Inducible Control of Virulence Gene Expression in *Listeria monocytogenes*: Temporal Requirement of Listeriolysin O during Intracellular Infection

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We have constructed a *lac* repressor/operator-based system to tightly regulate expression of bacterial genes during intracellular infection by *Listeria monocytogenes*. An *L. monocytogenes* strain was constructed in which expression of listeriolysin O was placed under the inducible control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-dependent promoter. Listeriolysin O (LLO) is a pore-forming cytolysin that mediates lysis of *L. monocytogenes*-containing phagosomes. Using hemolytic-activity assays and Western blot analysis, we demonstrated dose-dependent IPTG induction of LLO during growth in broth culture. Moreover, intracellular growth of the inducible-LLO (iLLO) strain in the macrophage-like cell line J774 was strictly dependent upon IPTG. We have further shown that iLLO bacteria trapped within primary phagocytic vacuoles can be induced to escape into the cytosol following addition of IPTG to the cell culture medium, thus yielding the ability to control bacterial escape from the phagosome and the initiation of intracellular growth. Using the iLLO strain in plaque-forming assays, we demonstrated an additional requirement for LLO in facilitating cell-to-cell spread in L2 fibroblasts, a nonprofessional phagocytic cell line. Furthermore, the efficiency of cell-to-cell spread of iLLO bacteria in L2 cells was IPTG dose dependent. The potential use of this system for determining the temporal requirements of additional virulence determinants of intracellular pathogenesis is discussed.

Listeria monocytogenes is a gram-positive, facultative intracellular bacterial pathogen of humans and animals (13). In humans, *L. monocytogenes* infections are typically foodborne and cause an invasive and often fatal disease in pregnant women, the elderly, newborns, and immunocompromised individuals (1, 9, 45). Furthermore, *L. monocytogenes* has been studied for more than 4 decades as a model system for understanding the development of acquired immunity to intracellular pathogens (23, 39). The cell biology of *L. monocytogenes* infection has also been well characterized (55). Following internalization, bacteria are initially contained within host vacuoles but rapidly escape from the vacuoles to enter the cytosol and begin replication. During intracytosolic growth, bacteria exploit a host mechanism of actin-based motility to move within the cytosol and spread from cell to cell without contacting the extracellular environment. Following cell-to-cell spread, a double-membrane vacuole, or secondary vacuole, is formed. Bacteria then lyse the secondary vacuole to continue intracellular growth.

A thorough understanding of the cell biology of infection, coupled with the development of genetic tools, in vitro tissue culture models, and the mouse model of infection, has made *L. monocytogenes* an ideal system for elucidating the molecular mechanisms of intracellular pathogenesis. Many of the bacterial determinants necessary for the intracellular growth, spread, and virulence of *L. monocytogenes* have been identified (40, 57). Since *L. monocytogenes* is amenable to genetic analysis, the use of transposon insertion mutants or nonpolar in-frame gene deletions has been the primary means for assigning functions to specific virulence determinants (7, 18, 37, 53). Many of these mutants cannot initiate intracellular growth or become arrested at specific stages of intracellular infection. Therefore, it has not been possible to use such mutants to determine the role of virulence determinants at subsequent stages of infection.

The ability to control the expression of an individual virulence determinant while a pathogen is contained within a host cell is an excellent means of determining the temporal requirement of a specific factor for continuous intracellular growth and spread of the pathogen. Here, we report the adaptation of the *Escherichia coli lac* repressor/operator system to construct a chromosome-based, tightly regulated system for inducible control of bacterial gene expression during the growth and spread of an intracellular bacterial pathogen. With this system, transcription of a gene(s) in single copy can be removed from the normal bacterial control mechanism and placed under the transcriptional control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter.

IPTG is a synthetic, nonhydrolyzable inducer of the *E. coli lac* repressor (3). Several studies have demonstrated that IPTG can be used in cultured mammalian cells to induce the expression of host cell genes controlled by the *lac* repressor/operator system (25, 54, 61). Therefore, the adaptation of the *lac* repressor/operator system to control bacterial gene expression during intracellular infection of host cells will allow pathogen-

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specific determinants to be induced at defined stages of infection and the effect on intracellular growth and cell-to-cell spread to be determined. This approach can be utilized to precisely define the role that determinants of virulence play throughout the intracellular infection process and yield a more complete understanding of the pathogenesis and cell biology of infection. A previous report has demonstrated the use of the *lac* repressor/operator system and IPTG to control plasmid-based virulence gene expression during intracellular infection by *Legionella pneumophila* (43). As *L. monocytogenes* is an ideal system for studying the molecular determinants of virulence and host response, we have adapted the *lac* repressor/operator system for use in *L. monocytogenes* as a model intracellular bacterial pathogen.

Listeriolysin O (LLO) is a principal determinant of *L. monocytogenes* pathogenesis. LLO is a member of the cholesterol-dependent family of related pore-forming cytolysins expressed by diverse species of gram-positive bacteria (2, 20). LLO is primarily responsible for lysing the primary phagocytic vacuole to allow bacteria access to the cytosol (18, 20, 32). LLO-negative mutants have been shown to be defective for growth in the majority of the cell types tested (17, 32, 41), and electron microscopy has revealed that LLO-negative mutants are unable to escape from the primary vacuole (17, 55). However, any role of LLO in subsequent stages of intracellular infection cannot be established by infection with LLO-negative mutants, as these bacteria fail to escape the primary vacuole and are unable to initiate intracellular growth.

With the newly constructed inducible system, a strain of *L. monocytogenes* was generated in which expression of LLO was placed under the inducible control of IPTG. We demonstrate that the production of LLO by the inducible-LLO (iLLO) strain during growth in broth culture is dependent upon the concentration of IPTG in the growth medium. Furthermore, intracellular growth of the iLLO strain in a macrophage-like cell line, J774, is dependent upon induction of LLO expression. Vacuolar escape and initiation of intracellular growth in J774 cells can be controlled by the addition of IPTG to host cells harboring iLLO bacteria contained within phagocytic vacuoles. Using the iLLO strain, we analyzed the temporal requirement of LLO expression subsequent to primary vacuole escape in L2 cells, a nonprofessional phagocytic fibroblast cell line, and demonstrate that continuous LLO induction is necessary for efficient intracellular growth and spread of *L. monocytogenes*.

MATERIALS AND METHODS

Bacterial strains and cell growth conditions. *L. monocytogenes* strains were typically grown in 3 ml of brain heart infusion broth (BHI; Difco, Detroit, Mich.) at 30°C in 15-ml conical tubes that were slanted without agitation. Prior to infection of host cells, bacteria grown overnight were diluted 10-fold in BHI and grown at 30°C for 2.5 h with agitation. *E. coli* strains used for molecular cloning were grown in Luria-Bertani (LB) medium at 37°C with agitation. All bacterial strains were stored at −80°C in BHI or LB plus 40% glycerol. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 10 μg/ml; gentamicin, 30 and 50 μg/ml; erythromycin, 0.04 and 1 μg/ml. Tissue culture cells were grown in Dulbecco modified Eagle medium (DMEM; Mediatech, Herndon, Va.) supplemented with 7.5% fetal bovine serum (HyClone, Logan, Utah) and 2 mM glutamine (Mediatech). Tissue culture cells were maintained at 37°C in a 5% CO$_2$–air atmosphere.

Construction of an inducible-expression vector for *L. monocytogenes*. Plasmid pLIV1 (Fig. 1A) was generated to facilitate chromosomal integration of IPTG-inducible expression constructs in *L. monocytogenes*. First, an intermediate
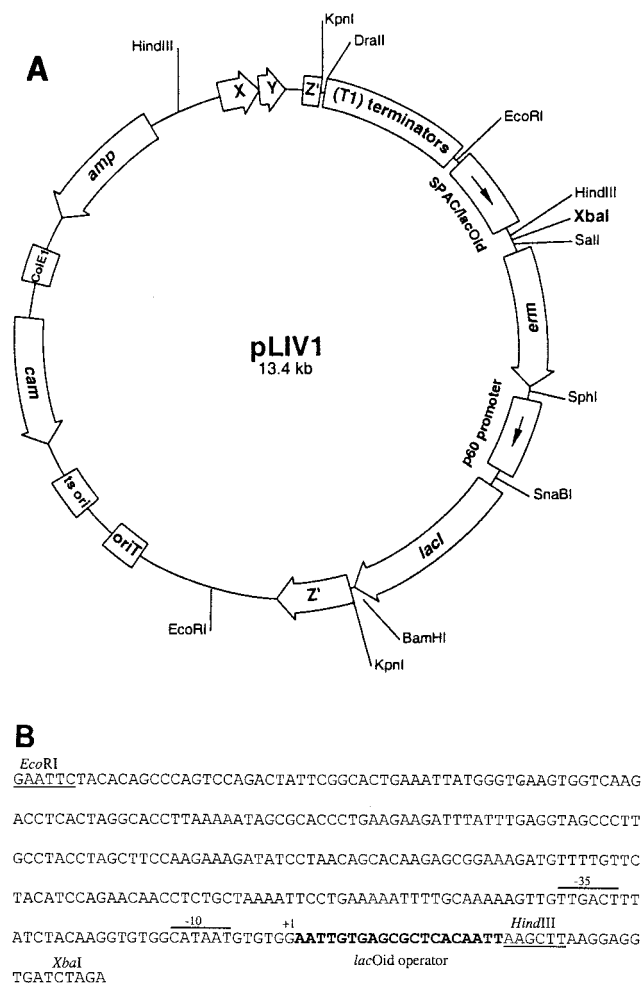

FIG. 1. Inducible-expression vector for *L. monocytogenes*. (A) The pLIV1 vector contains the following sequences: a temperature-sensitive origin of replication (ts ori) and a chloramphenicol resistance gene (*cam*) for plasmid selection in *L. monocytogenes*, the ColE1 origin of replication and an ampicillin resistance gene (*amp*) for cloning and selection in *E. coli*, an origin of transfer (oriT) to allow conjugal mating of plasmid derivatives from *E. coli* to *L. monocytogenes*, a unique *Xba*I restriction site (shown in bold type) for cloning of genes under the transcriptional control of the SPAC/*lac*Oid IPTG-inducible promoter, tandem copies of the *rrnB* T1 transcription terminator (T1 terminators) upstream of the SPAC/*lac*Oid region to ensure that transcription of cloned genes initiates only by the SPAC promoter, the *L. monocytogenes* p60 gene promoter to allow constitutive expression of the *lac* repressor gene (*lacI*), and an erythromycin resistance determinant within the expression cassette (*erm*) for selection of inducible constructs on the chromosome. The inducible gene expression cassette is placed within the *L. monocytogenes* orfZ gene (Z′), which is immediately followed by a putative transcription terminator and flanked by sufficient DNA to allow homologous recombination. Positions of restriction sites utilized for the construction of pLIV1 are indicated (see Materials and Methods). (B) Nucleotide sequence of the SPAC/*lac*Oid promoter/operator region within pLIV1. The −35 and −10 regions of the SPAC promoter are overlined. The transcription initiation site is indicated as +1. The *lac*Oid operator sequence is shown in bold. Restriction sites are noted and underlined.

pSPAC vector was constructed by cloning an *Eco*RI-*Bam*HI fragment containing the SPAC promoter/operator region, *lacI* gene, and bleomycin resistance determinant from plasmid pAG58-*ble*-1 (64) into pBluescript SK− (Stratagene, La Jolla, Calif.). Next, a *Dra*II-*Eco*RI fragment containing four tandem copies of the

rrnB T1 transcription terminator was subcloned from plasmid pTL61T (36) and ligated immediately upstream of the SPAC promoter/operator region in the intermediate pSPAC vector. The *lac*O1 operator sequence (63) within the SPAC promoter region was replaced with the *lac*Oid sequence (38). Primer DP-3730 (5′-CCG<u>GAATTC</u>TACACAGCCCAGTCCAGACTATTCG-3′), containing an upstream *Eco*RI site (underlined), and primer DP-3729 (5′-C TCCTT<u>AAGCTT</u>AATTGTGAGCGCTCACAATTCCACACATTATGCCA CACCTTGTAG-3′), containing the *lac*Oid sequence and a downstream *Hin*dIII site (underlined), were used to construct the SPAC/*lac*Oid region by PCR amplification from plasmid pAG58-*ble*-1. The 294-bp PCR fragment was digested with *Eco*RI-*Hin*dIII and ligated into the *Eco*RI-*Hin*dIII-digested pSPAC vector. The *L. monocytogenes* p60 promoter was then placed upstream of the *lacI* gene in the intermediate pSPAC vector. Primer DP-3727 (5′-GG<u>GCATGC</u>TCGATCATCATAATTCTGTC-3′), containing an upstream *Sph*I site (underlined), and primer DP-2858 (5′-GG<u>GGATCC</u>GATC TACTACTGGAGTTTC-3′), which are homologous to sequences within the coding region of p60, were used to amplify a 1,041-bp PCR fragment from chromosomal DNA of *L. monocytogenes* strain 10403S. The 1,041-bp fragment was digested with *Sph*I-*Sna*BI, generating a 409-bp fragment containing the p60 promoter (29). The *Sph*I-*Sna*BI-digested fragment was cloned upstream of the *lacI* gene in the *Sph*I-*Sna*BI-digested pSPAC vector. Finally, the bleomycin resistance gene in the intermediate pSPAC vector was replaced with a gene encoding erythromycin resistance. Primer DP-3725 (5′-ACGC<u>GTCGAC</u>CTAAAGTTATGGAAATAAGAC-3′), containing an upstream *Sal*I site (underlined), and primer DP-3726 (5′-GG<u>GCATGC</u>AT TCAAATTTATCCTTATTGTACAAA-3′), containing a downstream *Sph*I site (underlined), were used to PCR amplify the *ermAM* determinant from transposon Tn*917* (47). The 1,283-bp PCR product was digested with *Sal*I-*Sph*I and cloned into the *Sal*I-*Sph*I-digested intermediate pSPAC vector.

An integrational vector for *L. monocytogenes* was constructed by cloning the *orfXYZ* locus into gram-positive integrational vector pCON1 (15). Primer DP-3731 (5′-CCC<u>AAGCTT</u>TCAAGCAACGGAAGACATGGTAGCAAAAAGA-3′), containing an upstream *Hin*dIII site (underlined), and primer DP-3732 (5′-CCG<u>GAATTC</u>TCGGCGAAGTCATCACTGGCGTTAACTTTAT-3′), containing a downstream *Eco*RI site (underlined), were used to PCR amplify the *orfXYZ* locus from chromosomal DNA isolated from *L. monocytogenes* strain 10403S. The 1,578-bp PCR fragment was digested with *Hin*dIII-*Eco*RI and ligated into *Hin*dIII-*Eco*RI-digested pCON1. The complete pLIV1 vector, containing the entire modified SPAC/*lac*Oid expression cassette in the pCON1/*orfXYZ* integrational vector, was generated by using a two-step process. First, a *Sca*I-*Bam*HI fragment containing the modified SPAC/*lac*Oid expression cassette was cloned from the intermediate pSPAC vector into *Hin*cII-*Bam*HI-digested pUC18 (62). A 3,990-bp *Kpn*I fragment containing the modified SPAC expression cassette was then removed from the pUC18-derived vector and ligated into *Kpn*I-digested pCON1/*orfXYZ*, yielding plasmid pLIV1 (Fig. 1A).

Construction of an iLLO strain of *L. monocytogenes*. Primers DP-3033 (5′-GC<u>TCTAGA</u>AGAGAGAGGGGTGGCAAAC-3′) and DP-3137 (5′-GC<u>TCTA GAC</u>TAAAAAAAATTAAAAAATAAGC-3′) (*Xba*I sites are underlined) were used to PCR amplify a promoterless *hly* gene fragment from *L. monocytogenes* strain 10403S chromosomal DNA. The 1,798-bp PCR fragment contained the translation initiation signal and coding sequence for LLO, a downstream transcription terminator sequence, and *Xba*I sites at the 5′ and 3′ termini. pLIV1-LLO (see Fig. 7) was generated by ligating the *Xba*I-digested *hly* gene fragment into the unique *Xba*I site of pLIV1 (Fig. 1A).

pLIV1-LLO was integrated into the chromosome of *hly* deletion strain DP-L2161 (26) to generate strain DP-L3885. Integration and allelic exchange were performed as previously described (8). Briefly, DP-L2161 was transformed with pLIV1-LLO by electroporation. Plasmid-containing transformants were selected on BHI-chloramphenicol agar plates at 30°C. Chromosomal integration of plasmid sequences was selected by growth of bacteria in broth culture at 42°C, followed by selection on BHI-chloramphenicol agar plates at 42°C. To allow allelic exchange of plasmid sequences, cultures from a single colony were grown at 30°C in BHI broth for multiple passages in the absence of antibiotic. Allelic exchange was confirmed by plating aliquots of cultures on BHI agar without antibiotics and replica plating individual colonies on BHI and BHI-chloramphenicol agar. Chromosomal DNA from chloramphenicol-sensitive colonies was analyzed by Southern blot assay to verify loss of plasmid vector sequences and retention of the iLLO construct on the chromosome within the *orfXYZ* locus. A verified clone was designated DP-L3885. Retention of the iLLO construct in DP-L3885 was also verified by growth for 1 h in BHI broth containing 0.04 µg of erythromycin per ml, followed by continued growth in BHI containing 1 µg of erythromycin per ml.

L. monocytogenes strain DH-L616 was constructed by placing the iLLO ex-

pression cassette on the chromosome of DP-L2161 within the *L. monocytogenes* tRNA^Arg gene. Plasmid pDH618 (see Fig. 7) was generated by cloning the 5,779-bp *Kpn*I fragment from pLIV1-LLO, harboring the iLLO expression cassette, into *L. monocytogenes* site-specific integration vector pPL2 (33). Chromosomal integration of pDH618 and verification of the iLLO construct were performed as previously described (33) to yield strain DH-L616.

Determination of growth rates in BHI broth. *L. monocytogenes* was inoculated into 3 ml of BHI broth and grown overnight. One milliliter of overnight culture was added to 19 ml of BHI (with or without 1 mM IPTG), and cultures were grown for 6 h at 37°C with agitation. Immediately following dilution or at 30-min to 1-h intervals, 1 ml of culture was removed and the absorbance at 600 nm was measured in a DU 640 Spectrophotometer (Beckman Coulter, Fullerton, Calif.).

Hemolytic-activity assays. *L. monocytogenes* strains were grown overnight in BHI broth. One milliliter of overnight culture was added to 9 ml of BHI and grown for 5 h at 37°C. Cultures were grown in the presence or absence of various concentrations of IPTG. Determination of hemolytic activity contained in culture supernatants was performed as previously described (41), with slight modifications. Briefly, following incubation of culture supernatants with sheep red blood cells, samples were centrifuged (13,000 × *g*) for 1 min. A 100-µl volume of the supernatant was transferred to a 96-well flat-bottom microtiter plate (Nalge Nunc International, Rochester, N.Y.), and the absorbance at 541 nm of each well was measured in a VERSAmax microplate reader with SoftMax Pro v1.2 software (Molecular Devices, Sunnyvale, Calif.). Numbers of hemolytic units (HU) were defined as the reciprocal of the dilution of culture supernatant that yielded 50% lysis of sheep red blood cells.

IPTG dose-dependent induction of LLO. The iLLO strain was grown overnight in BHI broth. One milliliter of overnight culture was added to 9 ml of BHI broth in replicate flasks and grown for 5 h at 37°C in the presence of various concentrations of IPTG. The optical density at 600 nm (OD_{600}) of each culture was measured. A 1-ml aliquot was removed, and the hemolytic activity of culture supernatants was determined as already described. The remaining culture volume was centrifuged (6,000 × *g*) for 10 min at 4°C, and supernatants were precipitated for 1 h on ice in the presence of 10% (vol/vol) trichloroacetic acid. Samples were centrifuged (6,000 × *g*) for 10 min at 4°C, and an equivalent amount of each sample was resuspended in protein sample buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Western blotting with a rabbit polyclonal anti-LLO antibody as previously described (41).

Intracellular growth of *L. monocytogenes* in J774 cells. Intracellular growth of *L. monocytogenes* in J774 cells was performed essentially as previously described (41), with some modifications. Briefly, 1 ml of an overnight culture of *L. monocytogenes* was added to 9 ml of BHI with or without 1 mM IPTG. Bacteria were grown at 30°C with agitation for 2.5 h prior to infection of J774 cells. Approximately 10^6 bacteria were used to infect 1.5 × 10^6 J774 cells seeded 18 h previously onto circular 12-mm-diameter glass coverslips placed in a 60-mm-diameter petri dish. Infection of J774 cells was performed in DMEM with or without 10 mM IPTG. At 30 min postaddition of bacteria, J774 cells were washed with phosphate-buffered saline (PBS) and DMEM containing 50 µg of gentamicin per ml with or without 10 mM IPTG was added. Three coverslips were removed from the culture at appropriate time intervals and separately placed in 5 ml of sterile distilled H_2O in a 15-ml conical tube. Conical tubes were vortexed for 15 s to lyse J774 cells, and dilutions of lysates were plated on LB agar to determine the number of intracellular bacteria.

Temporal induction of vacuolar escape and intracellular growth. J774 cells seeded onto glass coverslips were infected with DP-L3885 as previously described, with the following modifications. One milliliter of an overnight culture was added to 9 ml of BHI without IPTG. Bacteria were grown at 30°C with agitation for 2.5 h prior to infection of J774 cells. Approximately 2.8 × 10^6 bacteria were used to infect 3.4 × 10^6 J774 cells seeded 18 h previously onto circular 12-mm-diameter glass coverslips placed in a 100-mm-diameter petri dish. At the time of infection or 2, 4, or 6 h after the initiation of infection, culture medium was removed and DMEM containing 10 mM IPTG was added. At appropriate time intervals, three coverslips were removed for determination of intracellular bacterial counts as previously described.

Analysis of plaque formation in L2 fibroblasts. Assays of plaque formation within L2 cell monolayers were performed essentially as previously described (53). Briefly, L2 cell monolayers were grown to confluency in six-well tissue culture plates. One milliliter of an overnight culture of *L. monocytogenes* was added to 9 ml of BHI broth and grown for 2.5 h at 30°C with agitation. Approximately 2 × 10^5 bacteria were used to infect L2 monolayers for 1 h in DMEM with or without IPTG. Monolayers were washed three times with 3 ml of PBS, and a DMEM–0.7% agarose overlay containing 30 µg of gentamicin per ml was added. The DMEM-agarose overlay contained various concentrations of IPTG. Plates were incubated for 3 days to allow plaque formation. Plaques were visu-

alized by addition of an additional DMEM-agarose overlay containing 6% neutral red and overnight incubation.

Nucleotide sequence accession numbers. The DNA sequences of the inducible-expression cassette region and the SPAC/*lac*Oid promoter/operator region described in this report have been deposited in the EMBL/GenBank/DDBJ databases under accession numbers AY126342 and AY126019, respectively. The DNA sequence of the pPL2 integration vector was previously deposited (33) under accession number AJ417449.

RESULTS

Construction of an inducible-expression system for *L. monocytogenes*. We chose to modify the *E. coli lac* repressor/operator system to generate an inducible system with which to control bacterial gene expression during intracellular infection by *L. monocytogenes*. To facilitate the generation of chromosome-based inducible gene constructs, an allelic-exchange vector, pLIV1, was constructed to allow the placement of a gene(s) on the *L. monocytogenes* chromosome in single copy under the transcriptional control of an IPTG-inducible promoter (Fig. 1A). The inducible gene constructs were placed on the chromosome within the *orfZ* locus, as previous studies indicated that complete deletion of the *orfXYZ* locus or insertion of DNA within this locus has no effect on the intracellular growth and spread of *L. monocytogenes* (49). Restriction enzyme- or PCR-generated DNA fragments containing the gene of interest were ligated into the unique cloning site of pLIV1 to place gene expression under the control of the SPAC/*lac*Oid promoter. The SPAC/*lac*Oid promoter (Fig. 1B) was constructed as a hybrid promoter/operator consisting of the *Bacillus subtilis* phage SPO-1 promoter and the *E. coli lac*Oid operator sequence (38, 63). The SPAC promoter/operator has been used to make gene expression inducible by IPTG in *B. subtilis* (5, 42, 63), a ubiquitous, nonpathogenic, gram-positive bacterium. Furthermore, the SPAC promoter/operator has been shown to function in *L. monocytogenes* for inducible expression of green fluorescent protein from a plasmid vector (16). The *lac*Oid operator sequence is a modified *lac* operator that is bound by the *lac* repressor protein with 8- to 10-fold higher affinity than the native *lac* operator sequence (38, 44). The *lac* repressor is produced constitutively from pLIV1 under the control of the *L. monocytogenes* p60 promoter (30), allowing efficient transcriptional repression of cloned genes in the absence of IPTG. To allow removal of the expression of virulence genes from the normal bacterial control, an *L. monocytogenes* strain with the virulence gene of interest deleted served as the background strain for recombination onto the chromosome. Integration of inducible-expression constructs onto the chromosome was performed by homologous recombination and allelic exchange as previously described (8).

Generation of an iLLO strain of *L. monocytogenes*. Transcription of many of the genes necessary for *L. monocytogenes* pathogenesis is controlled by the DNA binding activator PrfA (31, 40). To evaluate the inducible-expression system and gain further insights into the temporal requirement of LLO, a strain of *L. monocytogenes* was constructed in which transcription of the *hly* gene encoding LLO was removed from the control of PrfA and placed under the control of the inducible SPAC/*lac*Oid promoter. A PCR fragment containing the *hly* gene and the putative downstream terminator region was cloned behind the SPAC promoter in pLIV1 to yield plasmid pLIV1-LLO. *L.*
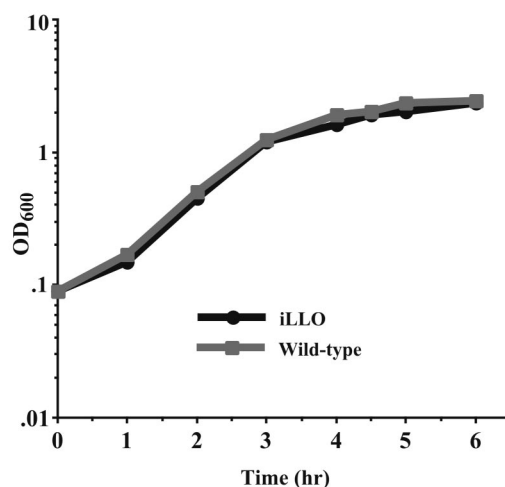

FIG. 2. Growth of *L. monocytogenes* strains in BHI broth. Overnight cultures of iLLO (DP-L3885) or wild-type *L. monocytogenes* strain 10403S were diluted 1:20 in BHI broth and grown in the presence of 1 mM IPTG for 6 h at 37°C. Culture aliquots were taken every 30 min to 1 h, and the OD_{600} was determined.

monocytogenes strain DP-L2161 served as the LLO deletion strain for recombination. DP-L2161 contains an in-frame deletion of the *hly* gene that removes all of the coding sequence of LLO except the last 16 amino acids (26, 27). However, the ribosome binding site and PrfA-controlled promoter remain, so as not to interfere with downstream gene expression. pLIV1-LLO was introduced into DP-L2161, and allelic exchange was performed to generate DP-L3885, an IPTG-iLLO strain of *L. monocytogenes*.

In vitro characterization of the iLLO strain. In vitro growth of the iLLO strain in BHI broth indicated no difference between its growth rate and that of wild-type *L. monocytogenes*. As shown in Fig. 2, iLLO bacteria grown in the presence of IPTG grew identically to wild-type *L. monocytogenes*. Furthermore, no difference in growth was observed when cultures were grown in the absence of IPTG in BHI broth (data not shown). We next characterized the iLLO strain to evaluate IPTG-inducible expression of LLO. To verify iLLO expression, the hemolytic activity present in culture supernatants was determined. As shown in Table 1, culture supernatants of the iLLO strain demonstrated hemolytic activity equivalent to that of wild-type *L. monocytogenes* when bacteria were grown in the presence of at least 0.5 mM IPTG (129.8 and 130.7 HU, respectively). Furthermore, in the absence of IPTG, only a small amount of hemolytic activity (2.8 HU) had accumulated in the culture supernatant of iLLO bacteria during the 5-h growth period. Taken together, these data indicated that the iLLO strain had no difference in growth rate during broth culture compared to wild-type *L. monocytogenes*, and hemolytic activity could be induced to levels similar to those of wild-type bacteria dependent upon the presence of IPTG in the culture medium.

We examined the dose-dependent induction of LLO by growing the iLLO strain in various concentrations of IPTG and assaying culture supernatants for LLO production. Hemolytic activity, as shown in Table 1, was IPTG dose dependent over a wide range of IPTG concentrations. Inducible hemolytic activ-

TABLE 1. Hemolytic activity[a]

Strain	Mean no. of HU (SD)
10403S (wild type)	130.7 (19.9)
DP-L2161 (LLO negative)	ND[b]
DP-L3885 (iLLO)	
IPTG concn (mM)	
0	2.8 (1.5)
0.001	5.2 (0.8)
0.005	12.5 (3.5)
0.01	23.5 (9.0)
0.02	47.5 (10.4)
0.03	62.4 (9.1)
0.04	70.5 (9.2)
0.05	81.4 (13.1)
0.1	107.9 (21.1)
0.25	120.8 (23.4)
0.5	129.8 (21.1)
1.0	132.9 (26.3)
10	133.3 (20.9)

[a] An overnight culture of iLLO strain DP-L3885 was diluted 1:10 in BHI broth into replicate flasks and grown for 5 h at 37°C in the presence of various concentrations of IPTG. The OD_{600} values of all of the cultures were identical. Wild-type strain 10403S and LLO-negative strain DP-L2161 were grown under similar conditions in the absence of IPTG. Hemolytic activity of culture supernatants was assayed as described in Materials and Methods. Numbers of HU are expressed as the reciprocal of the dilution of culture supernatant that yielded 50% hemolysis. The means of three experiments performed on separate days are shown.

[b] ND, not detectable (<1 HU).

ity was observed with concentrations as low as 0.001 mM IPTG, 5.2 HU, increasing to 133.3 HU with a concentration of 10 mM IPTG present in the culture medium. Induction of hemolytic activity with respect to IPTG concentration was most linear between 0.01 and 0.1 mM IPTG (Fig. 3A). In addition, Western blot analysis of LLO protein present in culture supernatants indicated a strict correlation of LLO protein with hemolytic-activity values over the range of IPTG concentrations examined (Fig. 3B).

Intracellular growth of iLLO bacteria. To be useful in determining the temporal requirement of *L. monocytogenes* virulence determinants, inducible gene expression must be functionally repressed in the absence of IPTG and induced to functional levels while bacteria are within host cells. We next examined the ability of the iLLO strain to grow intracellularly in the presence or absence of IPTG in the murine macrophage-like cell line J774. LLO-negative mutants fail to escape the primary vacuole and therefore do not grow in J774 cells (41). As shown in Fig. 4A, in the absence of IPTG, iLLO bacteria behaved identically to LLO-negative bacteria with respect to intracellular growth. Bacteria failed to escape the primary vacuole yet remained viable within the vacuole for several hours in the absence of intracellular growth. However, as shown in Fig. 4B, iLLO bacteria that were maintained in the presence of IPTG, prior to and during intracellular infection, were able to grow within J774 cells to levels comparable to that of wild-type bacteria. These data demonstrated that, in the absence of IPTG, the inducible system provided stringent repression of gene expression within infected host cells. Furthermore, IPTG induction of the iLLO strain was sufficient to allow bacteria to
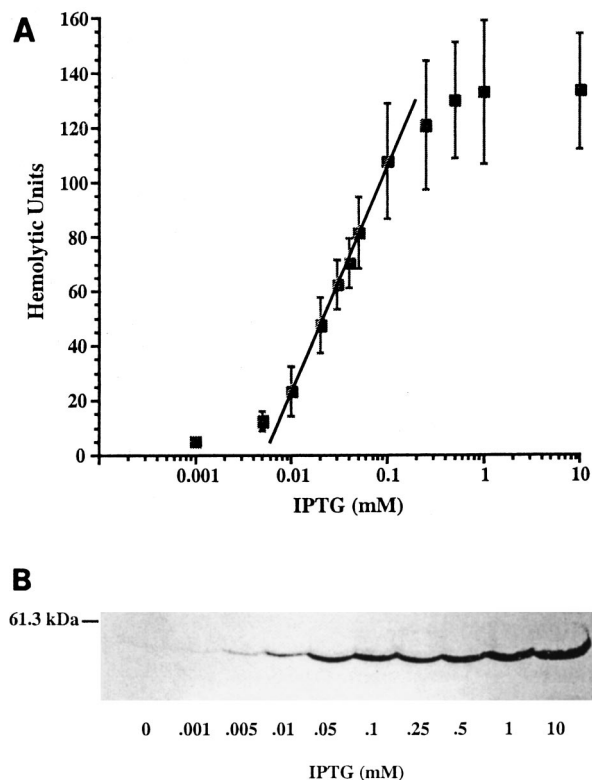

FIG. 3. IPTG induction of LLO is dose dependent. (A) The hemolytic-activity values listed in Table 1 for the iLLO strain (DP-L3885) were plotted versus the IPTG concentration. A best-fit line for linear induction of hemolytic activity is shown. (B) An overnight culture of iLLO strain DP-L3885 was diluted 1:10 in BHI broth into replicate flasks and grown for 5 h at 37°C in the presence of various concentrations of IPTG. The OD_{600} values of all of the cultures were identical. Culture supernatants were precipitated for 1 h on ice in the presence of 10% (vol/vol) trichloroacetic acid. Samples were centrifuged and resuspended in protein sample buffer. An equivalent amount of each sample was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Western blot assay with a rabbit polyclonal anti-LLO antibody. The position of the LLO bands (58 kDa) relative to that of a molecular size standard (61.3 kDa) is shown.

escape the primary vacuole and grow similar to wild-type *L. monocytogenes* in J774 cells.

Intracellular induction of LLO expression. In experiments shown in Fig. 4B, iLLO bacteria were induced with IPTG for 2.5 h prior to addition to J774 cells and then maintained in IPTG during infection. We next determined if LLO production could be initially induced while bacteria were trapped within primary vacuoles. iLLO bacteria were grown in BHI broth in the absence of IPTG and used to infect J774 cells. At various times postinfection, IPTG was added and maintained in the tissue culture medium and the effect on intracellular growth was determined. As shown in Fig. 5, IPTG added at the time of infection (0 h) and maintained in the tissue culture medium resulted in efficient intracellular growth of iLLO bacteria. Moreover, IPTG added to cell cultures up to 6 h postinfection allowed bacteria trapped within vacuoles to escape into the cytosol and grow intracellularly at rates comparable to those of bacteria induced at the time of infection. This indicated that, in addition to allowing tightly regulated intracellular induction of
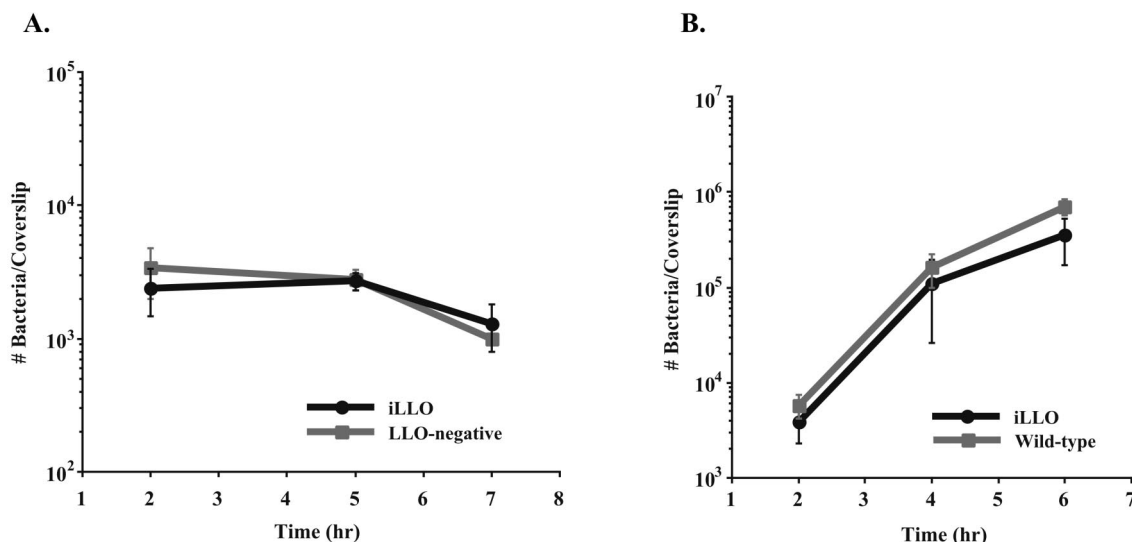
A.

B.

FIG. 4. Intracellular growth of the iLLO strain in J774 cells. Infection of J774 cells was done as described in Materials and Methods. (A) J774 cells were infected with iLLO (DP-L3885) or LLO-negative (DP-L2161) bacteria in the absence of IPTG. (B) iLLO (DP-L3885) or wild-type (10403S) bacteria were grown for 2.5 h in BHI broth containing 1 mM IPTG prior to infection of J774 cells and maintained in tissue culture medium containing 10 mM IPTG throughout the intracellular growth period.

LLO, the iLLO strain can be utilized to control vacuolar escape and initiation of intracellular growth subsequent to phagocytosis of bacteria.

LLO induction is required for cell-to-cell spread in L2 fibroblasts. A key component of *L. monocytogenes* pathogenesis is the ability of bacteria to spread from cell to cell during infection (10, 11, 22). In addition to macrophages, LLO is required to escape the primary vacuole in numerous nonprofessional phagocytic cell lines (17, 32, 41). We next examined
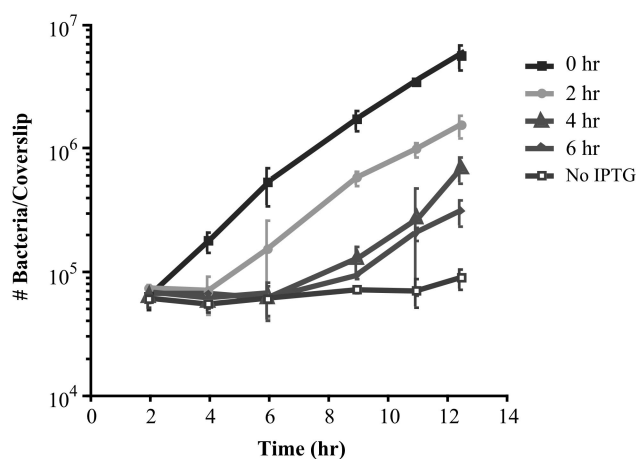

FIG. 5. Intracellular induction of LLO expression. iLLO (DP-L3885) bacteria were grown in BHI broth in the absence of IPTG and used to infect J774 cell cultures as described in Materials and Methods. Bacteria were added to J774 cells and maintained in medium containing 10 mM IPTG (0 h). Alternatively, bacteria were added to separate J774 cell cultures in the absence of IPTG. At 2-h increments (2, 4, or 6 h) postinfection, culture medium was removed and medium containing 10 mM IPTG was added to J774 cells and maintained throughout the intracellular growth period. One culture received no IPTG addition (No IPTG). Intracellular bacterial counts were determined as described in Materials and Methods.

the temporal requirement of LLO for intracellular growth and cell-to-cell spread following primary vacuole escape in a nonprofessional phagocytic cell line. We utilized the iLLO strain in plaque formation assays to evaluate the requirement of LLO for cell-to-cell spread in mouse L2 fibroblasts (53). As shown in Fig. 6A, the presence of IPTG had no effect on the efficiency of intracellular growth and cell-to-cell spread of wild-type *L. monocytogenes*, as indicated by the absence of differences in plaque formation (wild-type plates 1, 2, and 3). However, with iLLO bacteria, no plaques were formed in L2 cell monolayers infected in the absence of IPTG (iLLO plate 1). iLLO bacteria allowed to infect L2 monolayers for 1 h in medium containing IPTG, followed by removal of the medium and continued incubation in the absence of IPTG, formed small pinpoint plaques only visible upon magnification of the monolayer (Fig. 6A, iLLO plate 2, and B). Only when IPTG was present throughout the entire intracellular infection period were larger visible plaques formed in the iLLO-infected L2 monolayer (Fig. 6A, iLLO plate 3). However, the average diameter of iLLO plaques was 57% of that of plaques formed following infection with wild-type bacteria (Fig. 6A, compare wild-type and iLLO plates 3). These data indicated that, in the absence of IPTG, sufficient repression of LLO production was provided to prevent intracellular growth and cell-to-cell spread of *L. monocytogenes* in L2 fibroblasts. However, IPTG maintained in the L2 cell monolayer throughout infection by the iLLO strain mediated intracellular growth and cell-to-cell spread of bacteria, although not to the extent of wild-type bacteria. Furthermore, a brief incubation period with IPTG during the initial infection was sufficient to allow only the initiation of intracellular replication and minimum detectable spreading to immediately adjacent host cells (Fig. 6B).

IPTG dose-dependent induction of cell-to-cell spread. We next examined whether the concentration of IPTG present during assays of plaque formation in L2 fibroblasts could affect
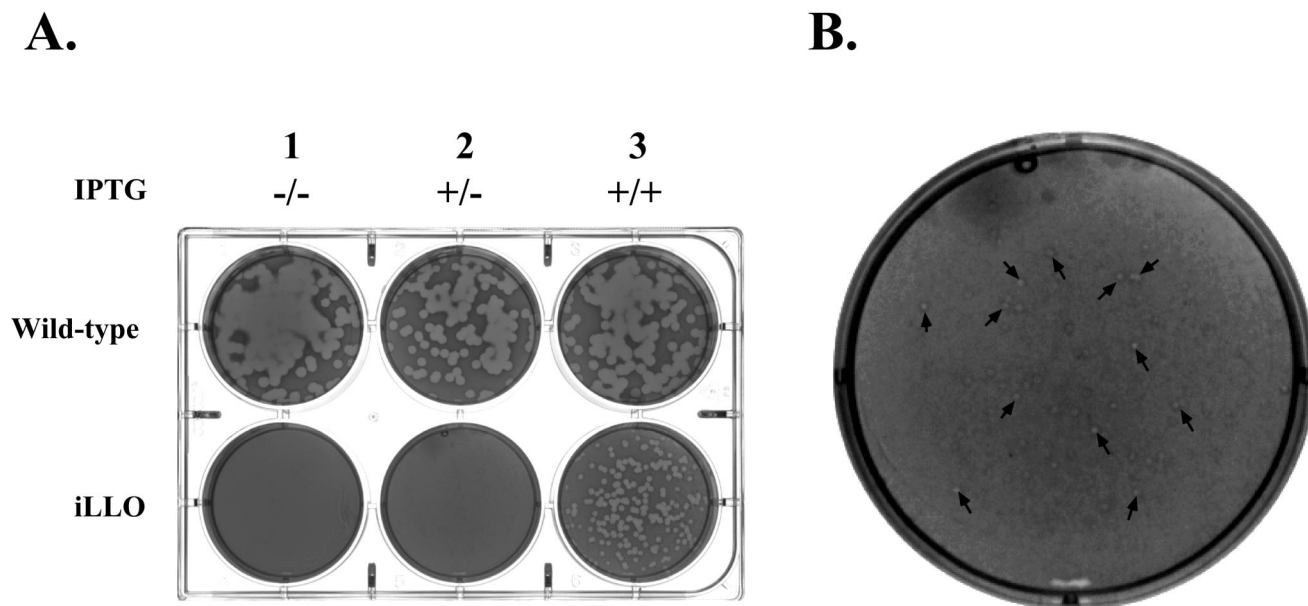
A.

B.

FIG. 6. LLO induction is required to mediate cell-to-cell spread in L2 fibroblasts. (A) iLLO (DP-L3885) or wild-type (10403S) bacteria were grown in BHI broth without IPTG and added to monolayers of mouse L2 fibroblasts, which were incubated for 1 h. Following washing of infected cell monolayers with PBS, a medium-agarose overlay containing gentamicin was added. Intracellular growth and cell-to-cell spread of bacteria were visualized after 96 h by the formation of clearing zones (plaques) within the L2 monolayers. +/− indicates, respectively, the presence or absence of 1 mM IPTG during the initial 1-h infection period or in the agarose overlay. (B) Magnification (×3) of iLLO plate 2 in panel A. Arrows point to representative pinpoint plaques present within the L2 monolayer.

the efficiency of intracellular growth and spread of iLLO bacteria. As shown in Table 2 for DP-L3885, the average diameter of plaques formed in L2 cell monolayers increased in an IPTG dose-dependent manner over a range of IPTG concentrations of 0 to 5.0 mM. The maximum plaque size observed was 69% of that achieved by wild-type infection at the 5 mM IPTG concentration. However, at higher IPTG concentrations, the plaque size decreased in a dose-dependent manner, resulting

TABLE 2. IPTG dose-dependent plaque formation in L2 fibroblasts[a]

IPTG concn (mM)	% Plaque size	
	DP-L3885	DH-L616
0	0	0
0.01	27	51
0.05	48	83
0.1	51	85
0.5	53	85
1.0	57	85
5.0	69	93
10	68	96
25	63	82
50	56	76

[a] iLLO (DP-L3885 and DH-L616) or wild-type (10403S) bacteria were grown in BHI broth without IPTG and added to monolayers of mouse L2 fibroblasts, which were incubated for 1 h in the presence or absence of IPTG. Following washing of the monolayers with PBS, a medium-agarose overlay containing gentamicin with or without IPTG was added. Cell-to-cell spread of bacteria was visualized after 96 h by the formation of clearing zones (plaques) within the monolayers. The concentration of IPTG present both during the initial 1-h infection period and in the agarose overlay is indicated. The average diameter of at least 10 individual plaques per sample was determined and is given as a percentage of that observed for wild-type infection.

in similar average plaque diameters at 50 and 1 mM IPTG (56 and 57% of the wild-type diameter, respectively). This decrease in plaque size is attributable to the toxicity of IPTG to host cells at higher IPTG concentrations (60), as a similar decrease in plaque size at IPTG concentrations of 25 and 50 mM was also observed with wild-type *L. monocytogenes* infection (data not shown).

We next examined whether the chromosomal locus in which the iLLO expression cassette was located could affect the efficiency of intracellular growth and cell-to-cell spread. *L. monocytogenes* strain DH-L616 was generated, which contains the iLLO expression construct in single copy within the *L. monocytogenes* tRNA[Arg] locus in the LLO-negative DP-L2161 background (Fig. 7). DH-L616 was identical to the original DP-L3885 strain with respect to in vitro growth in broth culture and LLO induction (Fig. 2 and 3 and data not shown). However, as shown in Fig. 8 and Table 2, DH-L616 yielded a plaque size that was 85% of the wild-type plaque size, compared to 57% for DP-L3885 (Fig. 6A), in plaque formation assays with 1 mM IPTG. The plaque size formed by DH-L616 bacteria increased to 93 and 96% of the wild-type size with IPTG concentrations of 5 and 10 mM, respectively. However, similar to infection with DP-L3885, further increasing the IPTG concentration in the plaque formation assay to 25 and 50 mM resulted in a decrease in plaque size (82 and 76% of the wild-type size, respectively). Taken together, these data indicated that the efficiency of cell-to-cell spread of iLLO bacteria in L2 fibroblasts was IPTG dose dependent. Furthermore, by altering the chromosomal location of the iLLO expression construct, no effect on LLO production during broth culture
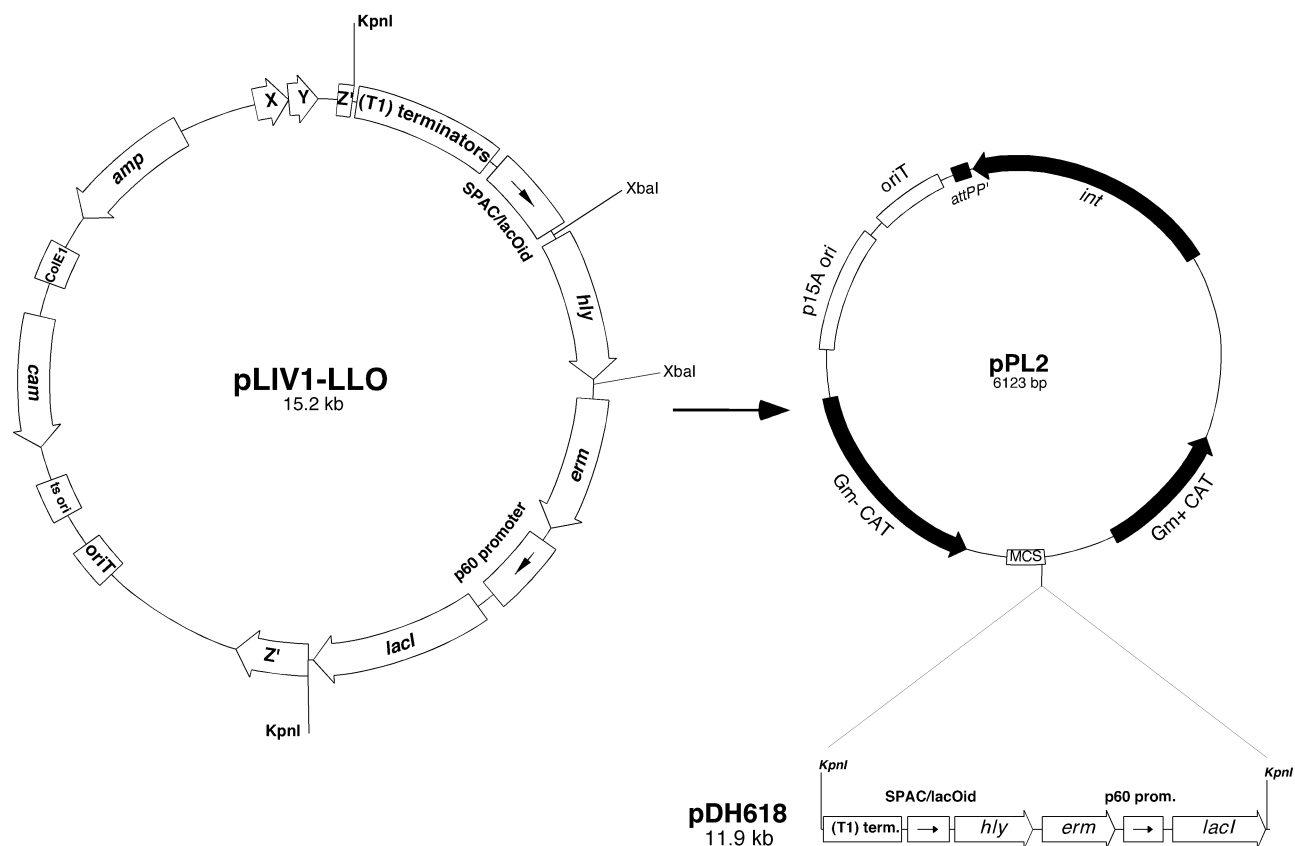
FIG. 7. Construction of iLLO strain DH-L616. *L. monocytogenes* strain DH-L616 was constructed by placing the iLLO expression cassette within the *L. monocytogenes* tRNA^Arg gene on the chromosome of LLO-negative strain DP-L2161. Plasmid pDH618 was generated by cloning the *Kpn*I fragment harboring the iLLO expression cassette from pLIV1-LLO into pPL2, an *L. monocytogenes* site-specific phage integration vector (33). Site-specific integration of pDH618 within the tRNA^Arg gene and verification of the iLLO construct were performed as previously described (33) to yield strain DH-L616.

was observed, yet the efficiency of cell-to-cell spread in L2 cells was significantly increased (Table 2 and Fig. 8).

DISCUSSION

L. monocytogenes is an excellent model for studying the molecular determinants of intracellular pathogenesis and cell-mediated immunity (6, 39, 40). The ability to control *L. monocytogenes* gene expression during intracellular infection will be extremely beneficial as a means by which to gain insights into the temporal requirements of virulence determinants necessary for intracellular growth and cell-to-cell spread. Here, we report the construction of a *lac* repressor/operator-based system to tightly regulate *L. monocytogenes* virulence gene expression during intracellular infection. With an IPTG-iLLO strain of *L. monocytogenes,* we demonstrated stringent, IPTG dose-dependent production of LLO in broth culture and within host cells. Furthermore, we showed the ability to control intracellular LLO expression following host cell entry by inducing vacuolar escape and intracellular growth of iLLO bacteria trapped within primary vacuoles. Data presented here also demonstrate that optimal intracellular growth and cell-to-cell spread of iLLO bacteria in L2 fibroblasts are only observed under continuous IPTG induction and that the efficiency of

cell-to-cell spread is IPTG dose dependent. Therefore, these data demonstrate a dose-dependent requirement of LLO expression following primary vacuole escape for efficient intracellular infection in a nonprofessional phagocytic cell line.

There are a number of regulatory systems available for inducible control of mammalian cell gene expression. These include heavy-metal ions (35, 56), steroid hormones (58), tetracycline induction (14, 51), and the *lac* repressor/operator system (4, 24). Of these, the *lac* repressor/operator system is one of the best studied and the specificity of the interactions between the *lac* repressor/operator and IPTG make it suitable for adaptation to the control of gene expression in mammalian cells. Additionally, the *lac* repressor/operator system has also been adapted to control mammalian cell gene expression in transgenic mice (12, 34, 59). Plasmid-based inducible-expression systems have been generated to control bacterial gene expression during intracellular infection of cultured mammalian cells. Arabinose-inducible and IPTG-inducible expression of bacterial plasmid-borne virulence genes has been previously reported for *Shigella flexneri* and *Legionella pneumophila* (43, 46), both gram-negative intracellular bacterial pathogens. However, because of the possibility of plasmid instability and reported variability within bacterial populations in expression levels of arabinose-induced protein from plasmid vectors (52),
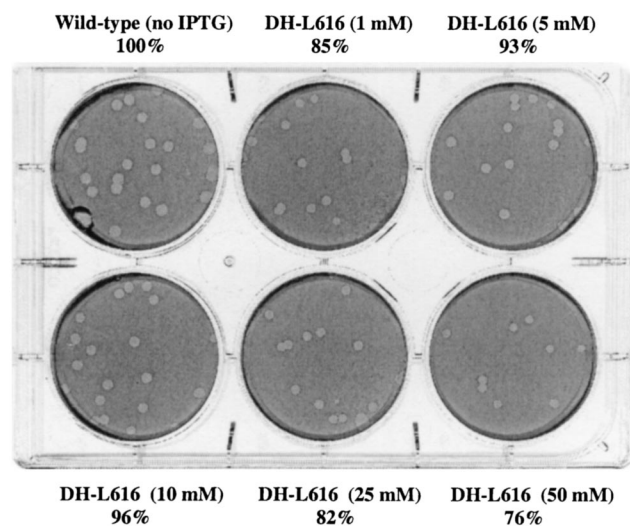
FIG. 8. IPTG dose-dependent plaque formation in L2 fibroblasts. iLLO (DH-L616) or wild-type (10403S) bacteria were grown in BHI broth without IPTG and added to monolayers of mouse L2 fibroblasts, which were incubated for 1 h. Following washing of the monolayers with PBS, a medium-agarose overlay containing IPTG and gentamicin was added to the monolayers. The concentration of IPTG present during both the initial 1-h infection period and in the agarose overlay is indicated. Cell-to-cell spread of bacteria was visualized after 96 h by the formation of plaques within the monolayers. The average diameter of 9 or 10 plaques per sample was determined and is given as a percentage of that observed for wild-type infection.

we chose to adapt the *lac* repressor/operator system to produce a chromosome-based inducible-expression system for *L. monocytogenes*.

The pLIV1 vector (Fig. 1A) was generated to allow integration of IPTG-inducible gene constructs into the *L. monocytogenes orfZ* locus. The salient features of pLIV1 that allow stringent *lac* repressor-inducible control in *L. monocytogenes* are the inclusion of multiple tandem copies of the *rrnB* T1 transcription terminator (36) immediately upstream of the inducible promoter to prevent aberrant readthrough transcription from the *orfXYZ* locus, the constitutive expression of the *lac* repressor by the *L. monocytogenes* p60 gene promoter (29, 30), and the modified SPAC/*lac*Oid promoter/operator sequence. With the modified *lac* repressor/operator system, the DP-L3885 strain generated here resulted in stringent, IPTG dose-dependent production of LLO (Table 1 and Fig. 3). When fully induced at 10 mM IPTG during broth culture, hemolytic activity was 47-fold higher than that of bacteria grown in the absence of the inducer. The IPTG dose dependency of LLO production was observed over a wide range of inducer concentrations, suggesting that it may be possible to utilize the inducible system to regulate the level of protein produced by bacteria during intracellular growth in an IPTG dose-dependent manner. Indeed, consistent with results for iLLO production in broth culture (Fig. 3), a dose-dependent increase in plaque size was also observed between 0.01 and 10 mM IPTG (Table 2). Also consistent with LLO production in broth culture, a maximum plaque size was observed with 5 or 10 mM IPTG. However, the maximum plaque size obtained

with the DP-L3885 iLLO strain was only 69% of the wild-type size.

Chromosomal integration of the iLLO construct in DP-L3885 was performed within the *orfZ* locus (48, 49). Initial studies indicated that integration of the native pLIV1 vector into *orfZ* of wild-type *L. monocytogenes* had no effect on intracellular growth and spread in any of the in vitro assays used here (data not shown). Therefore, the smaller-plaque phenotype exhibited by DP-L3885 appears to be due specifically to intracellular LLO production. The *orfZ* locus resides ~1.4 kb downstream of the *actA* gene on the *L. monocytogenes* chromosome. ActA expression is required for actin-based motility and, hence, cell-to-cell spread of bacteria (28). A recent report has indicated that intracellular expression of ActA is increased 150-fold compared to that in broth culture (50). Perhaps expression of iLLO from the *orfZ* locus prevents maximum intracellular induction of ActA, resulting in decreased efficiency of actin-based motility and a smaller plaque. Alternatively, intracellular induction of ActA in DP-L3885 may result in decreased intracellular LLO expression, resulting in the smaller-plaque phenotype, equivalent to the effect achieved by reducing the IPTG concentration in plaque formation assays. Nonetheless, plaque formation analysis with DH-L616, in which the iLLO construct is integrated within the tRNA[Arg] gene, resulted in a maximum plaque size that was 96% of that of wild-type bacteria (Fig. 8 and Table 2), indicating that the *orfZ* locus may not be suitable for optimal intracellular expression of all inducible gene constructs.

The IPTG dose dependency of plaque formation by iLLO bacteria (Fig. 8 and Table 2) indicates that a specific amount of LLO is required subsequent to primary vacuole escape to mediate efficient intracellular growth and cell-to-cell spread in L2 cells. In addition, infection of iLLO bacteria in L2 cells in the transient presence of IPTG resulted in an abortive infection (Fig. 6A, iLLO plate 2, and B). This result is consistent with a specific requirement for LLO to escape secondary vacuoles in L2 cells. We previously reported a specific requirement for LLO in mediating escape from secondary vacuoles formed during cell-to-cell spread in J774 cells (19). Electron microscopy analysis of bacteria at the outermost margins of L2 foci of infection is needed to confirm if bacteria are indeed trapped within secondary host cell vacuoles. An alternative explanation for the formation of pinpoint plaques is that once in the cytosol, bacteria can replicate and continue to spread in the complete absence of LLO but at a significantly reduced rate. Previous studies with J774 cells indicated no difference in the growth rate of intracellular bacteria in the absence of LLO (19). However, a recent report indicated that LLO-negative bacteria microinjected directly into the cytosol of Caco-2 cells replicated with 60% of the efficiency of wild-type bacteria in primary infected host cells yet also appeared to have a defect in replication in secondary host cells consistent with the inability to escape from secondary vacuoles (21). Microinjection of bacteria into host cells is fairly inefficient and is restricted to microscopic analysis (21). Therefore, further analysis of iLLO bacteria in intracellular growth and plaque formation assays will determine if LLO production is necessary for optimal intracellular growth in L2 cells and in a variety of host cell types. These studies, in addition to electron microscopy analysis of foci of infection, are under way.

To facilitate future studies of the temporal requirement of additional *L. monocytogenes* virulence determinants with our inducible system, gene expression should be inducible at any time while bacteria are localized to alternative subcellular locations within host cells. Results depicted in Fig. 5, in which iLLO bacteria were induced at time points following addition of bacteria to host cells, demonstrated the ability to induce LLO production while bacteria resided within primary vacuoles. One implication of the results shown in Fig. 5 is that the iLLO strain can be used to control *L. monocytogenes* escape from the primary vacuole and initiation of intracellular growth. This will be a significant asset for future studies addressing changes in host cell physiology observed during the transition of intracellular bacteria from residence in a vacuole to entry into the cytosol.

Understanding the functional roles that virulence determinants play in mediating growth and spread of intracellular pathogens could lead to a better understanding of the cell biological mechanisms involved and aid in the identification of specific inhibitors that would be an efficient means by which to control infection. Since *L. monocytogenes* has been a paradigm for understanding intracellular pathogenesis and the development of acquired immunity in the mouse infection model, we envision that the inducible-expression system may be an invaluable tool with which to achieve a more complete understanding of the temporal requirements for intracellular pathogenesis during infection of the host. Current studies are aimed at assessing the ability of the inducible system to control gene expression during *L. monocytogenes* infection of mice. Indeed, some of the most exciting possibilities are in controlling the extent of in vivo replication to examine the requirements for intracellular growth leading to protective immunity or generating attenuated vaccine strains that can initiate intracellular growth to prime an immune response but subsequently abort infection to avoid virulence. Furthermore, by combination of inducible gene expression constructs into defined mutation backgrounds, bacterial strains that localize to one cell or within a cellular compartment can be generated for functional complementation studies of inducible orthologous foreign gene products. We also envision the adaptation of this inducible genetic system for use in other pathogenic bacterial species, allowing direct analysis of host-pathogen interactions required for virulence in diverse pathogenic systems.

ACKNOWLEDGMENTS

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