Induced Biliary Excretion of *Listeria monocytogenes*

Jonathan Hardy,[1] Jeffrey J. Margolis,[2] and Christopher H. Contag[1,2,3]*

*Departments of Pediatrics,[1] Microbiology and Immunology,[2] and Radiology,[3] E150 Clark Center MC 5427,
Stanford University School of Medicine, Stanford, California 94305*

Listeria monocytogenes is a ubiquitous gram-positive bacterium that can cause systemic and often life-threatening disease in immunocompromised hosts. This organism is largely an intracellular pathogen; however, we have determined that it can also grow extracellularly in animals, in the lumen of the gallbladder. The significance of growth in the gallbladder with respect to the pathogenesis and spread of listeriosis depends on the ability of the bacterium to leave this organ and be disseminated to other tissues and into the environment. Should this process be highly inefficient, growth in the gallbladder would have no impact on pathogenesis or spread, but if it occurs efficiently, bacterial growth in this organ may contribute to listeriosis and dissemination of this organism. Here, we use whole-body imaging to determine the efficacy and kinetics of food- and hormone-induced biliary excretion of L. monocytogenes from the murine gallbladder, demonstrating that transit through the bile duct into the intestine can occur within 5 min of induction of gallbladder contraction by food or cholecystokinin and that movement of bacteria through the intestinal lumen can occur very rapidly in the absence of fecal material. These studies demonstrate that L. monocytogenes bacteria replicating in the gallbladder can be expelled from the organ efficiently and that the released bacteria move into the intestinal tract, where they pass into the environment and may possibly reinfect the animal.

Listeria monocytogenes, the causative agent of listeriosis, grows intracellularly in host macrophages and other cells. Its ability to replicate in the cytoplasm of the cells of the host is critical for virulence, as evidenced by the significant attenuation of *L. monocytogenes* when intracellular replication is compromised by mutation (17). Using in vivo bioluminescence imaging (BLI), we determined that this pathogen can also grow extracellularly, in the gallbladder lumen (12). BLI is a method in which the organism is genetically tagged with luciferase, which produces light that is detectable through the tissues of live anesthetized animals using an ultrasensitive charge-coupled-device camera (6, 8). When applied to murine listeriosis using *L. monocytogenes* genetically labeled with an integrated bacterial *lux* operon, this technique revealed strong signals from the gallbladder (12). *L. monocytogenes* in the gallbladder was neither associated with the cells of the gallbladder wall nor observed within the cells by microscopy, and over 98% of CFU were easily recovered from the lumenal contents of the organ directly without extensive washing of the excised tissue. Replication in the gallbladder lumen was apparently in situ, as the bacteria were observed in chains, indicative of many cell divisions. Chain morphology is not observed during intracellular replication or growth of wild-type bacteria in ordinary broth culture. The nature of bacterial growth in the hostile conditions of the gallbladder lumen has not been characterized, and the role of this organ in the pathogenesis and spread of listeriosis is unknown.

Extracellular replication in the gallbladder was unexpected because intracellular replication in the cytoplasm of host cells is generally thought to play the major role in the pathogenesis of listeriosis. Intracellular replication is dependent on the pore-forming protein listeriolysin O (LLO), encoded by the *hly* gene. LLO aids in the lysis of the phagocytic vacuole, permitting access to the cytoplasm of macrophages and other cells (11, 17). In the gallbladder, however, extensive extracellular replication was observed over many days when the highly attenuated Δ*hly* mutant was employed (12). This result indicates that the prolonged signals observed are due to extracellular bacteria persistently growing in this state that are apparently unaffected by the immune responses that would clear this attenuated mutant from other tissues such as the liver and spleen (11, 17). Thus, the gallbladder lumen was revealed as a novel niche for this pathogen, and we are undertaking efforts to characterize the growth of *L. monocytogenes* in this organ, which contains high concentrations of bile and has not been shown to be a site of growth for any other bacterium in an animal model. Whereas many bacteria infect and cause disease in the gallbladder tissue, none have been shown to reside in the lumen asymptomatically, with the exception of *Salmonella enterica* serovar Typhi in human carriers of typhoid fever. Because *L. monocytogenes* is carried asymptomatically in humans (1, 20), we are interested in whether a site of human carriage is the gallbladder lumen and how the observed growth of *L. monocytogenes* in the murine gallbladder may relate to such carriage. Aside from possible implications regarding human carriage, we are investigating the nature of bacterial growth in the hostile environment of the gallbladder lumen, as it represents an unusual and as-yet-uncharacterized aspect of the host-pathogen relationship in the context of a previously well-studied model.

Bile is highly toxic to most microorganisms, and the gallbladder lumen is usually sterile. *L. monocytogenes* has evolved to resist bile toxicity, as indicated by the gene *bshA*, encoding a bile salt hydrolase that confers resistance to bile (7), and the *bilE* locus, encoding an apparatus involved in bile exclusion

* Corresponding author. Mailing address: Department of Pediatrics, E150 Clark Center MC 5427, Stanford University School of Medicine, Stanford, CA 94305. Phone: (650) 725-8781. Fax: (650) 498-7723. E-mail: ccontag@cmgm.stanford.edu.

(21), as well as other loci. The presence of these genes may suggest adaptation to growth specifically in the gallbladder, presumably to take advantage of the organ as a reservoir in the animal, or alternatively, these and other factors may have evolved to ameliorate the antimicrobial effects of bile in the intestine (10), with replication in the gallbladder being merely a consequence of this adaptation. Bile resistance does not confer the ability to grow extracellularly in the lumen of the healthy gallbladder to *Salmonella enterica* serovar Typhimurium in animals, and BLI images of *Salmonella* do not display signals from the gallbladder (6).

Growth of *L. monocytogenes* in the gallbladder could be completely benign to the organ, with little or no effect on the gallbladder, or the bacterium may alter the tissue environment and affect the structure or function for the purpose of maximizing bacterial replication or avoiding bile toxicity. Bacterial persistence at this tissue site may represent a dangerous reservoir of infection. This organ is known to be recalcitrant to antibiotic therapy (19), and the bacterium may use this site to avoid the immune response, because there are no cells in the concentrated bile of the gallbladder lumen, and immunity to listeriosis is cell mediated (2, 4, 15).

While listeriosis is not thought to be spread directly by the fecal-oral route, fecal shedding by biliary excretion could result in the contamination of foods, as first posited by Briones et al. (3). We have previously observed intestinal signals on days following gallbladder signals, suggesting that the gut might be infected with bacteria discharged from the gallbladder (12). Thus, it is possible that bacteria in the gallbladder can be disseminated to other tissue sites via gallbladder contents being expelled into the intestine; however, the infection of these tissues cannot be observed in mice because of their extreme resistance to gastrointestinal inoculation. In mice, with oral 50% lethal doses (LD_{50}) above 10^9 CFU, the bacteria expelled from the gallbladder are unlikely to cause overt disease, but humans are susceptible to relatively lower oral doses (1, 14). Humans also have much larger gallbladders, which could harbor and release many more bacteria. The gallbladder is a dynamic organ that contracts in response to meals containing fat to expel bile through the bile duct into the small intestine to aid in the emulsification and digestion of lipids. Between meals, the gallbladder fills with bile produced by the liver and is distended. Contraction is due to the muscular outer layer of the organ, which responds to hormones such as cholecystokinin and secretin that are released by the small intestine when fats are encountered. We sought to use imaging to directly demonstrate biliary excretion of *L. monocytogenes* from the gallbladder and to test the hypothesis described previously by Briones et al. (3) that biliary excretion could lead to the release of the bacteria and the spread of the disease. Here, we describe the kinetics of food- and hormone-induced biliary excretion of *L. monocytogenes* from the gallbladders of asymptomatic animals by using BLI to monitor *lux*-tagged bacteria in mice over the course of infection. These studies demonstrate that *L. monocytogenes* bacteria replicating in the gallbladder are indeed efficiently expelled from the organ upon specifically induced contraction and that the bacteria so released penetrate deeply into the intestinal tract, where they may possibly reinfect the animal or pass into the environment. Because mice exhibiting signals from the gallbladder are often asymptomatic

even with the virulent strain (12), contamination via fecal material may occur from asymptomatic animals. Should asymptomatic human carriers (1, 20) harbor *L. monocytogenes* in the gallbladder in a manner similar to that of carriers of typhoid fever, these results suggest that listeriosis could be spread from such individuals.

MATERIALS AND METHODS

Bacterial strains. The construction of *L. monocytogenes* strain 2C and the *hly* deletion of this strain from parental *L. monocytogenes* strain 10403S have been described previously (8, 9, 12). Briefly, *L. monocytogenes* strain 10403S (a gift of Daniel Portnoy) was made bioluminescent using the *lux-kan* hybrid transposon of plasmid pAUL-A Tn*4001 luxABCDE* Km^r. Bacteria were initially selected on erythromycin, subsequent insertions of the promoterless hybrid *lux-kan* operon into listerial genes were selected on 200 μg/ml kanamycin, and the insertions were sequenced by inverse PCR and characterized for light production and stability. The insertion of clone 2C was found to lie 47 bp downstream of the start codon of the *flaA* locus. In the 10403S background, *flaA* is dispensable for virulence, so this insertion is not likely to affect the virulence of this strain (22). In addition, many other insertions exhibit identical patterns, including gallbladder signals (12). The 2C strain was used as a donor to transduce the *lux* operon into mutant backgrounds such as Δ*hly*, Δ*inlA/B*, and *prfA* mutants of 10403S (also kindly provided by Daniel Portnoy). Transduction was performed according to a method described previously by Lauer et al. using the generalized transducing phage U153 (13) and with the advice of Laurel Lenz (personal communication). Briefly, U153 lysates were prepared from the donor strain by incubation of 300 μl of overnight-aerated LB–10 mM MgSO$_4$–10 mM CaCl$_2$ broth culture with 10^3 to 10^4 PFU of U153 and plated in 3 ml of LB–0.7% agar overlays in 10-cm LB agar petri dishes. Plates with interlaced plaque patterns were scraped, centrifuged, and filtered for a donor stock, usually generating a titer of 2×10^8 PFU/ml. Two hundred microliters of the logarithmic recipient strain grown to an optical density of 0.2 was incubated with 50 μl and 100 μl of donor stock in brain heart infusion (BHI) broth–10 mM MgSO$_4$–10 mM CaCl$_2$ for 1 h and plated onto BHI–50 μg/ml kanamycin agar plates. The colonies were screened for bioluminescence using an IVIS system (Xenogen Corp., Alameda, CA) according to the instructions of the manufacturer.

Infection and imaging. Female inbred BALB/c and outbred CD1 mice, 8 to 20 weeks old, were injected intravenously with 200 μl of phosphate-buffered saline (PBS) containing 4×10^4 to 6×10^4 CFU of the virulent 2C strain or 1×10^9 CFU of the attenuated 2C(Δ*hly*) or 2C(*prfA*) strain. The two attenuated strains behaved identically with respect to BLI signal, and both exhibited a high degree of attenuation, with intravenous LD_{50} of approximately 2×10^9 CFU (5, 17). At 1×10^9 CFU/infection, the mice often showed symptoms of listeriosis, such as conjunctivitis of the eye, lethargy, hunched appearance, and matted fur, at day 1 postinoculation. On day 2 at this dose, the conjunctivitis was most often resolved and the animals were no longer lethargic or hunched in appearance. The mice generally recovered after 2 days and displayed no conjunctivitis or other symptoms thereafter, but gallbladder signals can persist from such infections for up to 2 weeks (12). In the present study, the mice were imaged each day postinoculation until high gallbladder signals were observed and matted fur and lethargy were absent in the case of the attenuated strains, whereupon fast-and-feed experiments were commenced. Images were obtained using IVIS 100 and 200 systems (Xenogen Corp., Alameda, CA) according to instructions of the manufacturer. Isoflurane gas anesthetic was administered at 2% in oxygen using the Matrix system (Xenogen Corp.), which enables mild anesthesia from which the mice recover within 2 min of removal from the gas. The images were obtained using an integration time of 3 to 5 min at a binning of 10 pixels for live animals and 10 and 2 pixels for excised intestinal tissues. Serial mode imaging was performed using Living Image version 2.5 software and the IVIS system, according to instructions of the manufacturer, employing 5-min exposures. Sequential images of live mice were acquired using single or multiple reference photographs as noted in the figure legends. For quantification of the signals originating from the gallbladder and abdomen, identical regions of interest were employed for all of the images. The circular region of interest of 1.2 cm^2 in diameter was drawn over a region corresponding to the gallbladder in all mice, and a 3- by 2-cm rectangular region of interest was used for the abdominal signal. The total photon count recorded in each region of interest was determined by Living Image software provided by the manufacturer (Xenogen Corp.). Camera settings and exposures were identical for all in vivo images, so these parameters were constant. Individual mice displayed different initial gallbladder signals, and all animals had slightly differ-

ent background levels of each region of interest in the absence of bacterial signals.

Fasting and feeding. Mice in which the gallbladder signal was strong were selected for experiments to image bacterial expulsion. In the case of attenuated strains given at doses that result in asymptomatic infection, most but not all mice displayed the desired signal (12). Food was withdrawn from the cages for periods of 4 to 8 h, a duration that minimizes contraction of the gallbladder, presumably by restricting the release of cholecystokinin and other food-induced hormones. Following the period of fasting, the animals were fed 200 μl of whole cow's milk, and 15 min later, the mice were anesthetized and imaged at the times indicated. For cholecystokinin administration, 100 μg/kg body weight of cholecystokinin Tyr-27-sulfated fragment 26-33 (Sigma C-2175) diluted to 200 μl in PBS was injected intraperitoneally. To obtain bioluminescence data from excised infected organs, the anesthetized mice were sacrificed by cervical dislocation when desired signals were observed, and the organs were removed and imaged in 10-cm petri dishes. It must be noted that images of excised organs may not be linear with respect to bacterial cell number, likely because oxygen saturation wanes after sacrifice (luciferase requires oxygen), and it cannot be assumed to be equally restored in all parts of all organs once they are excised and exposed to air. Nevertheless, the signal from excised organs indicates the presence of growing bacteria, and imaging of excised organs can be used to locate signals observed in the live animal just before sacrifice. For the ex vivo images, the liver, spleen, and intestine were imaged ex vivo with an IVIS system as described previously (12) using integration times of 30 s and a binning of either 2 by 2 or 10 by 10 pixels. No signals were detected from the liver or spleen (data not shown).

Ultrasound. Ultrasound images of cholecystokinin-induced gallbladder contraction were obtained using a VisualSonics model VEVO 660 small animal system as recommended by the manufacturer. For ultrasound, mice were anesthetized with isoflurane gas or 300 mg/kg intraperitoneal avertin and prepared for imaging by removal of hair with a shaver and depilatory cream. Cholecystokinin peptide fragment 26-33 (CCK8) was administered intraperitoneally after the gallbladder was located, and images were obtained using a model 603 probe at a frame rate of 2 or 30 Hz for a total of 300 frames per acquired sequence and 3 to 10 sequences per single administration of peptide.

Fecal CFU counts. Mice were subjected to the fast-and-feed imaging protocol described above and allowed to recover from anesthesia, whereupon they were separated into beakers, and fecal pellets were collected over a period of up to 5 h. All mice infected with *L. monocytogenes* shed the bacterium in their feces whether the infection was oral or intravenous (3). Because the mice subjected to fast-and-feed experiments had been starved and the infection resulted in dehydration, fecal production was greatly reduced in these animals, and fecal pellets were produced only sporadically, so regularly spaced time points were not possible. Pellets were collected, weighed, and then resuspended in proportional amounts of PBS, 1 ml of PBS per 20 mg of pellet, and processed simultaneously. Each pellet was manually disrupted with a pipette tip after a few minutes in PBS for softening and vortexed for 5 min, and dilutions were plated onto BHI agar plates with 50 μg/ml kanamycin. The next day, the plates were imaged using an IVIS-50 bioluminescence detection unit (Xenogen Corp.) according to instructions of the manufacturer.

RESULTS

Frequency of gallbladder signals. Extracellular replication of *L. monocytogenes* in the gallbladder is common during fatal and sublethal infection of highly resistant CD1 outbred mice as well as BALB/c mice. Figure 1 shows that elevated gallbladder signals occurred in 19 of 20 CD1 mice infected with 6×10^4 CFU of virulent *L. monocytogenes* strain 2C. Other sites, such as the abdomen, showed signals; however, the gallbladder signal dominated in almost all of the animals on day 2. *L. monocytogenes* is a systemic pathogen, and the other signals include those of the lymph nodes, intestine, liver, spleen, and brain (12). The central nervous system and the eye also exhibited strong signals upon imaging later in the course of the disease.

Fasting and feeding induction of gut signals. We have previously characterized the BLI patterns associated with fatal and sublethal infections by *L. monocytogenes* and determined that the gallbladder lumen is the site of extensive extracellular replication. Image and CFU data indicated that essentially all
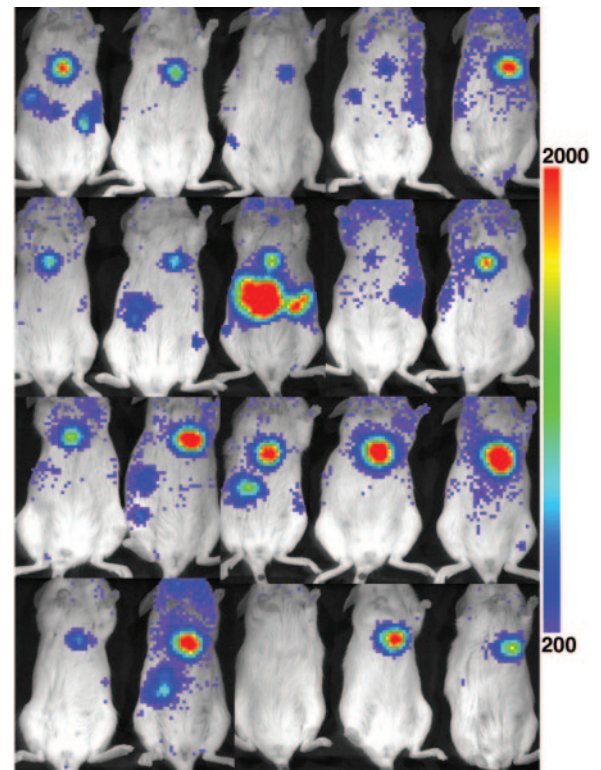

FIG. 1. Frequency of gallbladder signals upon intravenous infection with *L. monocytogenes*. Twenty female CD1 outbred mice (25 g) were infected intravenously via the tail vein with 6×10^4 CFU of virulent *L. monocytogenes* strain 2C and imaged 2 days later. This dose in 25-g CD1 female mice is fatal after 5 to 6 days. The scale bar indicates pseudocolor representation of photon counts detected during the 5-min exposure. The characteristic isotropic signal from the lower thorax of the animal originates from the gallbladder.

of the bacteria in the gallbladder are extracellular, and few if any bacteria can be recovered from the tissue of the organ. Thus, the gallbladder lumen represents a previously unrecognized source of extracellular *L. monocytogenes* within the body of the animal. To demonstrate that the bacteria were indeed extracellular, mutants defective in intracellular replication were used to show that replication could proceed in the lumen of the gallbladder. We sought to determine if the bacteria residing in the lumen of the gallbladder could be expelled or if they are trapped in the organ. This question is of interest for many reasons, partly because intracellular bacteria would be held in the organ upon contraction. We have found no evidence for invasion of this tissue and are interested in why the organ is resistant to invasion. Using BLI, we sought to image bacterial expulsion from the gallbladder and demonstrate biliary excretion from the organ to confirm the hypothesis, described previously by Briones et al., of biliary excretion of *L. monocytogenes* (3). To prevent the gallbladder from contracting, the mice were starved for 4 to 8 h (the fasting portion of the procedure), and to induce contraction of the organ, the mice were then fed a meal containing fat, which causes the release of cholecystokinin, secretin, and other hormones. These hormones cause the outer layer of the gallbladder to contract, expelling the lumenal contents into the small intes-
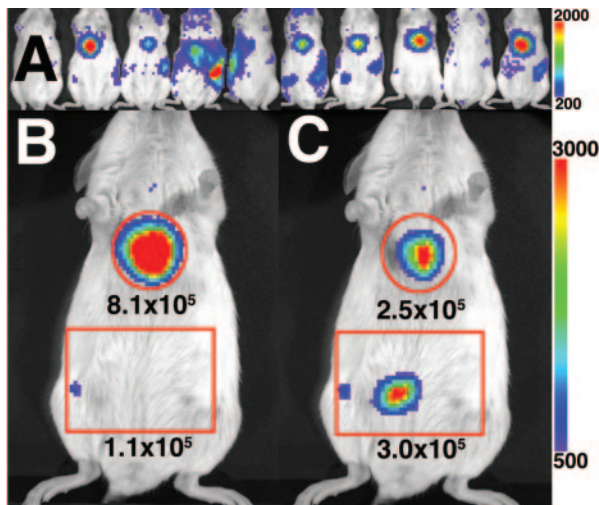
FIG. 2. BLI of *L. monocytogenes* in mice after fasting and feeding. Ten CD1 mice were infected intravenously with 4×10^4 CFU of the virulent 2C strain of *L. monocytogenes*. On day 4 postinfection, the mice were starved for 4 h and imaged (A), and the eighth mouse from the left was selected from the cohort for the fast-and-feed procedure. This animal was allowed to recover from the anesthetic for 10 min, fed 200 μl of whole cow's milk, and imaged 5 min (B) and 50 min (C) after feeding. Color bars indicate photon counts per pixel registered by the camera during the 5-min exposures, and the counts registered in the designated regions of interest (within the 1.2-cm^2 red circle and the 3-by 2-cm red square) are shown. The same reference photograph was used for both BLI images.

tine through the common bile duct. Mice infected with virulent *L. monocytogenes* (cohort shown in Fig. 2A) and subjected to fasting displayed strong signals from the gallbladder (Fig. 2B). When the mice were then fed whole cow's milk, the gallbladder signal was reduced and a new signal appeared over the abdomen (Fig. 2C). The change in signal intensity was quantified and revealed that the gallbladder signal decreased and the abdominal signal increased. The reduction of signal from the gallbladder did not equal the signal increase of the abdomen. This difference may be due to not all of the bacteria being expelled at once as a single bolus. Some of the signal may therefore be spread out in areas of the intestine that are too deep for signal detection.

Serial imaging of the induction of gut signal. The kinetics of the process were determined using sequential images, revealing the expulsion of *L. monocytogenes*. For this purpose, signal intensities needed to be high to reveal the nuances of bacterial movement. The signal from the virulent strain was sufficient for localizing the bacteria to the gallbladder and for monitoring expulsion into the intestine (Fig. 1 and 2); however, to more clearly monitor this process, large numbers of bacteria in the gallbladder were needed and could be obtained with bacterial strains of reduced virulence (12). Because doses of these strains that seed the gallbladder with more bacteria can be administered without causing any disease, the mice exhibited much greater signal intensities from the gallbladder than the virulent strains provided and did so asymptomatically for up to several weeks, during which many fast-and-feed experiments could be performed on the same animal. The low-virulence mutants that we have studied include *hly* deletion mutants (*hly*

encodes the LLO protein that lyses the phagocytic vacuole and permits intracytoplasmic growth in macrophages and other cells), *prfA* mutants (*prfA* is the master positive regulator of intracellular virulence factors), and strains with additional mutations in *inlA* and *inlB* (the genes encoding A and B internalins, which are cellular uptake proteins). Attenuated mutants provide a means of analyzing large numbers of intralumenal bacteria in the absence of symptoms and without extensive growth in other organs, such as the liver. The dynamic process of expulsion of bacteria from the gallbladder is easily visualized by imaging animals infected with attenuated strains, which show large gallbladder signals and little background from other organs. In a typical experiment of this type, the *prfA* mutant derivative of *L. monocytogenes* strain 2C was injected into three mice (Fig. 3, left), and the mice were imaged until the large signals appeared, in this case on day 2. At this dose, the mice displayed no symptoms at all, and about one in five animals showed the gallbladder signal over extended periods of up to 2 weeks (12). Mouse 1 was selected from the cohort shown in this experiment for a fast-and-feed procedure the next day. The mouse was starved for 4 h, imaged, allowed to recover from anesthesia for 10 min, fed 200 μl of milk, and imaged in serial mode as described in Materials and Methods. The postfeeding images revealed changes in location of abdominal signals, presumably due to expulsion of the bacteria into the intestine and their transit through the organ. In this case, the signal was observed to extend from the normally isotropic shape (12) to form a lobe toward the mouse's right, at or before 6 min postfeeding. This extension corresponds roughly to the direction of the bile duct observed when mice were sacrificed and dissected. The signal then traversed the abdomen of the mouse rapidly and reached a point in the mouse's lower left abdomen within 18 min, where it remained for the duration of the experiment. We hereafter refer to such signals as terminal signals. More than one expulsion event could be obtained from animals, as is apparent in the image acquired at 48 min postfeeding in Fig. 3. Quantification of the signals from the gallbladder and abdomen employing regions of interest identical to those of Fig. 2 (bottom), revealed that only modest increases in abdominal signal were observed. We therefore sought to increase the signal intensity and specificity of the procedure by inducing more complete contraction.

Cholecystokinin-induced gallbladder contraction. The images suggest that food-induced contraction of the gallbladder is responsible for the expulsion of the bacteria through the common bile duct and into the intestine, and the movement of signal through the intestine of the mouse subsequently occurs very rapidly. To demonstrate that gallbladder contraction and not some other process associated with feeding was responsible for the observations, and to induce more complete contraction of the organ, we used the hormone cholecystokinin, which is normally released from the intestine into the bloodstream upon the consumption of fat and specifically induces the gallbladder to contract. An 8-amino-acid-residue polypeptide derived from cholecystokinin has been shown to induce contraction of the gallbladder. This polypeptide (CCK8) was injected intraperitoneally at 100 μg/kg body weight to induce gallbladder contraction. To determine the kinetics of the contraction induced in this manner, uninfected female BALB/c mice were anesthetized, and ultrasound images were obtained after 4 to 8 h
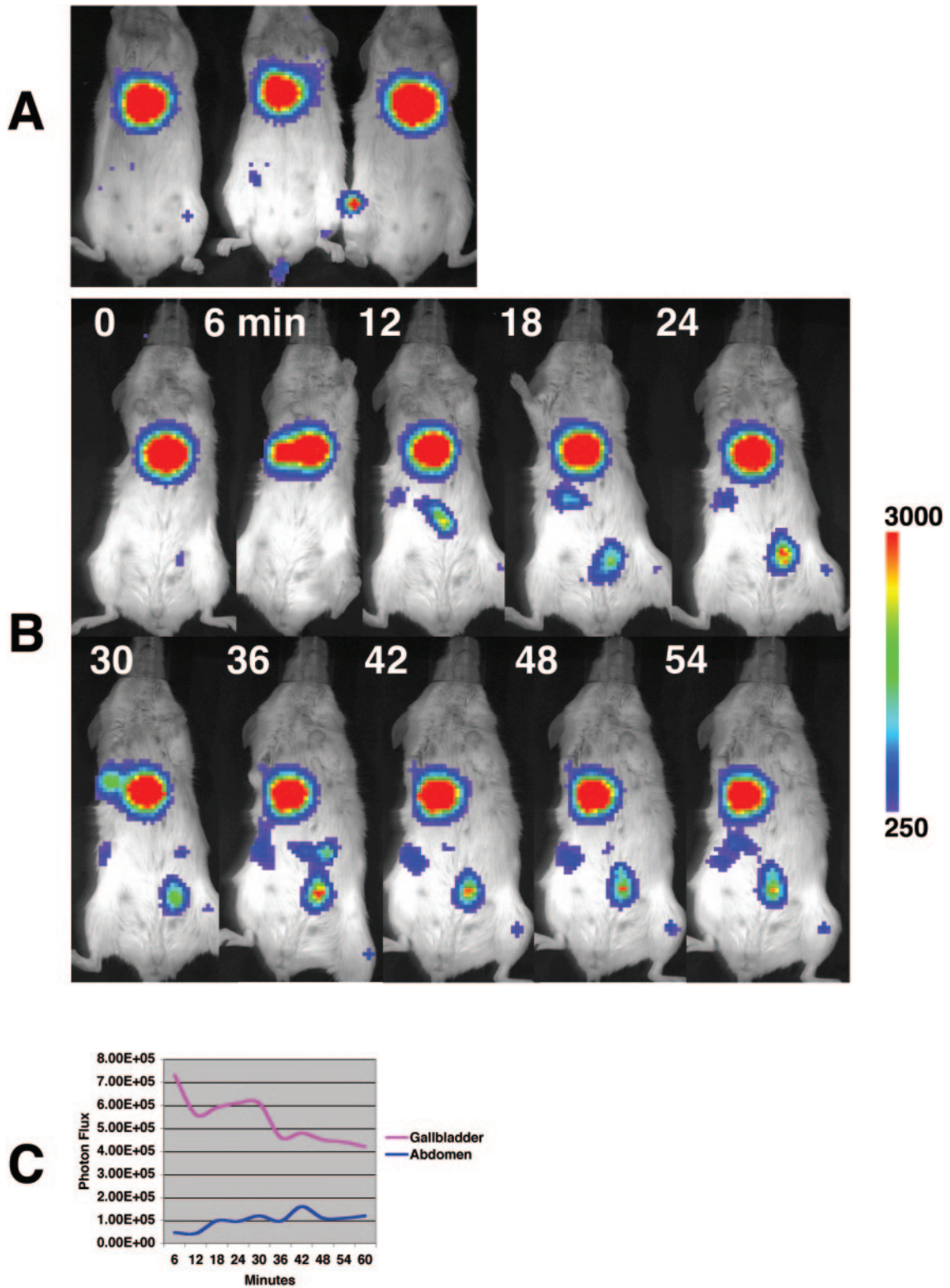
FIG. 3. Serial images of fasting and feeding. Three female BALB/c mice were infected intravenously with the 2C(Δ*prfA*) strain of *L. monocytogenes*, starved for 4 h, and imaged (A). Mouse 1 was then fed 200 μl whole cow's milk and imaged in serial mode every 6 min as indicated, with an exposure time of 5 min per image. Multiple reference photographs were employed for this series. Quantification of the signals over time using regions of interest identical to those of Fig. 2 is shown in panel C.
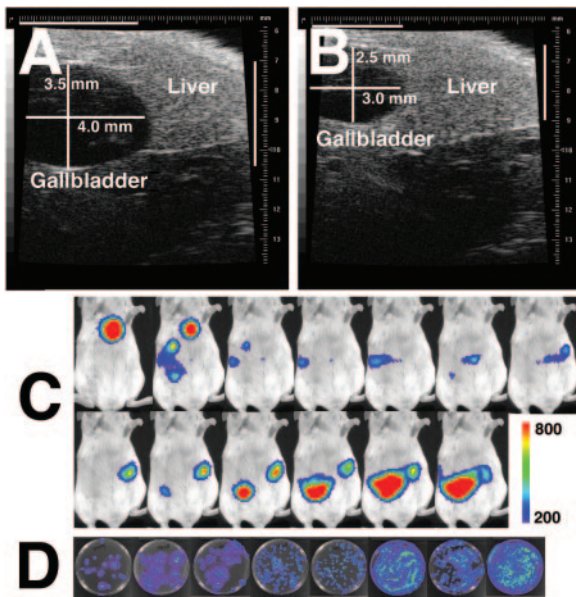
FIG. 4. Cholecystokinin-induced gallbladder contraction and fecal shedding. Normal, uninfected BALB/c mice were starved for 4 to 6 h and anesthetized, and the gallbladder was imaged using ultrasound. Cholecystokinin octapeptide fragment 26-33 (CCK8) was then injected intraperitoneally, and 300 images at 3 images per min were acquired to track the gallbladder size. Representative data from one animal are shown. (A) Image before administration; (B) image 400 s postadministration; (C) serial BLI of a representative CD1 mouse infected with virulent *L. monocytogenes* strain 2C and injected with CCK8; (D) imaging of petri plates of CFU from fecal pellets sequentially collected from the mouse shown in C for 2 h after the fast-and-feed procedure.

of fasting and subsequent peptide administration. Figure 4A shows an ultrasound image of the distended gallbladder of a normal, uninfected animal after 6 h of food deprivation and the contracted gallbladder 400 s after CCK8 administration. The serial images revealed that the gallbladder was beginning to contract within 3 min of injection. Contraction was observed to occur slowly over these first few minutes rather than all at once. The indicated measurements showed a 25% reduction in length and a 28% reduction in width 400 s after peptide administration. CCK8 also induces very efficient expulsion, a greater signal intensity in the abdomen, and an increase in fecal shedding, shown in Fig. 4B and C, using CD1 mice and virulent *L. monocytogenes* strain 2C.

Location of signals in excised intestines. To confirm that the abdominal signals originated from the gut, and to localize the signals along the length of the organ, we injected CCK8 into infected mice and excised the intestines at chosen times during the process. Mice were infected with 1×10^9 to 2×10^9 CFU of the 2C(Δhly) strain and imaged daily until they exhibited significant signals from the gallbladder, as described above for the previous experiments, and were then subjected to fasting and CCK8 administration. A representative experiment with the serial images and ex vivo images subsequently obtained is shown in Fig. 5. The in vivo images show that the administration of CCK8 was highly effective in expelling the bacteria, as judged by the reduced residual signal from the gallbladder several images after administration. After approximately 60 min, the signal appeared to stabilize over one area of the

abdomen of the animal shown for several images, which we define as a terminal signal. At this point, the animal shown in Fig. 5 was sacrificed, and the intestine was removed and imaged ex vivo to assess the extent of bacterial signal along the organ and to determine the source of the terminal signal. The ex vivo images of the intestine removed from the body are shown in Fig. 5B and C. By means of setting the binning of pixels on the detector at 2 by 2, the position and morphology of the most intense bacterial signal were discernible (Fig. 5B), and images obtained with binning at 10 by 10 were used to assess the presence of weak signals along the length of the organ (Fig. 5C). The images taken after the signal reached its terminal location in the series reveal that the bacteria were localized in a single area of the intestine, just proximal to the cecum. The signal does not appear to have been spread along the organ, at least within the limits of detection of the IVIS system using the setting for a high signal-to-noise ratio (10 by 10 binning). The position of the signal corresponded to the presence of fecal material in the intestine, i.e., the first fecal material that the bacteria would have encountered in the intestinal lumen in transit. During the process of transit, the signal was lost from the gallbladder and varied in the intestine (Fig. 5D).

Signal disappearance and reappearance in the animal. During the period of obtaining sequential images, the signal may completely disappear and then reappear as described above. To determine whether the signal was still being emitted by the bacteria and was obscured by transit into more dorsal regions of the abdominal cavity or if the bacteria were no longer emitting light, mice were infected and imaged as described above and then sacrificed during a period of signal reduction 60 min after CCK8 administration, and the intestines were removed and imaged. The results are shown in Fig. 6. The higher-resolution (2 by 2 binning) (Fig. 6B) image still reveals signal to be concentrated in one area, as in Fig. 5B, which we interpret as the previously detected signal seen in the live animal, indicating that the bacteria were still emitting light and suggesting that the in vivo loss of BLI signal results from the changing location of the labeled bacteria relative to the surface of the mouse. In the image with a higher signal-to-noise ratio (10 by 10 binning) (Fig. 6C), the signal was also distributed along the intestine, unlike the corresponding image taken of the excised intestine in Fig. 5C, which was from a mouse displaying a terminal signal. We interpret this result as bacteria distributed along the organ that have not yet moved to the terminal location. Again, a graphic representation of the signal intensity is shown in Fig. 6D.

DISCUSSION

Many enteric bacteria are bile resistant, yet none of these resistant species have been found to grow in the healthy gallbladder of any animal, with the exception of *Salmonella enterica* serovar Typhi in humans. Typhoid carriers harbor this pathogen in the gallbladder, and typhoid fever is thought to be transmitted from carriers by expulsion of contaminated bile from this organ into the intestine and out of the body in feces. To date, only *L. monocytogenes* has been demonstrated to grow in the asymptomatic gallbladder in an animal model. Figure 1 shows that many mice exhibit these signals upon intravenous
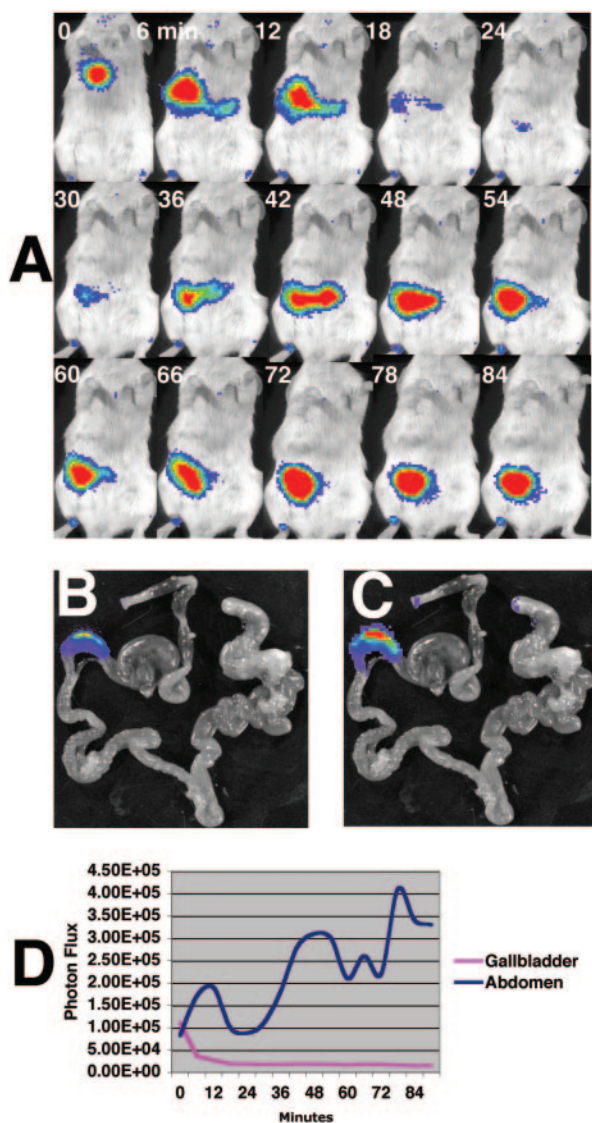
FIG. 5. Intestinal localization of terminal signal. The mouse shown was infected with attenuated strain 2C(Δhly) and imaged until symptoms were gone but the gallbladder signal was retained (day 3). After fasting for 6 h, the animal was then injected intraperitoneally with CCK8, and serial images were taken as described in the legend of Fig. 3. When the signal was stationary for several consecutive images, the animal was sacrificed and the intestine was removed for ex vivo BLI. Images of the excised organ were then taken at a higher resolution (binning, 2 by 2) (B) and a higher signal-to-noise ratio (binning, 10 by 10) (C). Multiple reference images were used for the in vivo series. Quantification of the signals using regions of interest identical to those of Fig. 2 is shown in the bottom panel.

infection. In this case, 19 of 20 CD1 mice showed increased signals from the gallbladder. We investigated this remarkable ability using imaging to assess bacterial burden and location in the individual animal over time. We sought to image bacteria induced to enter the intestine by causing the contraction of the gallbladder and to therefore directly demonstrate biliary excretion of *L. monocytogenes*, as first posited by Briones et al. (3), and support our previous data demonstrating extracellular replication.

After fasting for several hours, the gallbladder is filled with bile from the liver and becomes distended. The contraction of the organ can then be induced by a fat meal, which causes the small intestine to release cholecystokinin and secretin, hormones that enter the bloodstream and induce the contraction of the muscle layer on the outside of the gallbladder. This process results in the expulsion of bile, which aids in the emulsification of lipids. Mice exhibiting BLI signals from virulent *L. monocytogenes* in the gallbladder also often show signals from lower in the abdomen (Fig. 1), which suggests that excretion from the gallbladder could possibly result in intestinal infection. Such an infection may be extremely difficult to demonstrate in mice, because oral infection with *L. monocytogenes* is well known to be highly inefficient in these animals (14). The maximum number of CFU found in the gallbladder so far is 10^8 (data not shown), which is at least an order of magnitude less than the minimum infectious oral dose of *L. monocytogenes* in mice, and therefore, the bacteria expelled would not be sufficient to observe reinfection in the mouse model. Humans and larger animals, however, are much more susceptible to listeriosis orally and have gallbladders thousands of times the size of those in mice, so the colonized gallbladders of humans could therefore easily contain numbers of CFU that exceed infectious doses. The nature and origin of gut signals that follow signals from the gallbladder were unknown prior to this report. Lower abdominal signals could be due to sources of the bacteria other than the gallbladder, such as directly from the liver through the common bile duct (3). In addition, the blood and mesenteric lymph nodes often harbor *L. monocytogenes* and could be sources of the abdominal signal. To show a direct correlation between gallbladder contraction and the subsequent bacterial signal in the abdomen, we induced gallbladder distension and contraction using a fast-and-feed procedure. Mice infected with the virulent strain as described in the legend of Fig. 1 were starved for 4 to 8 h to prevent gallbladder contraction. When such animals were then fed whole cow's milk, signals from the abdomen subsequently appeared (Fig. 2). Quantification of the signal remaining in the gallbladder and the signal appearing in the lower abdomen could be demonstrated by selecting regions of interest. The results for the representative mouse shown in Fig. 2 reveal a decrease in gallbladder signal and a concomitant increase in the lower abdomen signal. To visualize this process in real time, serial images were obtained. These images revealed the rapid movement of *L. monocytogenes* through the abdomen of the animal, such that within 18 min of expulsion, a portion of the signal had reached a point in the lower abdomen where it remained for the duration of the experiment. We interpret this result to be due to the bacteria being prevented from further movement along the intestine, probably due to obstruction by fecal material. Quantification of the data using regions of interest identical to those of Fig. 2 showed that a decrease in the gallbladder signal was accompanied by only a modest increase in the signal from the lower abdomen in this case, although the abdominal signal was clearly evident.

As further evidence that gallbladder contraction was responsible for the observed signals and to increase the signal for serial imaging, we employed the synthetic tyrosine 27-sulfated peptide fragment 26-33 of cholecystokinin (CCK8), which is known to induce gallbladder contraction in a highly specific

manner. Using ultrasound, the contraction of the gallbladder was observed to begin within 4 min of intraperitoneal injection of the hormone-derived peptide. CCK8 administration was found to cause more complete contraction than feeding as judged by the greater reduction of gallbladder signal observed, as is clearly shown by the differences between the serial images of the two procedures. Using CCK8, the results showed kinetics of signal movement similar to that of feeding, however. Feeding took much longer to induce contraction, as expected, and was never as complete as CCK8 administration, so animals subjected to fasting and feeding retained most of the gallbladder signal after the procedure. CCK8, however, reduced gallbladder signals to background levels with the virulent strain in CD1 mice and the shedding of increased numbers of bacteria in the feces after the procedure (Fig. 4C and D). These data show that the signal from the gallbladders of these mice was almost entirely due to extracellular bacteria, supporting previous observations, because intracellular organisms would not be released by this procedure. The process does not appear to be entirely the same in all animals. This result is expected due to the well-known flexibility of the intestines of mice; however, the overall pattern is much the same in each animal. When the abdominal signal has the greatest intensity in the lower abdomen, the signal travels from the mouse's right to left, consistent with the anatomy of the transverse colon.

During the process of moving through the body, the signal was lost for periods of time corresponding to one or two images, whereupon it reemerged in a different location from that previous to the disappearance (Fig. 4C). This effect could be due to either the differential light output of the bacteria or their movement through different locations in the body. The former possibility would most likely be due to differences in oxygen availability in the different parts of the alimentary tract rather than other enzyme activity differences in the bacteria. Oxygen tension affects light output from a given amount of luciferase, because O_2 is required for the function of the enzyme. The most likely cause of the varying signal intensity in our estimation was the movement of the bacteria through the intestinal tract, which would result in differences in the depths of the signal within the mouse body. As the bacteria transit the intestine, some regions of this heavily folded organ will be closer to the ventral surface of the animal, resulting in a much greater signal intensity because the light has much less tissue to travel through. The effects of tissue depth on photon flux in mice have been carefully determined (18, 23), revealing that attenuation at different tissue depths can vary by orders of magnitude. The tissue overlaying the gallbladder is the liver, which is dark and attenuates the signal to a greater extent than the tissues overlaying many parts of the intestine. These factors may greatly change the signal as the bacteria move from one part of the body to another.

To determine the location along the intestine of the signal detected in live animals, mice exhibiting various signals during the process of expulsion were sacrificed, and the intestines were removed and imaged ex vivo. These images revealed that the terminal signal was largely localized to one area of the excised intestine (Fig. 5) but that at earlier time points, some distribution of bacteria proximal and distal to this concentrated signal was detectable at high-sensitivity settings (Fig. 6). We interpret these results to be that the bacteria are expelled over
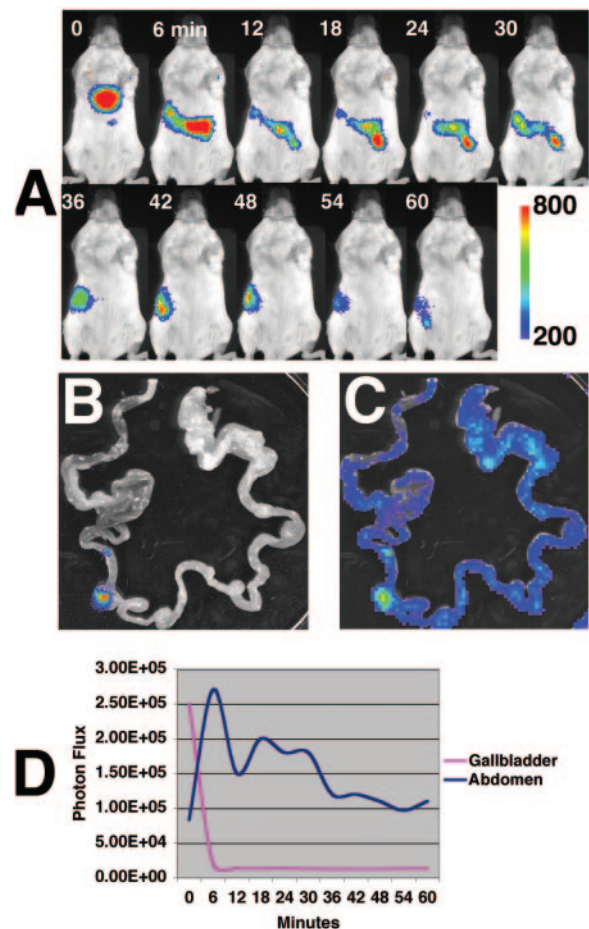

FIG. 6. Intestinal localization of bacteria in periods of low signal during biliary excretion. The mouse shown was infected with attenuated strain 2C(Δhly) and imaged as described in the legend of Fig. 4. After fasting for 6 h, the animal was then injected intraperitoneally with CCK8, and serial images were taken as described in the legend of Fig. 2. When a period of signal reduction occurred during the transit through the intestine, the mouse was sacrificed and the intestine was removed for ex vivo BLI. Images of the excised organ were then taken at a higher resolution (binning, 2 by 2) (B) and a higher signal-to-noise ratio (binning, 10 by 10) (C). A single reference image was used for the series. Focal signals in the excised intestine are designated with arrows. Quantification of the signals using regions of interest identical to those of Fig. 2 is shown in the bottom panel.

the period of contraction, as observed with ultrasound, but that a detectable bolus is expelled as well. This bolus could possibly be associated with mucus from the gallbladder. The transit of the bolus is impeded by fecal material at some point along the intestine, and the bacteria expelled later in the process catch up with those expelled earlier to form a more localized terminal signal, as shown in Fig. 5. During periods of reduced signal in the live animal, the subsequently excised intestines revealed signal concentrations (Fig. 6B and C) that we interpret as the previously detectable source in the live animal, indicating that the bacteria were still present and emitting light. Thus, it appears that the disappearance of the signal is the result of the location of the bacteria, i.e., deeper in the animal, where the signal is greatly reduced by the attenuation of the tissues. The

bacteria then move along the intestine until they reach a more ventral location and the light is detectable once more.

These results have implications for human listeriosis. It is currently unknown if the human gallbladder is a site of extensive replication, but the presence of gallbladder signals in all mice early in infection with 1 LD$_{50}$ of wild-type *L. monocytogenes*, albeit on different days in different animals (12), as well as the currently presented data shown in Fig. 1 indicate that this phenomenon is the rule in mice rather than a rare exception in some animals. The gallbladder, which is normally sterile and acellular, could provide the bacterium with an anatomical site in which to grow to large numbers without causing disease or eliciting an immune response. This phenomenon may occur in domestic animals as well, which could be particularly ominous if the gallbladder is ruptured during slaughter. In developed countries, listeriosis is often associated with processed foods contaminated at the point of processing, but this bacterium, which is ubiquitous in the environment, may be prevalent in the foods prepared domestically in impoverished areas. Due to the complete absence of careful studies, no data are available on the prevalence of listeriosis in the Third World, but the symptoms of the disease, fever and chills sometimes followed by convulsions and death, are common to a number of infectious diseases, including many viral infections. Thus, listeriosis may be much more common than is currently estimated because diagnosis is difficult. The recent report of the first outbreak of listeriosis in Japan (16) suggests that we do not know the extent of this disease. Many countries in the western part of Asia depend on milk products for a major portion of the normal diet, including yoghurts and other fermented substances that are known to support the growth of *L. monocytogenes*. People in many of these areas are in an almost constant state of hunger, which could cause their gallbladders to remain in the distended state, providing the bacteria with a location in which to multiply. When a meal containing milk fat is consumed, the gallbladder would then contract, and the bacteria would be expelled into the environment and spread via fecal contamination. Our data imply that such a scenario, while still speculative at this time, is possible in the physiological sense.

REFERENCES

1. Angelakopoulos, H., K. Loock, D. M. Sisul, E. R. Jensen, J. F. Miller, and E. L. Hohmann. 2002. Safety and shedding of an attenuated strain of *Listeria monocytogenes* with a deletion of *actA*/*plcB* in adult volunteers: a dose escalation study of oral inoculation. Infect. Immun. 70:3592–3601.
2. Bouwer, H. G., C. S. Nelson, B. L. Gibbins, D. A. Portnoy, and D. J. Hinrichs. 1992. Listeriolysin O is a target of the immune response to Listeria monocytogenes. J. Exp. Med. 175:1467–1471.
3. Briones, V., M. M. Blanco, A. Marco, N. Prats, J. F. Fernandez-Garayzabal, G. Suarez, M. Domingo, and L. Dominguez. 1992. Biliary excretion as possible origin of Listeria monocytogenes in fecal carriers. Am. J. Vet. Res. 53:191–193.
4. Busch, D. H., I. M. Pilip, S. Vijh, and E. G. Pamer. 1998. Coordinate regulation of complex T cell populations responding to bacterial infection. Immunity 8:353–362.
5. Chakraborty, T., M. Leimeister-Wachter, E. Domann, M. Hartl, W. Goebel, T. Nichterlein, and S. Notermans. 1992. Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the *prfA* gene. J. Bacteriol. 174:568–574.
6. Contag, C. H., P. R. Contag, J. I. Mullins, S. D. Spilman, D. K. Stevenson, and D. A. Benaron. 1995. Photonic detection of bacterial pathogens in living hosts. Mol. Microbiol. 18:593–603.
7. Dussurget, O., D. Cabanes, P. Dehoux, M. Lecuit, C. Buchrieser, P. Glaser, and P. Cossart. 2002. Listeria monocytogenes bile salt hydrolase is a PrfA-regulated virulence factor involved in the intestinal and hepatic phases of listeriosis. Mol. Microbiol. 45:1095–1106.
8. Francis, K. P., D. Joh, C. Bellinger-Kawahara, M. J. Hawkinson, T. F. Purchio, and P. R. Contag. 2000. Monitoring bioluminescent *Staphylococcus aureus* infections in living mice using a novel *luxABCDE* construct. Infect. Immun. 68:3594–3600.
9. Francis, K. P., J. Yu, C. Bellinger-Kawahara, D. Joh, M. J. Hawkinson, G. Xiao, T. F. Purchio, M. G. Caparon, M. Lipsitch, and P. R. Contag. 2001. Visualizing pneumococcal infections in the lungs of live mice using bioluminescent *Streptococcus pneumoniae* transformed with a novel gram-positive *lux* transposon. Infect. Immun. 69:3350–3358.
10. Gahan, C. G., and C. Hill. 2005. Gastrointestinal phase of Listeria monocytogenes infection. J. Appl. Microbiol. 98:1345–1353.
11. Gaillard, J. L., P. Berche, and P. Sansonetti. 1986. Transposon mutagenesis as a tool to study the role of hemolysin in the virulence of *Listeria monocytogenes*. Infect. Immun. 52:50–55.
12. Hardy, J., K. P. Francis, M. DeBoer, P. Chu, K. Gibbs, and C. H. Contag. 2004. Extracellular replication of Listeria monocytogenes in the murine gallbladder. Science 303:851–853.
13. Lauer, P., M. Y. Chow, M. J. Loessner, D. A. Portnoy, and R. Calendar. 2002. Construction, characterization, and use of two *Listeria monocytogenes* site-specific phage integration vectors. J. Bacteriol. 184:4177–4186.
14. Lecuit, M., S. Dramsi, C. Gottardi, M. Fedor-Chaiken, B. Gumbiner, and P. Cossart. 1999. A single amino acid in E-cadherin responsible for host specificity towards the human pathogen Listeria monocytogenes. EMBO J. 18:3956–3963.
15. Mackaness, G. B. 1962. Cellular resistance to infection. J. Exp. Med. 116:381–506.
16. Makino, S. I., K. Kawamoto, K. Takeshi, Y. Okada, M. Yamasaki, S. Yamamoto, and S. Igimi. 2005. An outbreak of food-borne listeriosis due to cheese in Japan, during 2001. Int. J. Food Microbiol. 104:189–196.
17. Portnoy, D. A., P. S. Jacks, and D. J. Hinrichs. 1988. Role of hemolysin for the intracellular growth of Listeria monocytogenes. J. Exp. Med. 167:1459–1471.
18. Rice, B. W., M. D. Cable, and M. B. Nelson. 2001. In vivo imaging of light-emitting probes. J. Biomed. Opt. 6:432–440.
19. Rodriguez-Noriega, E., J. Andrade-Villanueva, and G. Amaya-Tapia. 1989. Quinolones in the treatment of Salmonella carriers. Rev. Infect. Dis. 11(Suppl. 5):S1179–S1187.
20. Schuchat, A., K. Deaver, P. S. Hayes, L. Graves, L. Mascola, and J. D. Wenger. 1993. Gastrointestinal carriage of Listeria monocytogenes in household contacts of patients with listeriosis. J. Infect. Dis. 167:1261–1262.
21. Sleator, R. D., H. H. Wemekamp-Kamphuis, C. G. Gahan, T. Abee, and C. Hill. 2005. A PrfA-regulated bile exclusion system (BilE) is a novel virulence factor in Listeria monocytogenes. Mol. Microbiol. 55:1183–1195.
22. Way, S. S., L. J. Thompson, J. E. Lopes, A. M. Hajjar, T. R. Kollmann, N. E. Freitag, and C. B. Wilson. 2004. Characterization of flagellin expression and its role in Listeria monocytogenes infection and immunity. Cell. Microbiol. 6:235–242.
23. Zhao, H., T. C. Doyle, O. Coquoz, F. Kalish, B. W. Rice, and C. H. Contag. 2005. Emission spectra of bioluminescent reporters and interaction with mammalian tissue determine the sensitivity of detection in vivo. J. Biomed. Opt. 10:41210.