Relative Roles of Listeriolysin O, InlA, and InlB in 
*Listeria monocytogenes* Uptake by Host Cells

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**ABSTRACT** *Listeria monocytogenes* is a facultative intracellular pathogen that infects a wide variety of cells, causing the life-threatening disease listeriosis. *L. monocytogenes* virulence factors include two surface invasins, InlA and InlB, known to promote bacterial uptake by host cells, and the secreted pore-forming toxin listeriolysin O (LLO), which disrupts the phagosome to allow bacterial proliferation in the cytosol. In addition, plasma membrane perforation by LLO has been shown to facilitate *L. monocytogenes* internalization into epithelial cells. In this work, we tested the host cell range and importance of LLO-mediated *L. monocytogenes* internalization relative to the canonical invasins, InlA and InlB. We measured the efficiencies of *L. monocytogenes* association with and internalization into several human cell types (hepatocytes, cytotrophoblasts, and endothelial cells) using wild-type bacteria and isogenic single, double, and triple deletion mutants for the genes encoding InlA, InlB and LLO. No role for InlB was detected in any tested cells unless the InlB expression level was substantially enhanced, which was achieved by introducing a mutation (*prfA*) in the gene encoding the transcription factor PrfA. In contrast, InlA and LLO were the most critical invasion factors, although they act in a different manner and in a cell-type-dependent fashion. As expected, InlA facilitates both bacterial attachment and internalization in cells that express its receptor, E-cadherin. LLO promotes *L. monocytogenes* internalization into hepatocytes, but not into cytotrophoblasts and endothelial cells. Finally, LLO and InlA cooperate to increase the efficiency of host cell invasion by *L. monocytogenes*.

**KEYWORDS** InlA, InlB, internalin, *Listeria monocytogenes*, listeriolysin O, listeriosis, host cell invasion, pore-forming toxins

*Listeria monocytogenes* is a Gram-positive, facultative intracellular bacterium responsible for the foodborne disease listeriosis. Listeriosis is a life-threatening condition for elderly and immunocompromised individuals (1). In these populations, the bacterium can propagate from the intestines to the blood and further disseminate, causing septicemia and meningoencephalitis (1–3, 6). During pregnancy, susceptibility to *L. monocytogenes* infection is drastically increased and the bacterium can cross the placental barrier, leading to spontaneous abortion, preterm labor, stillbirth, and severe infections of the newborn (1a–1c). An important virulence attribute of *L. monocytogenes* is its ability to infect numerous cell types, from macrophages to normally nonphagocytic cells such as intestinal and placental epithelial cells, endothelial cells, and neurons...
The wide host cell range of this pathogen is thought to be critical for crossing the tightest barriers of the human host, i.e., the placental and blood-brain barriers. The expression of major virulence factors that mediate the *L. monocytogenes* intracellular life cycle is controlled by PrfA (8–10), which activates transcription in response to a variety of environmental signals, including temperature (11) and nutrient availability (12–14). Two of these virulence factors are the surface proteins InlA and InlB, depicted as the major invasins responsible for *L. monocytogenes* uptake by normally nonphagocytic cells (4, 15, 16). InlA (internalin) is covalently anchored to the peptidoglycan through its C-terminal LPXTG motif (16, 17), whereas InlB is retained noncovalently at the cell surface via electrostatic interaction between three C-terminal glycine and tryptophan (GW) repeat domains and lipoteichoic acids of the bacterial cell wall (18). The adherens junction protein E-cadherin has been identified as the sole InlA receptor (19), and several host surface proteins, c-Met (or HGF receptor) (20), gC1Q receptor (21), and surface glycosaminoglycans (22), have been identified as InlB receptors. The N-terminal leucine-rich repeat (LRR) domain of InlB binds to c-Met, whereas its C-terminal moiety binds to glycosaminoglycans and gC1Q receptor in addition to being the lipoteichoic acid anchor (21, 22). InlA mediates bacterial entry only into cells expressing E-cadherin, whereas InlB is a more versatile invasin, as its receptors are widely expressed. Importantly, InlA and InlB are species specific: humans and gerbils are permissive to both InlA and InlB, while rabbits/guinea pigs and mice are permissive only to InlA and InlB, respectively (24). It has been proposed that InlB acts as a facilitator of the InlA-dependent invasion pathway in enterocytes (25, 26) and that InlA and InlB, but not listeriolysin O (LLO), are the two most important invasion factors for crossing the intestinal barrier (6, 25, 26).

Upon ingestion by host cells, *L. monocytogenes* is confined within a vacuole or phagosome that is disrupted by the secreted pore-forming toxin LLO and phospholipases to release the bacterium into the cytosol, where it divides and from which it infects other cells by cell-to-cell spreading (27–30). The role of LLO in mediating vacuolar escape is certainly a major role of this toxin, as the absence of LLO leads to a marked deficiency in intracellular replication of phagocytosed bacteria (30). The role of LLO was considered to be specifically restricted to the disruption of the phagosome (31), but additional roles have been attributed to this toxin. In particular, it has been shown that LLO, secreted by extracellular bacteria, perforates the host cell plasma membrane during the early stage of infection; therefore, LLO secretion and membrane perforation precede the formation of the phagosome (32, 35). Perforation of the host cell plasma membrane activates several signaling pathways (28). One outcome of LLO-induced signaling is the internalization of *L. monocytogenes* into epithelial cell lines (HepG2, HeLa, and Hep2 cells) (33–35) and professional phagocytes (human neutrophils and murine bone marrow-derived macrophages) (36). However, once bacteria are opsonized, the contribution of LLO in bacterial uptake by professional phagocytes becomes negligible. In addition, LLO-mediated plasma membrane perforation by cytosolic bacteria was recently proposed to facilitate cell-to-cell spreading (37).

Because InlA and InlB are described as the most important factors controlling *L. monocytogenes* uptake by normally nonphagocytic cells, it was necessary to establish whether the role of LLO is significant in comparison to these two canonical invasins. It was also necessary to determine if LLO plays a general role in inducing *L. monocytogenes* internalization in all cell types. To address these questions, we used human hepatocytes and cytrophoblasts, because they are known to be infected by *L. monocytogenes* during listeriosis (1). It is also known that *L. monocytogenes* can infect endothelial cells *in vitro* and may infect these cells *in vivo* to cross the blood-brain and placental-fetal barriers (38–43). As such, endothelial cells were included in this work. Although enterocytes that make up the intestinal barrier are of critical importance for the establishment of listeriosis, previous work has convincingly shown that crossing the intestinal barrier is InlA dependent and LLO independent, so enterocytes were not included (6). To quantify and compare the roles of the three invasins, we used a
fluorescence-based microscopy assay that directly measures the efficiency of bacterial association with host cells and the efficiency of their internalization.

**RESULTS**

**LLO, InlA, and InlB expression levels in single and double deletion mutants.** To ensure that deletion of the virulence genes *hly*, *inlA*, and *inlB*, in the single and double deletion *L. monocytogenes* 10403S mutants, does not affect the expression of the others, the levels of mRNA and proteins of the three invasion factors were measured. Bacteria were grown under the same experimental conditions as for the cell invasion assay and reverse transcription quantitative real-time PCR (RT-qPCR) was used to measure *hly*, *inlA*, and *inlB* mRNA levels. As expected, deletion of one or two virulence genes does not significantly affect the expression of the other genes in comparison to the wild-type (WT) strain (Fig. 1). We then measured the protein expression levels by Western blotting, which required antibodies against LLO, InlB, and InlA. Anti-LLO antibodies are commercially available, but not anti-InlA and anti-InlB. Therefore, we cloned *inlA* and *inlB* genes (without the signal peptide-encoding sequence) into an expression vector (pET29b), purified the recombinant proteins, and obtained purified polyclonal rabbit anti-InlB and -InlA. The anti-InlB antibodies could efficiently detect InlB (see Fig. S1 in the supplemental material), but we were not successful with the anti-InlA antibodies. We then measured LLO and InlB protein expression levels by Western blotting. For rigorous evaluation, we analyzed serial dilutions of cell lysates and performed densitometry analyses of the corresponding bands. As expected, single and double deletions of the *inlA*, *inlB*, or *hly* genes do not significantly affect the expression levels of LLO or InlB (Fig. 2).

**InlA and LLO, but not InlB, control *L. monocytogenes* uptake by human hepatocytes.** To establish the relative roles of the three virulence factors in *L. monocytogenes* uptake by human hepatocytes, we used four human hepatocyte cell lines (HepG2, Hep3B, PLC5, and Huh7) to rule out any cell line-specific phenotype and draw conclusions that can generally apply to hepatocytes. Hepatocytes were incubated with *L. monocytogenes* (WT or Δ*hly*, Δ*inlA*, Δ*inlB*, Δ*inlAB*, Δ*inlB Δhly*, Δ*inlA Δhly*, or Δ*inlAB Δhly* mutants) for 30 min at 37°C and were processed for fluorescence microscopy analysis. Full data sets, including association and internalization efficiencies of the eight bacterial strains into the four cell lines, are presented in Fig. S2. We first focused on analyzing data obtained with the single and triple deletion mutants in comparison to WT *L. monocytogenes* (Fig. 3). Data show that LLO does not promote *L. monocytogenes* association with hepatocytes. In one of the hepatocyte cell lines (Hep3B), LLO even significantly decreases bacterial association. In contrast, InlA is the only factor that promotes bacterial association with hepatocytes, in three out of the four cell lines. The decreases in association of the *inlA* single deletion mutant and the triple deletion mutant were similar in all hepatocyte cell lines, confirming that among the three factors, InlA is the only adhesin. LLO and InlA, but not InlB, promote internalization of
L. monocytogenes, although the role of LLO was more prominent in that function than the role of InlA. In one cell line (Hep3B), single deletion mutants had no internalization phenotype, whereas the triple (ΔinlABΔhly) and double (ΔinlAΔhly) (Fig. S2) deletion mutants displayed a significant decrease in internalization. The latter result shows that LLO and InlA can exert a redundant role in L. monocytogenes internalization. To our surprise, no role for InlB was detected in L. monocytogenes association and internalization.

L. monocytogenes invasion of human hepatocytes. HepG2, Hep3B, PLC5, and Huh7 cells were infected with WT, InlA-deficient (ΔinlA), InlB-deficient (ΔinlB), LLO-deficient (Δhly), or InlA- and LLO-deficient (ΔinlAB Δhly) bacteria (MOI of 20) for 30 min at 37°C. Cells were washed, fixed, and labeled with fluorescent antibodies and DAPI. (A) The bacterial association efficiency was calculated as the total number of bacteria associated per host cell. The average bacterial association values for the WT strain before normalization were as follows: HepG2, 0.14; Hep3B, 3.13; PLC5, 1.34; Huh7, 0.77. (B) The bacterial internalization efficiency was calculated as the percentage of intracellular bacteria. The average percentages of internalization for the WT strain before normalization were as follows: HepG2, 26.45%; Hep3B, 38.77%; PLC5, 18.29%; Huh7, 33.12%. The minimum numbers of host cells counted were as follows: HepG2, 1,000; Hep3B, 150; PLC5, 600; Huh7, 2,000. The average numbers of WT bacteria counted per experiment were as follows: HepG2, 600; Hep3B, 4,000; PLC5, 2,000; Huh7, 3,000 (with a minimum count of 100 bacteria being required for any mutant with reduced association efficiency). Results are expressed as the mean ± SEM relative to the WT (n = 3). Statistical analyses compared each deletion strain to the WT strain and were performed on raw data before normalization (*, P < 0.01; **, P < 0.001).
InlB-mediated *L. monocytogenes* internalization is dependent on InlB expression level. The absence of a role for InlB led us to verify that its receptor, c-Met, was expressed and functional in the hepatocyte cell lines used in these studies. As expected, c-Met was expressed in all tested hepatocyte cell lines (Fig. 4A). Previous studies established that InlB activates c-Met-dependent Akt phosphorylation and F-actin remodeling (44–46). As expected, cell exposure to recombinant InlB induced a significant increase in Akt phosphorylation in all cell lines (Fig. 4B). As a second approach, live cell imaging showed that hepatocytes exposed to InlB formed dynamic membrane ruffles, which were not observed in the absence of InlB (see Movies S1 to S4 in the supplemental material). Finally, to evaluate if hepatocytes could undergo InlB-dependent phagocytic uptake, we exposed cells to polystyrene beads (1-μm diameter) that were covalently coated with saturating concentrations of InlB or bovine serum albumin (BSA), used as negative control. As shown in Fig. 4C, 80% of InlB-coated beads were internalized by hepatocytes. We then established if InlB produced by *L. monocytogenes* could stimulate c-Met. HepG2 cells were incubated with WT and ΔinlB 10403S strains for 30 min at a multiplicity of infection (MOI) of 20, as performed in the invasion assays. As shown in Fig. 4D, WT but not InlB-deficient bacteria induced Akt phosphorylation. Together, these results demonstrate that the hepatocyte cell lines express a functional c-Met and that InlB from 10403S is expressed in a sufficient amount to activate c-Met signaling. However, InlB produced by 10403S failed to induce significant bacterial entry. We then tested the hypothesis that InlB was not produced in sufficient amounts by strain 10403S to promote bacterial uptake. This hypothesis was based on the fact that the bead surface was coated with a saturating amount of recombinant InlB and the fact that laboratory strains used to show a role for InlB in bacterial internalization express high levels of InlB (15, 18, 20, 39, 40, 44, 47–51). Indeed, the commonly studied

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**FIG 4** The InlB/c-Met signaling pathway is functional in hepatocytes. (A) HepG2, Hep3B, PLC5, and Huh7 cell lysates were subjected to Western blot analysis using anti-c-Met and anti-actin (loading control) antibodies. (B) Cells were exposed, or not, to 1.25 nM InlB for 5 min, and cell lysates were subjected to Western blot analysis using anti-Akt and anti-phospho-Akt antibodies. A representative Western blot is presented (n = 3). (C) HepG2 cells were incubated with BSA- or BSA/InlB-coated beads for 30 min at 37°C (MOI of 5). Results are expressed as the average percentage of internalization ± SEM (n = 4; *, P < 0.01; **, P < 0.001). (D) After infection with WT or ΔinlB bacteria (MOI of 20) for 30 min, HepG2 cells were lysed and lysates were subjected to Western blot analysis using anti-Akt and anti-phospho-Akt antibodies. A representative Western blot is shown (n = 3). MW, molecular weight.
laboratory strain EGD expresses a constitutively active variant of the transcriptional regulatory factor PrfA, known as a PrfA* (G145S) variant, which is responsible for high production levels of InlB and other PrfA-regulated virulence factors (14, 47, 52–57). To test if an increase in InlB production in the 10403S background would result in InlB-mediated internalization of L. monocytogenes, we generated prfA* and ΔinlB prfA* strains in the 10403S background by phage transduction (47, 57, 58). We compared the production of InlB between 10403S WT and prfA* strains and report a marked increase in InlB production, as expected (47) (Fig. 5A). The replacement of WT prfA with prfA* led to a 5-fold increase in bacterial association (Fig. 5B) and a 7-fold increase in bacterial entry into host cells (Fig. 5C). A comparison of prfA* and ΔinlB prfA* strains showed that, in the prfA* background, InlB plays a significant role in bacterial entry (Fig. 5C), while a comparison of the WT and ΔinlB strains shows no difference in either bacterial association or bacterial entry (Fig. 5B and C). Collectively, these data show that a bacterial strain such as 10304S produces enough InlB to activate c-Met, but this amount is not sufficient to affect L. monocytogenes internalization.

Only InIA, not InIB or LLO, controls L. monocytogenes uptake by human cytotrophoblasts. We next determined the role of LLO, InIA, and InIB in L. monocytogenes uptake by human cytotrophoblast-like BeWo cells. Cytotrophoblasts are cells of fetal origin located at the interface between maternal and fetal tissues. Invasion of the placenta requires traversal of the cytotrophoblast barrier. No role for LLO in L. monocytogenes association and entry was detected in BeWo cells. Two other cytotrophoblast-like cells, Jeg-3 and JAR, were also tested, leading to the same conclusion (data not shown). Only InIA plays a major role in L. monocytogenes association with BeWo cells, but it does not affect the efficiency of internalization (Fig. 6). Finally, no role for InIB was observed in the invasion of BeWo cells, as previously reported by others using the same bacterial strain (59).

Uptake of L. monocytogenes by HUVECs is independent of the three invasion factors. We next assessed the role of LLO, InIA, and InIB in the uptake of L. monocytogenes by human umbilical vein endothelial cells (HUVECs). We used the low MOI of 5 because HUVECs are severely damaged at higher MOIs due to LLO activity, as we have observed and as recently reported (42). Our data showed no role for InIA, InIB, or LLO in the invasion of HUVECs (Fig. 7). This is congruent with the most recent report in the literature regarding L. monocytogenes strain 10403S and HUVECs that supports the notion that bacterial uptake is largely independent of InIA, InIB, and LLO (42).
Establishing cooperation between LLO and InlA in *L. monocytogenes* invasion of hepatocytes. Hepatocyte infection data indicated an important role for both LLO and InlA in *L. monocytogenes* host cell invasion. This infection model was therefore appropriate for establishing whether LLO and InlA cooperate to potentiate the efficiency of host cell invasion. The biological expectation for positive cooperation between the two proteins, also referred to as synergism, is that the biological response when both proteins are expressed (when both genes are present) will be greater than the sum of their individual responses (when one of the corresponding genes is deleted) (60). To establish if InlA and LLO display positive cooperation in bacterial association with host cells on entry into host cells, we established four groups: InlA and LLO are both expressed (WT strain), LLO is expressed alone (ΔinlA strain), InlA is expressed alone (Δhly strain), and neither of the two proteins is expressed (ΔinlA Δhly double deletion).
TABLE 1 Invasion factor cooperation analysis

| Invasion factor combination tested | Cooperation in internalization | Cooperation in association |
|-----------------------------------|--------------------------------|---------------------------|
|                                   | Estimate | SE  | P value | Estimate | SE  | P value |
| InlA/LLO                          |          |     |         |          |     |         |
| HepG2                             | 13.996   | 5.9587 | 0.0232 | -0.04043 | 0.0379 | 0.2917 |
| Hep3B                             | -16.8597 | 9.9539 | 0.0995 | -1.1548 | 0.6499 | 0.0848 |
| PLC5                              | 6.5958   | 2.9439 | 0.0332 | -0.3497 | 0.4422 | 0.4357 |
| Huh7                              | 7.0611   | 6.8443 | 0.3114 | 0.01674 | 0.2237 | 0.9409 |
| InlA/InlB                         |          |     |         |          |     |         |
| HepG2                             | 7.6389   | 6.0995 | 0.2168 | -0.02705 | 0.03911 | 0.4928 |
| Hep3B                             | -2.8172  | 10.1881 | 0.7838 | -0.4551 | 0.6875 | 0.5126 |
| PLC5                              | 3.645    | 3.3279 | 0.2827 | -0.6872 | 0.4973 | 0.1779 |
| Huh7                              | -1.0749  | 7.8543 | 0.8922 | -1.0749 | 7.8543 | 0.8922 |
| InlB/LLO                          |          |     |         |          |     |         |
| HepG2                             | 8.3178   | 5.4892 | 0.1365 | -0.07209 | 0.03502 | 0.0452 |
| Hep3B                             | -3.0958  | 9.4808 | 0.746  | 0.4169 | 0.6232 | 0.5082 |
| PLC5                              | 0.9946   | 2.9439 | 0.738  | 0.04456 | 0.4422 | 0.9205 |
| Huh7                              | 2.6579   | 7.1474 | 0.7129 | 2.6579 | 7.1474 | 0.7129 |

*The estimate is the result of the synergistic interaction tests described in Results. Statistically significant *P* values (<0.05) indicate positive (synergistic) cooperation. SE, standard error. Boldface indicates statistically significant *P* values.

A linear mixed-effects model was used to test this hypothesis: \((\mu_{\text{both}} - \mu_{\text{neither}}) > [(\mu_{A} - \mu_{\text{neither}}) + (\mu_{B} - \mu_{\text{neither}})], \) i.e., \(\mu_{\text{both}} - \mu_{A} - \mu_{B} + \mu_{\text{neither}} > 0,\) where both is the WT, neither is the double deletion mutant, and \(\mu\) is the mean outcome for each group (60). If the *P* value for this test is significant, we claim that there is significant synergistic interaction (positive cooperation) between the two proteins. We used this analytical method to test whether InlA and LLO work synergistically to affect bacterial association and internalization of *Listeria monocytogenes*. Similar analyses were performed to test for potential positive cooperation between InlB and LLO and between InlB and InlA. Estimates and accompanying statistics are included in Table 1. In the process of bacterial association, no pattern of positive cooperation was observed (Table 1). This is consistent with InlA being the sole contributor to association among the tested invasins. In the process of bacterial internalization, no synergistic effect was observed between InlB and the two other invasins (Fig. S2 and Table 1), confirming that InlB does not affect the uptake of *L. monocytogenes* (strain 10403S) into human hepatocytes. Only LLO and InlA interact in a synergistic manner to potentiate *L. monocytogenes* internalization into HepG2 and PLC5 cells.

**DISCUSSION**

This work focused on establishing the relative roles of LLO, InlA, and InlB in *L. monocytogenes* (strain 10403S) association with and internalization into normally nonphagocytic human cells. The data show that LLO activity is cell type dependent, as LLO plays a significant role in *L. monocytogenes* internalization into hepatocytes but not into cytotrophoblasts or endothelial cells. InlA and LLO are the two virulence factors that significantly contribute to the invasion of human hepatocytes, with InlA playing a significant role as an adhesin and LLO as an invasin. To our surprise, no role for InlB was detected unless the *prfA* gene was replaced by a constitutively active *prfA* mutant, indicating that higher expression levels of InlB are required for InlB-mediated bacterial internalization.

Studies that identified the *L. monocytogenes* virulence factors controlling host cell invasion have traditionally used the gentamicin survival assay. This assay robustly measures bacterial intracellular survival but presents some limitations. First, it indiscriminately and collectively reports the efficiencies of bacterial association and internalization. Second, host cell perforation by LLO allows for diffusion of gentamicin and potential targeting of intracellular bacteria (35). Finally, this assay generally involves long incubation times, which can be sufficient for intracellular bacterial division or killing. Because of these limitations, we analyzed cells infected for only 30 min at a low...
MOI and in the absence of gentamicin, using a fluorescence microscopy approach (61). Microscope automation allows for rapid acquisitions of a high number of images, and software-assisted analytical tools considerably decrease the time for analysis. Importantly, this approach specifically quantifies with sensitivity and accuracy the efficiencies of bacterial attachment and association with host cells (61).

No role for InlB was initially detected in the present work. This result was unexpected, because numerous studies report that InlB promotes host cell invasion (15, 20, 40, 44, 48, 49). Using the hepatocyte model, we showed that the InlB receptor, c-Met, was expressed and functional. In addition, the amount of InlB produced by L. monocytogenes 10403S under our experimental conditions was sufficient to activate c-Met-dependent signaling but not bacterial internalization (Fig. 3 and 4). Furthermore, hepatocytes could massively internalize polystyrene beads coated with high concentrations of recombinant InlB (Fig. 4C). Studies that characterized the role of InlB in host cell invasion mostly used strain EGD, which carries a mutation in the gene coding for the master regulator of the virulence gene prfA (designated prfA*), leading to high expression levels of InlB among other virulence factors (47). Among all sequenced L. monocytogenes strains analyzed, the prfA* mutation is very rarely observed (47). When the prfA* mutation was introduced into EGD-e, inlB transcription was increased over 40-fold (47). This led us to hypothesize that the strain used in our study, 10403S, may not produce enough InlB for productive bacterial internalization. To test this hypothesis, we replaced the WT prfA allele with a prfA* allele in the 10403S background and consequently observed a marked increase in InlB production and a statistically significant role for InlB in bacterial internalization. Together, these data support the idea that the level of expression of InlB is critical for bacterial internalization. Therefore, it is reasonable to extrapolate that any conditions, including different bacterial cell growth conditions or environmental conditions, that substantially increase InlB expression would favor InlB-dependent internalization. For example, the transcription level of inlB in strain EGD-e is increased in human blood and the murine intestine (62). One should also consider that the role of InlB observed at later time points of infection may be related to bacterial intracellular survival and/or multiplication and not to bacterial internalization.

As expected, InlA promotes invasion of cells that express its receptor, E-cadherin (19). Importantly, the role of InlA was substantial even in strain 10403S expressing wild-type prfA. Few studies have focused on distinguishing the role of E-cadherin in anchoring the bacterium to the host cell surface from its role in stimulating bacterial internalization. It was initially proposed that the InlA–E-cadherin interaction promotes both anchoring and internalization, since the intracellular domain of E-cadherin and its association with the F-actin cytoskeleton were necessary for InlA-dependent L. monocytogenes uptake by fibroblasts (63). More recent work studying L. monocytogenes invasion of MDCK epithelial cells expressing wild-type E-cadherin or the glycosylphosphatidylinositol (GPI)-anchored extracellular domain of E-cadherin concluded that the InlA–E-cadherin interaction anchors the bacterium to the host cell surface but is dispensable for F-actin-dependent internalization of the bacterium (64). Our results are in accordance with both studies. We report that the primary function of the InlA–E-cadherin interaction is to anchor the bacterium to the host surface, but this interaction can also control the efficiency of bacterial internalization in some, but not all, cell lines.

LLO plays a critical role in L. monocytogenes internalization into hepatocytes. Other studies established that the formation of LLO pores on the plasma membrane activates the following signaling cascade: influx of extracellular Ca^{2+}, activation of Ca^{2+}-dependent conventional protein kinase C upstream from the Rho GTPase Rac1, and Arp2/3-dependent formation of F-actin-rich membrane projections that promote internalization of the bacterium (34, 35, 65). Because LLO targets all membranes that contain cholesterol, it was expected that LLO would activate bacterial internalization in all animal cells, including cytotrophoblasts and HUVECs, but to our surprise, this was not the case. However, hepatocytes are not the only cells thus far identified to undergo LLO-dependent L. monocytogenes internalization, as this was also reported in HeLa cells,
Hep2 cells, human neutrophils, and macrophages (34). Furthermore, LLO-dependent internalization has been demonstrated for *L. monocytogenes* strains 10403S, L028, and EGD (33, 34). The difference in host cell response to LLO should be investigated further to understand what makes some cell types permissive to the LLO-dependent entry pathway. This would be useful for understanding how pathogens can generally take advantage of plasma membrane perforation to gain entry into host cells (66, 67).

We report that InlA and LLO cooperate in an additive or synergistic fashion depending on the cell line. Though the mechanism by which LLO and InlA cooperate is still unknown, two non-mutually exclusive hypotheses can be envisioned. First, by anchoring *L. monocytogenes* to the host cell, InlA increases local LLO concentration and thereby LLO-dependent internalization. Along this line, InlA likely served as the adhesin and LLO promoted the signaling cascade for bacterial internalization into MDCK cells expressing GPI-anchored E-cadherin (64). Second, LLO- and InlA-induced signaling cascades may potentiate the activation of common transducers for the remodeling of F-actin and bacterial engulfment (65, 68).

Most studies that addressed the roles of InlA, InlB, and LLO utilized the laboratory strain EGD, EGD-e, or 10403S, which all belong to serovar 1/2a. EGD is derived from the strain of *L. monocytogenes* isolated from guinea pigs in 1926 (69). EGD-e is thought to be a derivative of strain EGD (47, 70). 10403S is a derivative of strain 10403, a strain initially isolated from a human skin lesion (71). Of these strains, EGD-e is the most virulent in mice and has been shown to express high levels of some of the PrfA-stimulated genes despite the absence of the *prfA* allele (47). *L. monocytogenes* strains associated with clinical cases and outbreaks of listeriosis belong predominantly to serovars 1/2a, 1/2b, and 4b, with greater than 50% of isolates belonging to serovar 4b (72, 73). Characterization of virulence factors in clinical strains seems to be lacking. A role for InlA in the invasion of Caco-2 cells has been demonstrated with a clinical isolate (Scott A, serotype 4b) from an outbreak of listeriosis in Massachusetts in 1983 (74, 75). One epidemiological study reported that 96% of clinical isolates, and only 65% of food isolates, express full-length InlA (76), and other studies have similarly found a higher prevalence of full-length InlA in strains associated with human and animal infections, with more strains expressing truncated InlA in food isolates (77–79). Other work has found that LLO- and InlB-encoding genes are highly prevalent in clinical strains (80). However, these studies emphasize the importance of InlA, InlB, and LLO as virulence factors but do not directly inform on their mechanism of action in vivo. In vivo studies using animal models also established a role for these three virulence factors. Of the three factors, LLO is the most important for virulence, as LLO-deficient strains are avirulent, so dissecting its role in vivo is challenging. In mice infected with 10403S or EGD-e, InlB does not affect liver and spleen colonization or the 50% lethal dose (*LD*$_{50}$) (81, 82). One recent study infecting E-cadherin-humanized mice and gerbils with EGD (prfA*) showed that neither InlA nor InlB affected infection of the liver (7). The same study also showed that InlA is important for infection of the intestines, colon, and cecum and that both InlA and InlB contribute to infection of the placenta and fetus.

In conclusion, to successfully cross the host barriers and invade multiple tissues, *L. monocytogenes* uses a collection of virulence factors that collectively facilitate bacterial anchoring to host cells and successive internalization. It appears that InlA is the major adhesin, while InlA, LLO, and InlB can stimulate bacterial internalization alone or in concert with InlA. Collectively, the three factors are conserved among clinical strains, but their roles likely vary in a tissue- and strain-dependent fashion.

**MATERIALS AND METHODS**

**Bacterial strains and culture.** *Escherichia coli* XL1-Blue and BL21(DE3) were grown in Luria-Bertani (LB) broth under agitation at 37°C. Plasmids were maintained with either ampicillin (50 μg/ml) or kanamycin (30 μg/ml), as indicated. Wild-type (WT) *L. monocytogenes* (EGD-e) was a gift from Pascale Cossart (Pasteur Institute, Paris, France) (Table 2). WT *L. monocytogenes* (10403S) and Δ*hly* (DP-L2161), Δ*inlA* (DP-L4405), Δ*inlB* (DP-L4406), and Δ*inlAB* (DP-L4404) isogenic mutants were gifts from Daniel Portnoy (UC Berkeley, CA, USA). Strain 10403S, a member of lineage II and serotype 1/2a, is a streptomycin-resistant derivative of strain 10403 (47, 71), which was originally isolated from a human skin lesion in 1968 (83). The Δ*inlAB* Δ*hly* triple deletion mutant was developed previously (34). Δ*inlA Δhly* and...
ΔinlB Δhly double deletion mutants were constructed using DP-L4405 and DP-L4406, respectively, by knocking out the hly gene via allelic exchange using the pKSV7 integration shuttle vector and primers listed in Table 3, as described previously (34, 84). The deletion of hly was confirmed by PCR using primers listed in Table 3.

L. monocytogenes strains were grown overnight under agitation at 37°C in brain heart infusion (BHI) (BD Biosciences). For invasion assays, overnight cultures were diluted 20-fold in BHI and grown at 37°C until an optical density at 600 nm (OD600) of 0.7 to 0.8 was reached. Cells were washed three times in sterile, 37°C phosphate-buffered saline (PBS) and diluted to the indicated multiplicity of infection (MOI) in appropriate mammalian cell culture medium without serum or antibiotic.

Transduction and prfA* mutant isolation. U153 bacteriophage (85) was used to infect L. monocytogenes strain NF-L1177 (prfA* G145S actA-gus-neo-plcB), and the phages were recovered and used to transduce the prfA* (leading to G145S) actA-gus-neo-plcB to the target strains, WT 10403S and the ΔinlB mutant, as previously described (U153 bacteriophage and strain NF-L1177 were gifts from Nancy Freitag [University of Illinois, Chicago, IL] (52, 58). Transductants were selected by plating the mixture of phage and bacteria on BHI/agar plates (5 μg/ml neomycin) for 2 days at 37°C. Neomycin-resistant mutants were further screened by plating on BHI/agar plates containing 5 μg/ml neomycin plus 50 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galacturonic acid (X-gluc) to confirm the prfA* mutation and the downstream

### Table 2 L. monocytogenes strains used in this study

| Strain   | Genotype                        | Source or reference |
|----------|---------------------------------|---------------------|
| EGD-e    | Wild type                       | 70                  |
| 10403S   | Wild type                       | 87                  |
| DP-L2161 | 10403S Δhly                     | 88                  |
| DP-L4405 | 10403S ΔinlA                    | 59                  |
| DP-L4406 | 10403S ΔinlB                    | 59                  |
| DP-L4404 | 10403S ΔinlAB                   | 59                  |
| SL33     | 10403S ΔinlA Δhly               | This study          |
| SL40     | 10403S ΔinlB Δhly               | This study          |
| SL20     | 10403S ΔinlAB Δhly              | This study          |
| NF-L1177 | 10403S prfA G145S actA-gus-neo-plcB | 89               |
| SL64     | DP-L4406 prfA G145S actA-gus-neo-plc | This study |

### Table 3 Primers used in this study^a^

| Purpose of constructs | Oligonucleotide sequence (5’-3’) | Reference |
|----------------------|----------------------------------|-----------|
| Construction of Δhly strains | Forward: GGG AAT TCA ATT GTT GAT ACA ATG ACA TC  
Reverse: GGC TGC AGG GTC TTT TTG GCT TGT GTA T | 88 |
|                      | Forward: CCG TCG GAT CCA TGA AAA AAA TAA TGC TAG TTT TTATTACAC  
Reverse: ATC CGC GCT GCA GTT CGA TTG GAT TAT CTA CTT TAT TAC | 88 |
|                      | Forward: AAC GTG CAT ATG GAG ACT ATC ACC GTA ACA TAA TAT GCC  
Reverse: ATT CTC GAG TTT TCG CTC CCT TAA ATT AAG TGC | This study |
|                      | Forward: GCA GTC AAT TAA TAT GCC GAC CG  
Reverse: CAG CTA ACA ATT GTT GTT ACT GCC | This study |
|                      | Forward: GGC AAA GAA ACA ACC AAA GAA A G  
Reverse: GGG CATCAA ACC ACC AAA CAA | This study |
|                      | Forward: ATG TGA CTG CCG GAA TGT ACC ACC ACT TTA T  
Reverse: ATG TCA CGG CCA ATA AAG CTG GC | This study |
|                      | Forward: TCA TCT GTG CTC ATT CAA GAC CGG  
Reverse: TTA TCT GCT ACC GGG ACT TTA A | This study |
|                      | Forward: ATG TCA CGG CCA ATA AAG CTG GC  
Reverse: ATG TCA CGG CCA ATA AAG CTG GC | This study |
|                      | Forward: TCA CAG CGC AAG ACA AAC  
Reverse: GGG CAT CAA ACC ACC AAA CAA | This study |
|                      | Forward: ATC TCG GAT AAA GCC TGG TG  
Reverse: ATC TCG GAT AAA GCC TGG TG | This study |
|                      | Forward: TGA TGA CGG CAA TGT ACC ACT TGT GA  
Reverse: ATG TCA CGG CCA ATA AAG CTG GC | This study |
|                      | Forward: TCA CAG CGC AAG ACA AAC  
Reverse: ACT GTT CTA GTT CCG ACT TTA T | This study |
|                      | Forward: TGA TGA CGG CAA TGT ACC ACT TGT GA  
Reverse: ATC TCG GAT AAA GCC TGG TG | This study |
|                      | Forward: TCA CAG CGC AAG ACA AAC  
Reverse: ACT GTT CTA GTT CCG ACT TTA T | This study |
|                      | Forward: TGG AAT ATA TCG ACG GCA TCG T  
Reverse: GCT GTT TGA ATC TCA ATT AAG TTT GG | 90 |
|                      | Forward: CT GAT TCG CGC AAA ACT TCT ACG CG  
Reverse: CTG GAT GCT AAT TCA ATT AAG TTT GG | 90 |

^a^All probes have a 5’ FAM reporter dye and a 3’ Iowa Black FQ quencher.
actA-gus-neo-plc8 transcription fusion. The actA-gus-neo-plc8 insertion was then confirmed by PCR, and the praG145S mutation was confirmed by sequencing using primers described in Table 3.

RNA purification, reverse transcription, and RT-qPCR. For RNA purification, L. monocytogenes was cultured in BHI under agitation at 37°C to an OD\textsubscript{600} of 0.7 to 0.8. RNA was purified from 10\textsuperscript{8} bacteria and subsequently treated with RNase-free DNase as described previously (12). RNA concentration and purity were measured via a NanoDrop ND-1000 spectrophotometer. RNA integrity was determined on a 1.2% agarose gel. Reverse transcription was performed using a high-capacity RNA-to-cDNA kit (Applied Biosystems) according to the manufacturer’s instructions. Duplicate reaction mixtures lacking the reverse transcriptase enzyme were performed in parallel, and these samples were used in RT-qPCR to test for residual DNA contamination. RT-qPCR was performed using a CFX96 real-time system and a C1000 thermal cycler (Bio-Rad). All reactions were performed in 96-well plates using 1.5 ng of converted cDNA, iQ Supermix (Bio-Rad), forward and reverse oligonucleotide primers, and hydrolysis probes (Table 3). No-reverse-transcriptase (NRT) samples were used as negative controls. inlB, inlA, and hly gene expression was normalized to housekeeping genes gap and rpoB. Fold changes in gene expression are relative to that of WT L. monocytogenes. Primer and probe concentrations were optimized by testing a concentration gradient of all oligonucleotides as described previously (86). All primer/probe sets yielded reaction efficiencies of ~100%. All RT-qPCR hydrolysis probes include a 5′ FAM reporter dye and a 3′ Iowa Black FQ quencher. Samples were analyzed in triplicate by RT-qPCR.

InlB purification and generation of anti-InlB rabbit polyclonal antibodies. The inlB gene, excluding the signal sequence (bp 106 to 1890), was amplified from genomic DNA of L. monocytogenes strain EGD-e using primers (Table 3) that contain NdeI and XhoI restriction sites. This DNA fragment was ligated into the pET29b expression vector upstream of the C-terminal 6His tag sequence. The resulting expression vector, pET29b-inlB, was transformed into Escherichia coli strain BL21(DE3). For expression of recombinant InlB-6His, the strain was grown at 37°C until an OD\textsubscript{600} of 0.6 was reached, and expression of recombinant InlB-6His was induced by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (48). After 5 h of induction, the bacteria were pelleted and suspended in binding buffer (5 mM imidazole, 500 mM NaCl, and 50 mM HEPES, pH 7.9) and lysed with a French press. The crude lysate was centrifuged, and the supernatant was incubated with Ni-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen). After washes, the protein was eluted and dialyzed overnight. Purified recombinant InlB was sent to GenScript (Piscataway, NJ, USA) to generate rabbit anti-InlB polyclonal antibodies. To immunize rabbits, recombinant InlB and complete Freund’s adjuvant were administered via subcutaneous injection. After the primary immunization, three boosts were performed over the course of 66 days. InlB-specific IgG antibodies were purified from serum by affinity chromatography using a Sepharose 4B gel coupled to recombinant InlB. The specificity of the antibodies was ensured by Western blotting of WT and inlB deletion mutant L. monocytogenes strains (see Fig. S1 in the supplemental material).

Mammalian cell culture. The human hepatocyte cell line HepG2 (HB-8065) was purchased from ATCC. The human hepatocyte cell lines Hep3B (HB-8064; ATCC), PLC5 (CRL-8024; ATCC), and Huh7 (Health Science Research Resources Bank, Osaka, Japan; JCRB0403) were gifts from Ching-Shih Chen (The Ohio State University, OH, USA). HepG2, Hep3B, and PLC5 cells were grown in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS; Atlanta Biologicals), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Huh7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% HI-FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. The human choriocarcinoma cell line BeWo (ATCC CCL-98) was a gift from John Mitchell Robinson (The Ohio State University, OH, USA). BeWo cells were grown in DMEM-F12 medium (1:1) supplemented with 10% HI-FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Human umbilical vein endothelial cells (HUVECs; ScienCell Research Laboratories, San Diego, CA, USA) were cultured in endothelial cell medium (ECM) with 5% HI-FBS, endothelial cell growth supplement (ECGS; ScienCell), 100 U/ml penicillin, and 100 µg/ml streptomycin. All plates and flasks used for HUVEC culture were coated with 2 µg/cm\textsuperscript{2} human fibronectin (BD Biosciences).

Western blotting (LLO, InlB, c-Met). Bacterial lysates were loaded at several dilutions (8 × 10\textsuperscript{7}, 4 × 10\textsuperscript{7}, and 2 × 10\textsuperscript{7} bacteria loaded for LLO, and 3.2 × 10\textsuperscript{6}, 1.6 × 10\textsuperscript{6}, and 8 × 10\textsuperscript{5} bacteria loaded for InlB) and subjected to SDS-PAGE and Western blot analysis using polyvinylidene difluoride (PVDF) membranes and anti-LLO antibody (rabbit polyclonal from Abcam), anti-InlB antibody (rabbit polyclonal from GenScript), and secondary anti-rabbit IgG antibody conjugated to horseradish peroxidase (Cell Signaling). For detection of InlB production in prf\textsuperscript{A} mutants, 1.6 × 10\textsuperscript{9} cells were used. We also probed for p60 as a loading control (Adipogen). Signal detection was performed using an Amersham ECL select reagent kit (GE Healthcare) and a ChemiDoc XR imaging system (Bio-Rad). Densitometry analysis was performed by enclosing each protein band within a region of standard size, and the intensity of each band was measured using ImageJ gel analysis. Results were the average intensities calculated from three independent experiments. All intensities were set relative to that of WT bacterial lysates. For detection of c-MET, hepatocytes were grown to 80% confluence under the same experimental conditions as those used for infection assays. Cell lysates were subjected to SDS-PAGE and Western blot analysis using PVDF membranes with anti-c-MET (4F8.2; Millipore) antibodies and secondary anti-mouse IgG antibodies conjugated to horseradish peroxidase (Cell Signaling). Signal detection was performed as described above.

Measuring bacterial association and internalization. HepG2 (10\textsuperscript{5} cells/well), Hep3B (0.75 × 10\textsuperscript{5} cells/well), PLC5 (0.75 × 10\textsuperscript{5} cells/well), Huh7 (0.75 × 10\textsuperscript{5} cells/well), and HUVECs (2 × 10\textsuperscript{4} cells/well) were cultured in 24-well tissue culture plates on glass coverslips at 37°C in a 5% CO\textsubscript{2} atmosphere for 48 h before infection. BeWo cells (0.85 × 10\textsuperscript{4} cells/well) were cultured in 24-well tissue culture plates on glass coverslips coated in 0.2% gelatin for 72 h before infection. The hepatocyte cell lines were infected.
with *L. monocytogenes* at an MOI of 20 and HUVECs at an MOI of 5; BeWo cells were infected with 10^6 bacteria/well. Infection of hepatocytes with *prfA* bacterial strain was performed at an MOI of 5 to avoid toxicity of LLO in *prfA* strains. Plates were centrifuged for 5 min (500 × g) at room temperature and incubated for 30 min at 37°C. Cells were washed three times with PBS, fixed with 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature, and blocked for 1 h in 0.1 M glycine and 10% HI-FBS in PBS, pH 7.4. Extracellular bacteria were labeled with anti-L. *monocytogenes* rabbit polyclonal antibodies (GeneTex) and with anti-rabbit secondary antibodies conjugated to Alexa Fluor 488 (Molecular Probes). Samples were permeabilized with 0.5% Triton X-100, and total (extracellular and intracellular) bacteria were labeled with anti-*L. monocytogenes* antibodies and secondary antibodies conjugated to Alexa Fluor 568 (Molecular Probes). Slides were mounted in ProLong gold antifade mountant containing DAPI (4',6-diamidino-2-phenylindole; Molecular Probes) to stain nuclei. To quantify the number of cells, images (phase contrast, DAPI, Alexa Fluor 488, and Alexa Fluor 568) were automatically acquired for each experimental condition using the 20× objective. MetaMorph analysis software was used to enumerate the total numbers of bacteria (N_b), extracellular bacteria (N_e), and mammalian cells (N_c) (61). The efficiency of bacterial internalization was calculated as follows: internalization = ([N_e] − [N_b]) / [N_c] × 100. The efficiency of bacterial association was calculated as follows: association = N_b / N_c. For each experimental condition, a minimum of 100 bacteria were counted (this applies to bacterial mutants with the lowest association efficiency) and a minimum of 150 mammalian cells (this applies to Hep3B, which are the largest cells and the cells with which *L. monocytogenes* associates the most effectively). The average numbers of WT bacteria and corresponding mammalian cells counted in each experiment are indicated in the figure legends. Because BeWo cells clustered in a fashion that made individual cell nuclei challenging to enumerate, we quantified the cell surface area by tracing plasma membrane outlines in MetaMorph and determined the surface area in μm^2. We then calculated the efficiency of bacterial association as follows: association = N_b / cell surface area (μm^2).

**Polystyrene bead coating with recombinant InlB and invasion assay.** Blue fluorescent carboxylate-modified latex beads (1-μm diameter; Molecular Probes) were covalently mixed with a recombinant InlB (5 mg/ml) and BSA (5 mg/ml) according to the manufacturer’s instructions. Control, BSA-coated beads were prepared with 10 mg/ml BSA under the same conditions. The beads were then washed three times with 0.33× PBS, pH 7.4, and stored at 4°C. To assess the capacity for InlB-coated beads to be ingested by hepatocytes, HepG2 cells were seeded in 24-well plates on cover glasses for 48 h, as described for bacterial invasion assays. Cells were washed with MEM, and InlB/BSA- or BSA-coated beads were added to the wells at an MOI of 5. Plates were centrifuged for 3 min at 500 × g and incubated for 30 min at 37°C in 5% CO₂. Cells were washed with PBS, fixed with 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature, and washed and blocked for 1 h in 0.1 M glycine and 5% blotting-grade blocker (Bio-Rad) in PBS, pH 7.4. Extracellular beads were labeled with rabbit anti-BSA antibodies (Sigma-Aldrich; B1520), followed by anti-rabbit secondary antibodies conjugated to Alexa Fluor 488 (Molecular Probes) to stain Alexa Fluor 488. Slides were mounted in ProLong gold antifade mountant containing DAPI to stain the nuclei. The percentage of intracellular beads was determined by fluorescence microscopy. The percentage of intracellular beads was calculated as the number of intracellular beads divided by the total number of beads, multiplied by 100.

**Live-cell imaging to assess hepatocyte response to InlB.** Hepatocytes were seeded (HepG2, 4 × 10^5 cells/dish; Hep3B, PLC5, and Huh7, 3 × 10^6 cells/dish) in 35-mm-diameter imaging dishes (Matek; P35G-1.5-10-C) and cultured at 37°C in 5% CO₂ for 48 h. Cells were placed on the 37°C microscope stage and analyzed using an automated stage, 20× Plan Neofluar (numerical aperture [NA] = 0.5), 40× Plan Neofluor (NA = 1.3), and 63× Plan Apochromat (NA = 1.4) objectives, a high-speed Xenon fluorescence emission device (Lambda DG-4, 300 W; Sutter Instrument Company), a Lambda 10-3 optical emission filter wheel for the fluorescence imaging, a SmartShutter to control the illumination for phase-contrast and DIC imaging (Sutter Instrument Company), a back-illuminated, frame-transfer electron-multiplying charge-coupled device (EMCCD) camera (Cascade II 512; Photometrics); and an ORCA-Flash 4.0 CMOS camera (Hamamatsu). The filter sets for fluorescence were purchased from Chroma Technology Corporation and were as follows: DAPI (49000), Alexa Fluor 488 (49002), Alexa Fluor 568 (49005), and Cy5 (49006). Images were acquired and analyzed using MetaMorph imaging software (Molecular Devices).
Statistical methods. All experimental work involved at least three biological replicates, each performed on different days. Data obtained each day include different treatment conditions, which are considered a cluster. Data within the same cluster are more correlated to each other than to data from clusters obtained on different days. Linear mixed-effects models were used to account for the correlation among observations from a same cluster. Linear mixed-effects models were used to analyze data from invasion assays (bacterial entry and association), studies of the interaction between invasion proteins, RT-qPCR, and quantitative Western blot analyses. For RT-qPCR and Western blot analyses, data were first normalized to internal controls or the loading standard to reduce standard deviation before analysis. Holm’s procedure was used to adjust for multiple comparisons such as comparisons of each L. monocytogenes deletion mutant to the WT. SAS 9.4 was used for all analyses (SAS Institute, Inc., NC). Although normalized data were presented in some figures for a clearer visualization of results, all statistical analyses were performed on raw data before normalization.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00555-18.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.
SUPPLEMENTAL FILE 2, AVI file, 16.6 MB.
SUPPLEMENTAL FILE 3, AVI file, 16.6 MB.
SUPPLEMENTAL FILE 4, AVI file, 16.6 MB.
SUPPLEMENTAL FILE 5, AVI file, 16.6 MB.

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