
Bioluminescence signal intensity correlated with *Salmonella* inoculum dose. Photon flux signals from invasion assays of HT29 cells across a 20-fold dilution range of invasion-competent (*invC*+) bioluminescent *Salmonella*. Because eukaryotic cell numbers per well were constant, this also corresponds to 20-fold range of multiplicity of infection. In wells lacking eukaryotic cells, photon flux signals approach ambient background. The results are from quadruplicate samples, with photon flux data (in units of photons/second) shown as means (± SD).

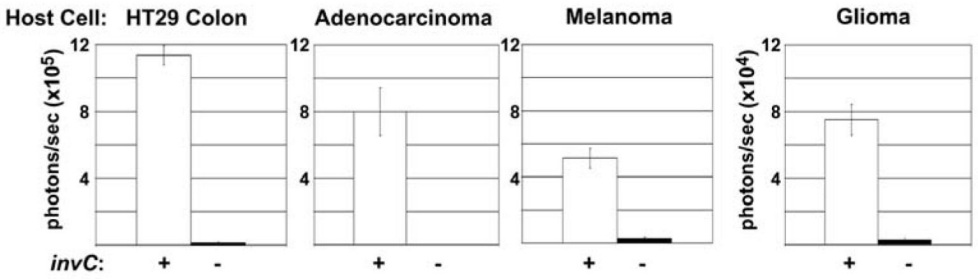

Figure 5.

Invasion of bioluminescent *Salmonella* into eukaryotic cell lines of diverse origins. Photon flux data represent invasion of wild-type *invC*+ and *invC*− *Salmonella* into human intestinal HT29, mouse colon adenocarcinoma MC38, mouse melanoma B16F10, or rat glioma C6 cell lines. For *invC*+ bioluminescent *Salmonella*, the photon signal following exposure to glioma eukaryotic cells was approximately one-tenth that seen with adenocarcinoma cells (note the different *y*-axis scale for glioma cells). Data are from triplicate wells, shown as means (± SD), in photons/second.

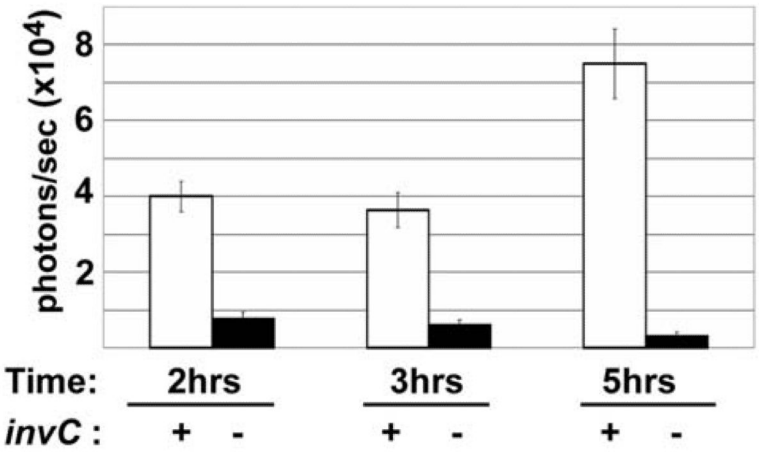

Figure 6.

Kinetics of bioluminescence during *Salmonella* infection. Photon flux data of bacteria serially measured from the same invasion assay wells. The times indicated hours after initiation of a 90-minute gentamicin treatment followed by replacement with medium. *Salmonella invC+* and *invC−* strains and C6 glioma eukaryotic cells are as described above. Data are from triplicate wells, shown as means (± SD), in units of photons/second.

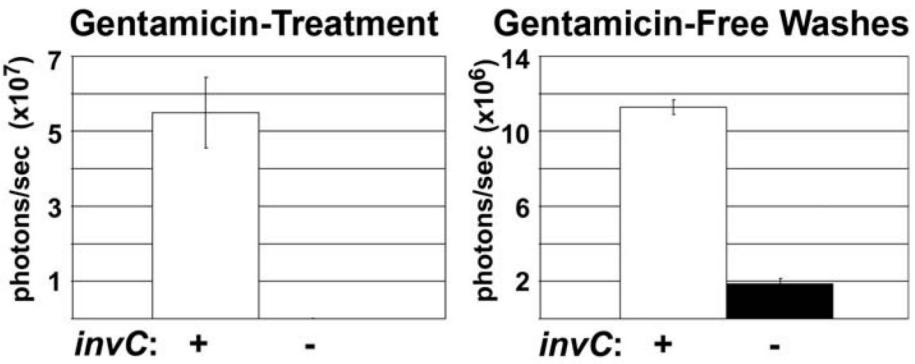

Figure 7.

Bioluminescence monitoring of *Salmonella* interactions with eukaryotic cells, using gentamicin-containing and gentamicin-free media. Photon output from *invC+* and *invC−* *Salmonella* invasion of Henle cells, from quadruplicate samples, with photon flux data shown in units of photons/second, expressed as means (± SD). Gentamicin-treatment conditions represented 90 minutes of gentamicin incubation, following replacement with phenol red free DMEM and imaging at 5 hours after initiating gentamicin addition. Gentamicin-free conditions represented use of phenol red-free DMEM lacking gentamicin throughout, followed by three washes; imaging occurred 3 hours after washing.

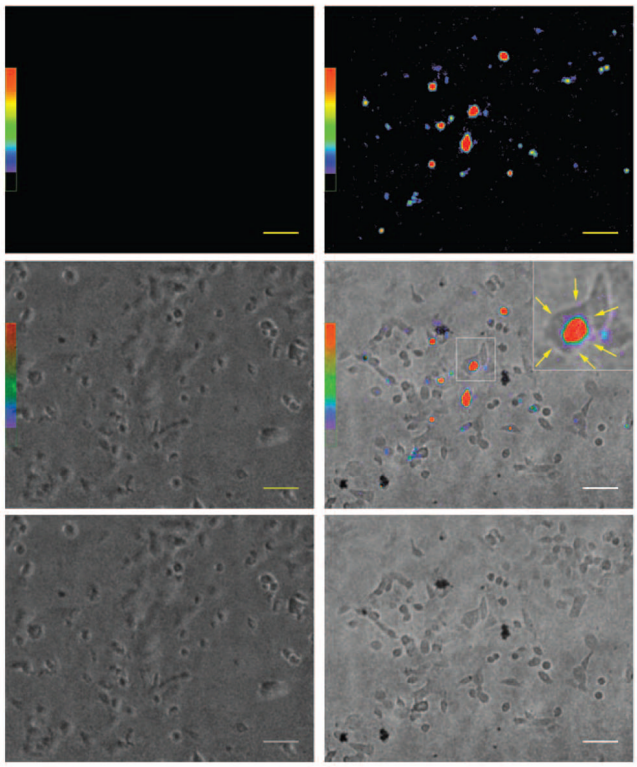

Figure 8.
Microscopic detection of bioluminescence from *luxCDABE+ Salmonella* in eukaryotic cell cultures. Henle cell monolayers are shown either alone (*left panels*) or following inoculation with *luxCDABE invC+ Salmonella* (*right panels*). For each sample, images show the bioluminescence signal alone (*top*), the phase contrast (*bottom*), and a merged image of bioluminescence and phase contrast (*middle*). Within the merged image of the Henle cells inoculated with *luxCDABE Salmonella*, a box demarcates the image area enlarged in the upper right corner (*inset*). *Yellow arrows* outline a Henle cell; maximal intensity foci occur near or within eukaryotic cells. Spectral scales denote pseudo–color representation of bioluminescence signal intensity in relative light units (RLU), ranging from 1 to 50 RLU for samples without *Salmonella* and 1 to 100 RLU for samples with *Salmonella*. Processing of these samples followed the same methods as gentamicin protection assays described in the text; images were obtained 2 to 3 hours after inoculation of *Salmonella*. Bar = 50 μM.

Table 1

Strains and Eukaryotic Cell Lines Used in the Study

Strain or Cell Line	Description	Source or Reference
Salmonella SB300A1	Parent strain, not bioluminescent; contains *araC*-P_{BAD}-regulated T7 RNA polymerase	11
Salmonella SB300A1FL6	SB300A1, modified by chromosomal integration of *luxCDABE* to be constitutively bioluminescent	This study
Salmonella SB300A1FL6AM1	SB300A1FL6, modified by in-frame excision of *invC* nucleotides between 506 and 590	This study
Escherichia coli S17-1	Donor strain in conjugation with SB300A1, for delivery of pUT mini-Tn5 *lux Km2*	12
E. coli SM10(λpir)	Donor strain in conjugation with SB300A1FL6, for delivery of plasmid pCVD442(*invC*Δ506–590)	17
Henle 407	Human epithelial cell line	ATCC: CCL-6
B16F10	Murine melanoma cell line	ATCC: CRL-6475
HT29	Human colon carcinoma cell line	ATCC: HTB-38
C6	Rat glioma cell line	ATCC: CCL-107
MC38	Murine colon adenocarcinoma cell line	Gift: N.O. Davidson

ATCC = American Type Culture Collection.