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inlA Premature Stop Codons Are Common among *Listeria monocytogenes* Isolates from Foods and Yield Virulence-Attenuated Strains That Confer Protection against Fully Virulent Strains▽†

K. K. Nightingale,[1,2]* R. A. Ivy,[1] A. J. Ho,[1] E. D. Fortes,[1] B. L. Njaa,[3,4] R. M. Peters,[3] and M. Wiedmann[1]

Department of Food Science, Cornell University, Ithaca, New York 14853[1]; Department of Animal Sciences, Colorado State University, Fort Collins, Colorado 80523[2]; Department of Biomedical Sciences, Cornell University, Ithaca, New York 14853[3]; and Department of Veterinary Pathology, Oklahoma State University, Stillwater, Oklahoma 74078[4]

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Previous studies showed that a considerable proportion of *Listeria monocytogenes* isolates obtained from foods carry a premature stop codon (PMSC) mutation in *inlA* that leads to production of a truncated and secreted InlA. To further elucidate the role these mutations play in virulence of *L. monocytogenes*, we created isogenic mutants, including (i) natural isolates where an *inlA* PMSC was reverted to a wild-type *inlA* allele (without a PMSC) and (ii) natural isolates where a PMSC mutation was introduced into a wild-type *inlA* allele; isogenic mutant sets were constructed to represent two distinct *inlA* PMSC mutations. Phenotypical and transcriptional analysis data showed that *inlA* PMSC mutations do not have a polar effect on the downstream *inlB*. Isogenic and natural strains carrying an *inlA* PMSC showed significantly reduced invasion efficiencies in Caco-2 and HepG2 cell lines as well as reduced virulence in oral guinea pig infections. Guinea pigs were also orally infected with a natural strain carrying the most common *inlA* PMSC mutation (vaccinated group), followed by challenge with a fully virulent *L. monocytogenes* strain 15 days postvaccination to probe potentially immunizing effects of exposure to *L. monocytogenes* with *inlA* PMSC mutations. Vaccinated guinea pigs showed reduced bacterial loads in internal organs and improved weight gain postchallenge, indicating reduced severity of infections in guinea pigs exposed to natural strains with *inlA* PMSC mutations. Our data support that (i) *inlA* PMSC mutations are causally associated with attenuated virulence in mammalian hosts and (ii) naturally occurring virulence-attenuated *L. monocytogenes* strains commonly found in food confer protective immunity.

Listeria monocytogenes is a human food-borne pathogen that can cause a severe invasive disease known as listeriosis in high-risk populations, including young children, the elderly, pregnant women, neonates, and other immunocompromised individuals (45). In 1999, Mead and coworkers estimated that approximately 2,500 cases of invasive listeriosis, including 500 deaths, occur each year in the United States alone (29). The infectious dose for *L. monocytogenes* appears to be high (49), and the current *L. monocytogenes* risk assessment by the U.S. Food and Drug Administration, USDA Food Safety and Inspection Service, and the Centers for Disease Control and Prevention (48) estimated that the average individual in the United States is frequently exposed to *L. monocytogenes* without developing clinical symptoms. Specifically, the risk assessment estimated that the average person in the United States consumes a food serving containing low ($<1 \times 10^3$ CFU) or intermediate (between 1×10^3 and 1×10^6 CFU) levels of *L. monocytogenes* 19 and 2.4 times per year, respectively. The risk assessment also projected that, on average, an individual consumes a food serving contaminated with high levels (between 1×10^6 and 1×10^9 CFU) of *L. monocytogenes* every 2 years and very high levels ($>1 \times 10^9$ CFU) of *L. monocytogenes* every 3 years (48).

Following a multilocus enzyme electrophoresis study published in 1989 (40), a number of phylogenetic and molecular subtyping studies demonstrated that *L. monocytogenes* isolates represent two major genetic lineages (lineages I and II) that include most natural isolates as well as at least one additional lineage (lineage III) that is uncommon (30, 32, 50, 52). Previous studies (16, 31, 50) also found that *L. monocytogenes* isolates grouping into lineage I, which predominantly contains serotypes 1/2b and 4b (designated as division I by Pifaretti et al. [40]), are significantly overrepresented among human clinical cases compared to their presence in foods. Lineage I also contains two highly clonal serotype 4b strains (epidemic clones I and II) that have been linked to multiple listeriosis epidemics worldwide (21) and also are significantly overrepresented among sporadic listeriosis cases (16). In contrast, several *L. monocytogenes* strains that group into lineage II (designated division II by Pifaretti et al. [40]), which is predominantly composed of serotypes 1/2a and 1/2c, are isolated from foods significantly more frequently than from human listeriosis cases (16).

Internalin A, encoded by *inlA*, is a bacterial cell wall-anchored protein that plays a critical role in *L. monocytogenes* virulence and, in particular, invasion of human intestinal epithelial cells (26). Interestingly, a number of different mutations leading to premature stop codons (PMSCs) in *inlA* have been observed among *L. monocytogenes* isolates collected from multiple countries, including the United States (33, 39), France (18, 20, 35, 36,

* Corresponding author. Mailing address: Department of Animal Sciences, Colorado State University, 108B Animal Sciences Building, Fort Collins, CO 80523-1171. Phone: (970) 491-1156. Fax: (970) 491-5326. E-mail: kendra.nightingale@colostate.edu.

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TABLE 1. Description of *L. monocytogenes* strains used for cell culture and animal infection experiments

Strain (previous name)	Isogenic mutant pair no.[a]	Isogenic mutant set no.[b]	Genotype or description	Reference
10403S	1	1	Wild-type laboratory control strain encoding a full-length InlA	Bishop et al. (3)
EGD-e			Laboratory control strain encoding a full-length InlA that carries a homologous 3′ *inlA* allelic type to ribotype DUP-1062A isolates with the exception of the mutation leading to *inlA* PMSC type 3	Glaser et al. (15)
FSL W3-084 (HEL-399)	1	1	Isogenic mutant where premature *inlA* stop codon mutation type 3 was introduced in a 10403S background	Nightingale et al. (33)
FSL K4-006			Δ*inlA* null mutant in a 10403S background	Kim et al. (22)
FSL K4-007			Δ*inlB* null mutant in a 10403S background	Kim et al. (22)
FSL K4-009			Δ*inlAB* null mutant in a 10403S background	Kim et al. (22)
FSL F2-515	2	1	Natural ribotype DUP-1062A isolate with premature *inlA* stop codon mutation type 3	Nightingale et al. (33)
FSL N4-730	2	1	Isogenic mutant where premature *inlA* stop codon mutation type 3 was reverted in a FSL F2-515 background	This paper
FSL F2-563	3	2	Natural ribotype DUP-1052A isolate with premature *inlA* stop codon mutation type 1	Nightingale et al. (33)
FSL N4-734	3	2	Isogenic mutant where premature *inlA* stop codon mutation type 1 was reverted in a FSL F2-563 background	This paper
FSL F2-245	4	2	Wildtype DUP-1052A isolate with *inlA* homologous to FSL F2-563 with the exception of premature *inlA* stop codon mutation type 1 and thus encoding a full-length InlA	Nightingale et al. (33)
FSL N4-733	4	2	Isogenic mutant where premature *inlA* stop codon mutation type 1 was introduced in a FSL F2-245 background	This paper

[a] Paired isogenic mutants that only differ by the presence or absence of each respective premature stop codon mutation in *inlA*.
[b] A set of four isogenic mutants assembled to evaluate the virulence of each respective premature stop codon in *inlA*. Each mutant set includes a natural isolate carrying a PMSC mutation and its revertant along with a wild-type strain encoding a full-length *inlA* and its paired mutant, where a given premature stop codon in *inlA* was introduced into its parent wild-type strain.

44), Portugal (12), and Japan (17). Most naturally occurring *inlA* PMSCs have been shown to result in the production of a truncated and secreted form of InlA (18, 20, 33), and at least one *inlA* PMSC located in the 5′ end of the gene abolishes InlA production (39). Studies in both France and the United States showed that *L. monocytogenes* isolates carrying *inlA* PMSCs constitute a significant proportion (>30%) of *L. monocytogenes* isolated from food but are only rarely isolated from human listeriosis cases (18, 33). *L. monocytogenes* isolates that carry *inlA* PMSCs also demonstrate an impaired ability to invade Caco-2 human intestinal epithelial cells (12, 33, 35, 36, 39, 44). While *inlA* PMSCs are found in *L. monocytogenes* isolates representing both lineages, they are most commonly found among lineage II serotype 1/2a and 1/2c isolates (18, 33), providing a possible explanation for the underrepresentation of lineage II isolates among human clinical cases. *L. monocytogenes* populations in food thus include both a considerable subpopulation of strains carrying PMSC mutations in *inlA* (18, 33) as well as a subpopulation of fully virulent strains, including epidemic clones and other subtypes commonly linked to human listeriosis cases (16, 21). Humans thus appear to be commonly exposed to both subpopulations through consumption of contaminated foods.

While one previous study used isogenic mutants to evaluate whether *inlA* PMSCs are solely responsible for virulence attenuation (34), only tissue culture and chicken embryo infection assays were used for phenotypic characterization and only a single *inlA* PMSC was evaluated. The initial goal of this study was thus to construct and characterize a number of isogenic *L. monocytogenes* mutants to determine (i) if *inlA* PMSC mutations alone are fully responsible for attenuated virulence, by using both human tissue culture cells as well as an oral infection route in guinea pigs (which express an isoform of the human InlA receptor E-cadherin that permits InlA binding), and (ii) if *inlA* PMSC mutations have a polar effect on expression of the downstream *inlB*, which encodes another internalin that plays a role in virulence (27). Since our initial data showed that *inlA* PMSC mutations are responsible for virulence attenuation and previous studies reported a common occurrence of these strains in the food supply (16, 18, 33), we used an oral guinea pig infection model to determine whether *L. monocytogenes* carrying an *inlA* PMSC can function as a natural vaccine by conferring protective immunity against subsequent exposure to fully virulent *L. monocytogenes*. The potential for *L. monocytogenes* strains carrying *inlA* PMSCs to function as a natural vaccine would suggest a complex epidemiology of human listeriosis, including regular vaccination through natural exposure to virulence-attenuated *L. monocytogenes* in foods.

MATERIALS AND METHODS

Bacterial isolates and strains. Two naturally occurring *L. monocytogenes* strains with an *inlA* PMSC, FSL F2-515 (ribotype DUP-1062A; *inlA* PMSC mutation type 3) and FSL F2-563 (ribotype DUP-1052A; *inlA* PMSC mutation type 1), were used in this study (Table 1). These specific *inlA* PMSC mutations were evaluated because

they represent the two most common strains (EcoRI ribotypes) known to carry these mutations among food isolates as well as both major *L. monocytogenes* genetic lineages (16, 33). EGD-e and FSL F2-245 were used as parent strains carrying an *inlA* allelic type that encodes a full-length InlA, as the 3′ *inlA* sequences for these isolates were identical (with the exception of each PMSC mutation) to FSL F2-515 and FSL F2-563, respectively (33) (Table 1).

Isogenic *inlA* mutants were constructed using a standard allelic exchange mutagenesis procedure (6) with the suicide shuttle vector pKSV7, which carries a temperature-sensitive *ori*R for *Listeria*. Sequences for all primers used to construct mutants are listed in Table S1 of the supplemental material. Mutants constructed included two revertant mutants, where the *inlA* PMSC allelic types in isolates FSL F2-515 and FSL F2-563 were replaced by otherwise-homologous *inlA* alleles (which encode a full-length InlA) from EGD-e and FSL F2-245, respectively (Table 1); this procedure generated isogenic mutants that differed only by the presence/absence of each respective PMSC. Additionally, we constructed two mutant strains in which we introduced the 3′ *inlA* sequences from FSL F2-563 (*inlA* PMSC mutation type 1) and FSL F2-515 (*inlA* PMSC mutation type 3) into isolates FSL F2-245 and EGD-e, respectively (Table 1); this approach yielded two strains that differed from their parent strains only by the presence of an *inlA* PMSC mutation. All *inlA* isogenic allelic types generated by allelic exchange mutagenesis were verified by sequencing the 3′ region of *inlA* as detailed previously (33). Our strategy yielded two sets of four strains each for evaluating the effects of *inlA* PMSC mutations types 1 and 3 on *L. monocytogenes* virulence; each set included a natural isolate with or without an *inlA* PMSC as well as two corresponding isogenic mutants that differed from their respective parent strains only by the presence of a single nucleotide polymorphism responsible for a given PMSC in *inlA*.

Bacterial growth conditions. Bacterial cultures for invasion assays were grown in brain heart infusion (BHI; Becton Dickson, Sparks, MD) broth under two conditions: (i) growth at 30°C without shaking to stationary phase as described previously (33) and (ii) growth at 37°C with shaking to early stationary phase as detailed previously (22). Bacterial cultures used for guinea pig infection experiments were grown to early stationary phase at 37°C with shaking and frozen at −80°C in phosphate-buffered saline (PBS) with 20% glycerol until use in animal infection experiments as previously described (14); bacterial viability was determined by plating appropriate serial dilutions on BHI agar prior to guinea pig infection experiments.

Cell culture invasion assays. Invasion assays were performed in both human intestinal epithelial Caco-2 and human hepatic HepG2 cell lines, as efficient invasion of both of these human cell lines requires internalin A (22). Internalin B plays a role in the invasion of HepG2 cells, which can be differentiated from InlA-mediated invasion when bacteria are grown at 37°C with shaking (22). Invasion assays were performed essentially as previously described (22, 33). Briefly, semiconfluent monolayers were inoculated with approximately 2×10^7 *L. monocytogenes* cells/well in triplicate wells, and the exact inoculum was determined by plating on BHI agar. Inoculated monolayers were incubated at 37°C for 30 min, followed by three washes with PBS to remove nonadherent bacteria and the addition of fresh medium without antibiotics. Medium containing 150 μg/ml gentamicin was added 45 min postinoculation to kill extracellular bacteria. At 90 min postinoculation, tissue culture cells were washed three times with PBS and lysed by addition of cold sterile deionized water with vigorous pipetting. Intracellular *L. monocytogenes* cells were enumerated by spread-plating appropriate dilutions of the lysed cell suspensions on BHI agar. The standard control strain *L. monocytogenes* 10403S (3) and uninoculated BHI broth were included as controls in each invasion assay. Three independent assays for each strain were performed in each of the two cell lines. Invasion efficiency was reported as the natural log of the percentage of initial inoculum recovered after enumeration of intracellular bacteria.

Statistical analysis of cell culture invasion assays. Natural log-transformed Caco-2 and HepG2 invasion efficiency data for *L. monocytogenes* grown at 30°C were not normally distributed due to the bimodal nature of invasion efficiency for *L. monocytogenes* strains with and without *inlA* PMSC mutations. The Wilcoxon rank sum test (a nonparametric approximation of the *t* test) was thus used to compare the differences in invasion efficiencies between *L. monocytogenes* strains with and without *inlA* PMSC mutations. Transformed invasion efficiency data for HepG2 invasion assays, where *L. monocytogenes* was grown at 37°C with shaking, were distributed normally and were compared by one-way analysis of variance using Tukey's studentized residuals to correct for multiple comparisons as implemented in the general linear model procedure in SAS (Statistical Analysis Systems Software, Cary, NC). *P* values of <0.05 were considered statistically significant.

qRT-PCR. To determine if PMSC mutations in *inlA* have a polar effect on *inlB*, quantitative reverse transcriptase PCR (qRT-PCR) was performed to determine transcript levels for *inlA* and *inlB* in paired isogenic *L. monocytogenes* strains with and without *inlA* PMSC mutation type 3 (i.e., strains 10403S, FSL W3-084, FSL F2-515, and FSL N4-7300) (Table 1). In addition to *inlA* and *inlB*, transcript levels were also determined for two housekeeping genes, *gap* and *rpoB*. Total RNA for qRT-PCR was collected from *L. monocytogenes* cells grown to early stationary phase at 37°C with aeration (shaking at 250 rpm) as previously described (46). RNA isolation was performed using RNA Protect and the RNeasy Midi kit (Qiagen) as previously described (46). RNA concentration and purity were determined by measuring absorbance with an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). qRT-PCR was performed using previously described TaqMan primers and probes for *inlA*, *inlB*, *gap*, and *rpoB* (28, 46), TaqMan One Step RT-PCR Master Mix, and the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) as detailed previously (46). Transcript levels for *inlA* and *inlB* were reported as normalized transcript levels relative to the geometric mean of the transcript levels for the housekeeping genes *gap* and *rpoB* (28).

Intragastric guinea pig infection. As humans and guinea pigs, but not mice or rats, express a form of E-cadherin that serves as a receptor for the *L. monocytogenes* internalin A protein (25), a guinea pig oral infection model (24) was used to evaluate the virulence of paired isogenic *L. monocytogenes* strains with and without a PMSC mutation in *inlA*. Animal infection experiments were conducted as approved by Cornell University Institutional Animal Care and Use Committee (protocol 2002-0060) and were performed essentially as described previously (14). Briefly, male juvenile (300-g) pathogen-free Hartley guinea pigs (purchased from Elm Hill, Chelmsford, MA) were housed in individual cages and acclimated for a minimum of 5 days prior to infection. Animals were fasted for 12 h prior to infection and then anesthetized with isoflurane. Anesthetized animals were gavaged using a rubber catheter (Viagon, Norristown, PA), which was used to deliver 1.5 ml of a calcium carbonate solution (125 g/liter) to buffer stomach pH, 1 ml of inoculum (containing 1×10^{10} log CFU/ml of a given *L. monocytogenes* strain suspended in PBS), followed by 1 ml of PBS. Animals were weighed daily after infection and were euthanized by CO_2 asphyxiation at 72 h postinfection.

Oral guinea pig infection experiments were also conducted to evaluate the potential of oral infection with a natural *L. monocytogenes* isolate carrying a PMSC mutation in *inlA* to confer protective immunity against subsequent challenge with a fully virulent *L. monocytogenes* strain. For these experiments, guinea pigs were either infected with 1×10^9 CFU of *L. monocytogenes* FSL F2-515, a natural lineage II serotype 1/2a isolate carrying *inlA* PMSC mutation type 3 (vaccinated), or received a mock infection with PBS (unvaccinated). Fifteen days later, vaccinated and unvaccinated animals were challenged orally with 1×10^{10} CFU of either the standard laboratory strain 10403S (lineage II serotype 1/2a) or the lineage I serotype 4b outbreak strain FSL J1-116 (13); these animals were also euthanized at 72 h postchallenge. FSL J1-116 was selected as a challenge strain because this strain belongs to ribotype DUP-1042B, a ribotype previously estimated to have a lower infectious dose than other subtypes (8).

Microbiological analysis of guinea pig organs. Infection of guinea pigs was evaluated by testing select organs (i.e., liver, spleen, mesenteric lymph nodes, and small intestine) to detect and quantify the presence of *L. monocytogenes* as previously described (14). Small intestine (ileum) samples were washed and treated with gentamicin (150 μg/ml) prior to homogenization and *L. monocytogenes* enumeration in order to eliminate extracellular microflora. Organs were homogenized in PBS using sterile blending units (Semi-micro; Eberback, Ann Arbor, MI), and bacterial numbers were determined by plating appropriate dilutions on BHI agar plates. Enrichment of organ homogenates (10 ml) in 90 ml of *Listeria* enrichment broth (Difco) was also performed (at 37°C for 48 h) to allow for detection of *L. monocytogenes* in samples having low bacterial numbers; enrichments were streaked onto Oxford (Oxoid, Hampshire, United Kingdom) plates after both 24 and 48 h of enrichment. A previously described PCR-restriction fragment length polymorphism assay (33) was used to characterize up to five colonies from each *L. monocytogenes*-positive organ for vaccinated animals to differentiate the vaccine strain with an *inlA* PMSC from the challenge strain.

Histopathology and immunohistochemistry. Histopathology was performed on selected tissues from infected guinea pigs as detailed previously (14). Tissue samples from the liver, mesenteric lymph nodes, small intestine, spleen, and brain of euthanized guinea pigs were fixed in 10% buffered formalin and subsequently processed and embedded in paraffin using Tissue Tek equipment (Sakura Finetek USA, Inc.). Paraffin-embedded tissues sectioned at 6-μm thickness were stained with hematoxylin and eosin for evaluation on an Olympus BX41 microscope. Pathologist-assigned numeric values ranged from 1 to 11 for each tissue, with 1 indicating no sign of infection and 11 indicating signs of severe infection (see Table S2 in the supplemental material).

The tissues detailed above were also used for immunohistochemical detection

of *L. monocytogenes*, performed as detailed previously (19) with minor modifications. Two slides were prepared for each tissue sample; one slide was stained with polyclonal anti-*L. monocytogenes* antibody (Becton Dickinson, Sparks MD), while the second slide was stained with a nonspecific antibody. Slides were then stained with a secondary anti-immunoglobulin G antibody; the avidin-biotin system was used to detect bound antibodies, and diaminobenzidine was used as the chromogenic substrate. Slides were either scored as 0 (i.e., no detectable *L. monocytogenes* antigen) or 1 (i.e., detectable *L. monocytogenes* antigen) by the pathologist (see Table S3 in the supplemental material). Samples for histopathology and immunohistochemistry were blinded (i.e., the pathologist was not informed of the treatment received by each animal prior to examination of tissues), and tissues from uninfected control animals were also submitted for analyses.

Serology. Serology for *L. monocytogenes* antibodies was performed on serum from blood samples collected from the saphenous vein immediately prior to infection with the vaccine strain and immediately following euthanization at 72 h postinfection. Serum was obtained from blood samples by centrifugation of blood samples at 2,700 × *g* for 5 min; serum was aspirated and stored at −80°C. Bacterial agglutination assays were performed essentially as described by Wesley et al. (51). To obtain antigens for the agglutination assay, *L. monocytogenes* 10403S was grown to log phase (optical density at 600 nm, 0.27) at 37°C with aeration (shaking at 250 rpm), and bacterial cells were collected by centrifugation. The cell pellet was washed with PBS, fixed with 10% buffered formalin at 25°C for 1 h, washed again with PBS, and resuspended in PBS with 1% bovine serum albumin (4). In each agglutination assay, rabbit anti-*L. monocytogenes* antiserum (Difco) and serum obtained from a negative control animal which was not infected with *L. monocytogenes* were included as positive and negative agglutination controls, respectively.

Statistical analysis of guinea pig infection experiments. For the oral guinea pig infection experiments, designed to evaluate the virulence of *L. monocytogenes* strains with and without *inlA* PMSC mutations, three animals were infected with each of the four *L. monocytogenes* strains evaluated. The recovery levels (in log CFU/g) of *L. monocytogenes* from organs (i.e., liver, spleen, mesenteric lymph nodes, and small intestine) were used as the main measure of virulence. *L. monocytogenes* numbers recovered were normally distributed, and statistical analyses were performed using a one-way analysis of variance (ANOVA), as implemented using the general linear model procedure in SAS (Statistical Analysis Systems Software, Cary, NC). Tukey's studentized range test was used to correct for multiple comparisons. Mean weights for guinea pigs infected with *L. monocytogenes* strains with and without a PMSC in *inlA* postinfection were also normally distributed, and final weights at 72 h postinfection were compared using a one-way ANOVA and Tukey's studentized range test.

For animal infection experiments conducted to evaluate the ability of *L. monocytogenes* strains carrying an *inlA* PMSC mutation to confer protective immunity, three animals were used in each of the four treatment groups, including (i) vaccinated animals (i.e., animals first infected with a strain carrying an *inlA* PMSC mutation) challenged with *L. monocytogenes* 10403S at 15 days postvaccination; (ii) vaccinated animals challenged with *L. monocytogenes* FSL J1-116; (iii) unvaccinated animals challenged with *L. monocytogenes* 10403S; and (iv) unvaccinated animals challenged with *L. monocytogenes* FSL J1-116. Statistical analyses were performed on *L. monocytogenes* numbers recovered in internal organs (i.e., liver, spleen, mesenteric lymph nodes, and small intestine) as well as animal weights, similar to the analyses described above. Analyses were performed separately for guinea pigs challenged with the two different strains, as the challenge strain had a significant effect on *L. monocytogenes* numbers recovered from the organs of unvaccinated animals. A number of organ samples were either (i) negative by direct plating but positive by enrichment or (ii) negative by both direct plating and enrichment. As a result, bacterial numbers for these samples were thus reported as either (i) the detection limit of the direct plating procedure or (ii) the detection limit of the enrichment procedure, respectively. Normal probability plots indicated these data did not fit a normal distribution, and a nonparametric approximation to the *t* test (Wilcoxon rank sum test) was thus used to compare *L. monocytogenes* numbers recovered from each organ of vaccinated and unvaccinated guinea pigs. Final animal weights at 72 h postchallenge for vaccinated and unvaccinated animals were compared using a one-way ANOVA with Tukey's correction. Histopathology and immunohistochemistry data were also analyzed for differences among vaccinated and unvaccinated animals. Histopathological observations were treated as a continuous dependent response variable and were analyzed by one-way ANOVA, while immunohistochemistry results were subjected to a chi-square test of independence. In all cases, P values of <0.05 were considered statistically significant.

RESULTS

***L. monocytogenes* strains with PMSC mutations in *inlA* show reduced invasion efficiency in Caco-2 and HepG2 cells.** When *L. monocytogenes* strains were grown to stationary phase at 30°C (without shaking), median natural log-transformed Caco-2 cell invasion efficiencies of *L. monocytogenes* strains that encode a full-length InlA ranged from 0.52 to 0.89, whereas median log-transformed invasion efficiencies of strains carrying a *inlA* PMSC mutation ranged from −3.60 to −4.24 ($P < 0.0001$; Wilcoxon rank sum test) (Fig. 1a). Since we created two sets of paired mutants each containing four isogenic strains (one set each for *inlA* PMSC mutation 1 and 3), we were also able to evaluate the specific effects of each *inlA* PMSC on Caco-2 invasion efficiency. Both a natural strain with *inlA* PMSC mutation type 3 (FSL F2-515) and the isogenic strain where PMSC mutation type 3 was introduced in wild-type strain 10403S (i.e., FSL W3-084) showed similar low invasion efficiencies (−3.87 and −3.97, respectively), compared to the parent strain 10403S and FSL N4-730, an isogenic mutant of FSL F2-515 where PMSC type 3 was reverted to encode a full-length InlA (0.52 and 0.63 invasion efficiencies, respectively). Similarly, both a natural strain with PMSC mutation type 1 (FSL F2-563) and strain FSL N4-733, an isogenic mutant created by inserting PMSC type 1 into FSL F2-245, showed similar low invasion efficiencies (−4.24 and −3.60, respectively) compared to the parent strain FSL F2-245 (which encodes full-length InlA) and FSL N4-734, an isogenic mutant of FSL F2-563 where PMSC type 1 was reverted to encode a full-length InlA (0.88 and 0.89 invasion efficiency, respectively).

When invasion assays with the two sets of isogenic mutants described above were performed in the human hepatocyte cell line HepG2 (using bacteria grown at 30°C without aeration), results similar to those obtained in Caco-2 cells were observed (Fig. 1b). Median natural log-transformed HepG2 invasion efficiencies for *L. monocytogenes* strains encoding a full-length InlA ranged from −0.49 to −1.65, whereas median invasion efficiencies of strains carrying an *inlA* PMSC mutation ranged from −4.43 to −5.81 ($P < 0.001$; Wilcoxon rank sum test). As observed in Caco-2 invasion assays, natural strains with *inlA* PMSC mutation types 1 or 3 and strains where these PMSC types were introduced into a parent strain with full-length InlA showed similar low invasion efficiencies (Fig. 1b). Furthermore, strains where *inlA* PMSC mutation types 1 and 3 were reverted to *inlA* alleles that encode a full-length InlA showed significantly increased invasion efficiencies, which did not differ from those for wild-type strains encoding a full-length InlA. These data suggest that *inlA* PMSC mutations types 1 and 3 are fully responsible for attenuated invasion efficiency for both Caco-2 and HepG2 cells.

***inlA* premature stop codons do not have a polar effect on *inlB* expression.** Nonsense mutations can have a strong polar effect on downstream genes located in the same operon (9). Since *inlA* and *inlB* are organized in an operon that can be transcribed bicistronically and *inlB* is downstream of *inlA* (27), we surmised that the presence of a PMSC in *inlA* may have a polar effect on expression of *inlB*. This hypothesis was tested using both transcriptional profiling of *inlA* and *inlB* and a phenotypic assay to assess InlB functionality (i.e., an invasion

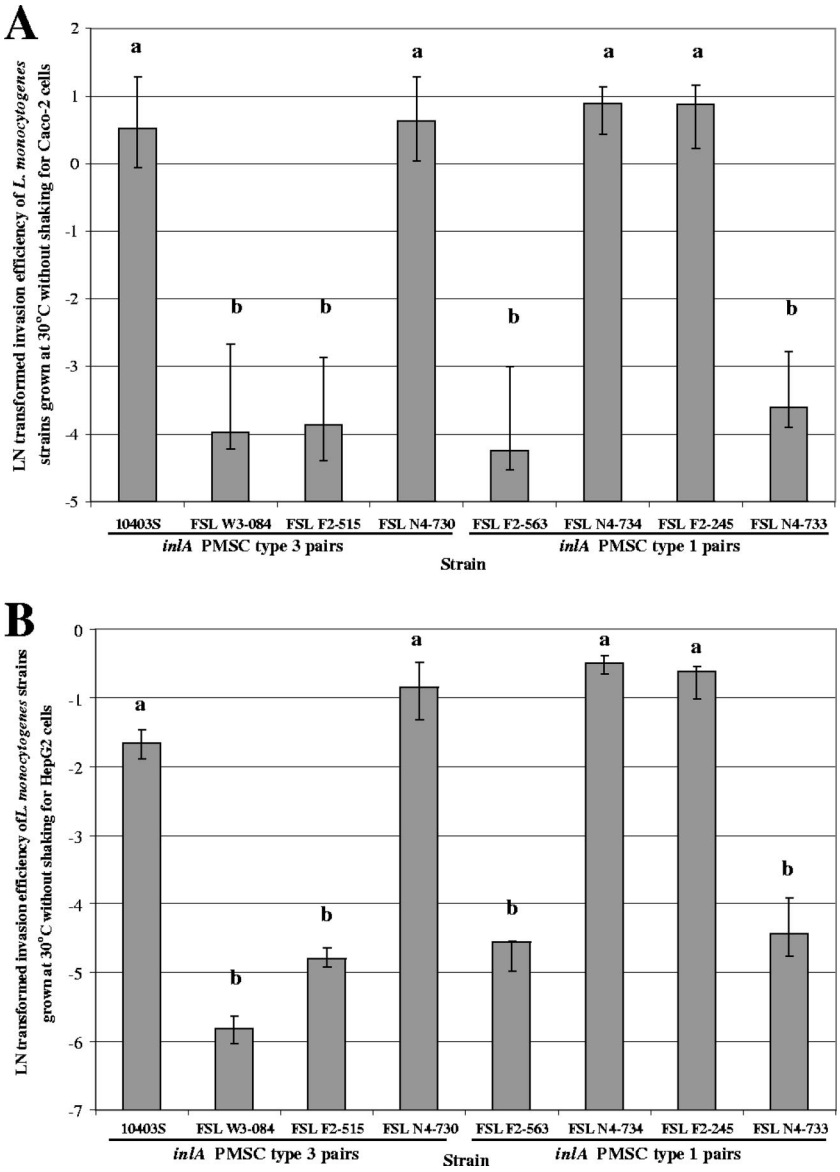

FIG. 1. Natural log (LN)-transformed Caco-2 (A) and HepG-2 (B) cell invasion efficiencies for paired isogenic *L. monocytogenes* strains with and without a PMSC in *inlA* that were grown at 30°C without shaking. The two sets of strains used to evaluate the effects of the most common *inlA* PMSC mutations on invasion are indicated on the *x* axis. Strains in the set representing *inlA* PMSC mutation type 3 include wild-type *L. monocytogenes* strain 10403S encoding a full-length InlA, an isogenic mutant where *inlA* PMSC mutation type 3 was introduced into a 10403S background (FSL W3-084), a natural isolate carrying *inlA* PMSC mutation type 3 (FSL F2-515), and its paired revertant mutant (FSL N4-730) encoding a full-length InlA. Strains included in the set representing *inlA* PMSC mutation type 1 include a natural isolate carrying *inlA* PMSC type 1 (FSL F2-563), its paired revertant mutant (FSL N4-734) encoding a full-length InlA, a wild-type isolate encoding a full-length InlA that belongs to the ribotype associated with PMSC mutation type 1 (FSL F2-245), and an isogenic mutant where PMSC mutation type 1 was introduced in an FSL F2-245 background (FSL N4-733). The natural log-transformed invasion efficiency of each *L. monocytogenes* strain is indicated on the *y* axis. Each strain was assayed in triplicate in each independent experiment, and three independent invasion experiments were performed for each strain. Columns represent median invasion efficiencies (data did not fit a normal distribution), and error bars indicate the minimum and maximum invasion efficiencies observed for each strain. Different letters denote strains with significantly different invasion efficiencies at the $P < 0.05$ level. A Wilcoxon rank sum test showed that naturally occurring and isogenic *L. monocytogenes* strains carrying a PMSC in *inlA* demonstrated significantly reduced invasion efficiencies in Caco-2 ($P < 0.0001$) and HepG-2 ($P < 0.001$) cells compared to wild-type and revertant mutant strains carrying an *inlA* that encodes a full-length InlA.

assay in HepG2 cells). Determination of *inlA* and *inlB* transcript levels using qRT-PCR for (i) wild-type *L. monocytogenes* strain 10403S, (ii) an isogenic strain that carries *inlA* PMSC mutation type 3 in a 10403S background (FSL W3-084), (iii) a natural isolate with *inlA* PMSC mutation type 3 (FSL F2-515), and (iv) an isogenic mutant of FSL F2-515 where *inlA* PMSC mutation type 3 was reverted to encode a full-length InlA (strain FSL N4-730) showed similar *inlA* and *inlB* transcript levels. Specifically, normalized log-transformed *inlA* transcript levels for 10403S, FSL W3-084, FSL F2-515, and FSL N4-740

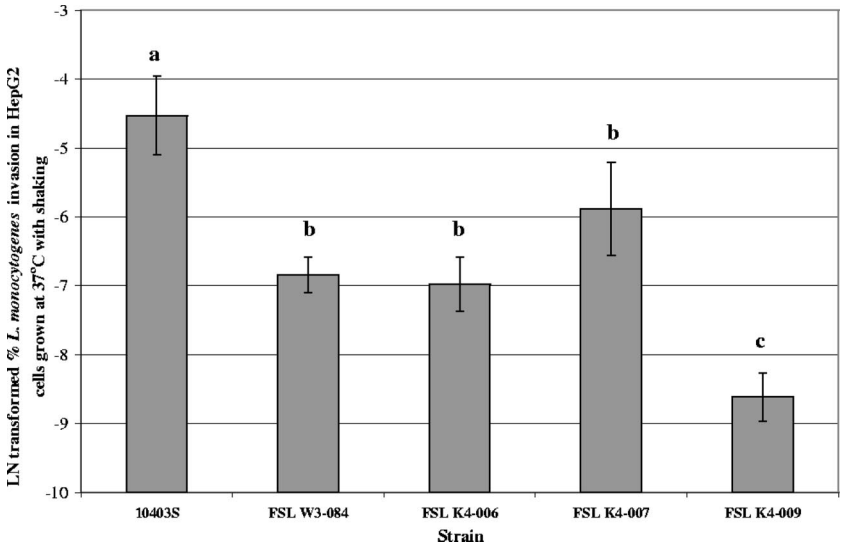

FIG. 2. Natural log (LN)-transformed HepG2 cell invasion efficiencies for paired isogenic *L. monocytogenes* strains with and without a PMSC in *inlA* and null mutant control strains in a laboratory control strain (10403S) background. Bacteria were grown at 37°C with shaking, a condition shown to differentiate InlB-mediated invasion from InlA-mediated invasion in the cell line (22). Strains evaluated are indicated on the *x* axis and include wild-type *L. monocytogenes* strain 10403S encoding a full-length InlA, an isogenic mutant in which we introduced *inlA* PMSC mutation type 3 into a 10403S background (FSL W3-084), and three control null mutant strains in a 10403S background, including Δ*inlA* (FSL K4-006), Δ*inlB* (FSL K4-007), and Δ*inlAB* (FSL K4-009). Invasion efficiency for each *L. monocytogenes* strain is indicated on the *y* axis. Each strain was assayed in triplicate in each experiment, and three independent invasion assays were performed for each strain. Columns represent natural log-transformed mean invasion efficiencies, and error bars indicate the standard deviations around the mean invasion efficiency observed for each strain. Transformed invasion efficiencies were analyzed by one-way analysis of variance, and different letters denote strains with significantly different invasion efficiencies at the $P < 0.05$ level. The isogenic strain where PMSC mutation type 3 was introduced into a 10403S background (FSL W3-084) showed significantly greater invasion efficiency in HepG2 cells compared to Δ*inlAB* (FSL K4-009) in the same background, supporting that PMSC mutations in *inlA* do not have a polar affect on InlB-mediated invasion of human hepatic cells.

were −0.67, −0.51, −0.79, and −0.77, respectively, while *inlB* transcript levels for 10403S, FSL W3-084, FSL F2-515, and FSL N4-730 were −0.84, −0.79, −1.05, and −0.90, respectively (based on two independent experiments). Overall, these data support that (i) the presence of a PMSC mutation in *inlA* does not affect transcription of *inlA* or *inlB*, since transcript levels of both *inlA* and *inlB* were similar for isogenic pairs with and without a PMSC mutation in *inlA*, and (ii) that *inlA* PMSCs do not have a polar effect on *inlB* transcription.

HepG2 invasion assays were performed essentially the same, using *L. monocytogenes* strains grown at 37°C with shaking, as previous work showed that InlB-mediated invasion of HepG2 cells for *L. monocytogenes* grown under these conditions can be differentiated from InlA-mediated invasion (22). HepG2 invasion assays were performed using a specific set of isogenic *L. monocytogenes* strains in a 10403S background, including Δ*inlA* (FSL K4-006), Δ*inlB* (FSL K4-007), Δ*inlAB* (FSL K4-009), and a 10403S strain where *inlA* PMSC mutation type 3 was introduced (FSL W3-084). In these assays, the strain carrying an *inlA* PMSC mutation in a 10403S background and the Δ*inlA* strain showed similar ($P > 0.05$) invasion efficiencies (−6.84 and −6.97, respectively), indicating that an *inlA* PMSC mutation has the same effect on HepG2 invasion efficiency as an *inlA* null mutation. Consistent with our previous study (22), the Δ*inlAB* strain showed lower invasion efficiency than the Δ*inlA* strain ($P = 0.01$) (Fig. 2); in addition, the isogenic mutant with the *inlA* PMSC (FSL-W3-084) showed higher HepG2 invasion efficiency than the Δ*inlAB* strain ($P = 0.007$). Overall, these data suggest that a PMSC in *inlA* does not have a polar effect on *inlB*, as such an effect would be expected to lead to lower invasion efficiency for the isogenic strain with the *inlA* PMSC than seen for the Δ*inlA* strain and an invasion efficiency similar to the Δ*inlAB* strain.

***L. monocytogenes* strains with *inlA* PMSC mutation type 3 show reduced virulence in an oral guinea pig infection model.** As *inlA* is critical for invasion of intestinal epithelial cells in humans as well as guinea pigs, we evaluated the virulence of both natural and isogenic strains with *inlA* PMSC mutation type 3 in an oral guinea pig infection model. Both FSL F2-515, a natural strain with PMSC mutation type 3, and FSL W3-084, an isogenic strain where PMSC mutation type 3 was introduced in the wild-type strain 10403S, showed significantly ($P < 0.05$) lower *L. monocytogenes* numbers in liver (Fig. 3a) and small intestine (Fig. 3c) compared to their corresponding isogenic strains encoding a full-length InlA (i.e., 10403S and FSL N4-730, respectively). Overall, lower *L. monocytogenes* numbers were recovered from the spleens and mesenteric lymph nodes (Fig. 3b and d) of animals infected with a strain carrying a PMSC in *inlA*, even though only *L. monocytogenes* counts for the natural isolate with *inlA* PMSC mutation type 3 (i.e., FSL F2-515) were significantly ($P < 0.05$) lower compared to the isogenic revertant mutant of FSL F2-515 (strain FSL N4-730).

Postinfection weight changes in guinea pigs infected with natural and isogenic strains with *inlA* PMSC mutation type 3 also supported that strains carrying a mutation leading to a PMSC in *inlA* show reduced virulence. While all animals gained weight 24 h postinfection (Fig. 4), only uninfected (negative control) animals continued to gain weight at 48 and 72 h

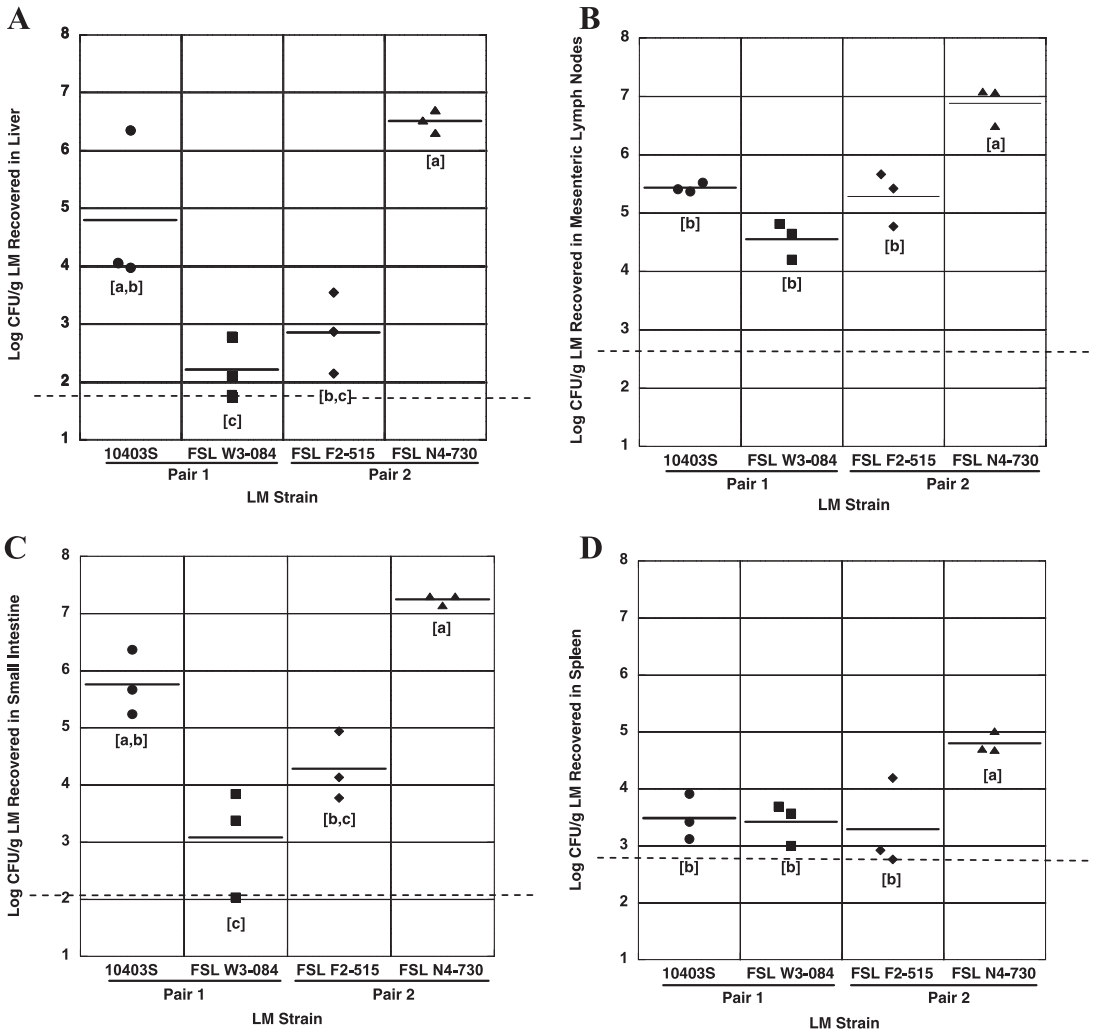

FIG. 3. Dot plot of *L. monocytogenes* populations recovered from organs of guinea pigs orally infected with paired isogenic strains with or without PMSC type 3 in *inlA* at 72 h postinfection. *L. monocytogenes* strains evaluated are indicated on the *x* axis and include the following: the wild-type standard laboratory control strain 10403S encoding a full-length InlA (●); FSL W3-083, an isogenic mutant of parent strain 10403S carrying *inlA* PMSC mutation type 3 (■); FSL F2-515, a natural isolate carrying *inlA* PMSC mutation type 3 (◆); and FSL N4-730, an isogenic mutant of FSL F2-515, where *inlA* PMSC mutation type 3 was reverted to encode a full-length InlA (▲). The *y* axis denotes *L. monocytogenes* populations (in log CFU/g) recovered from the liver (A), mesenteric lymph nodes (B), small intestine (C), and spleen (D) of three guinea pigs infected with each of the four strains evaluated. Mean *L. monocytogenes* populations observed for animals infected with each strain are represented by each of the four short solid horizontal lines in each panel. The limit of *L. monocytogenes* detection by direct plating for each organ (based on the average weight observed for each organ) is denoted by the dashed horizontal line near the bottom of each panel. For organs in which *L. monocytogenes* was not detected by direct plating but that were positive for *L. monocytogenes* after selective enrichment, these data were reported at the detection limit for plating. Overall, one-way analysis of variance indicated a highly significant effect by strain ($P < 0.0001$) but not animal or day when experiments were performed ($P > 0.05$). Different letters indicate significance at the $P < 0.05$ level with respect to *L. monocytogenes* populations recovered from each organ.

postinfection. Animals infected with *L. monocytogenes* strains encoding a full-length InlA (i.e., 10403S and FSL N4-730) showed the greatest weight loss, while animals infected with *L. monocytogenes* strains carrying an *inlA* PMSC showed reduced (FSL F2-515) or limited (FSL W3-084) weight loss (Fig. 4). Specifically, animals infected with FSL W3-084 (an isogenic mutant generated from 10403S by introducing *inlA* PMSC type 3) showed weight gain at 72 h postinfection that differed significantly ($P < 0.05$) from that observed for animals infected with the parent strain 10403S, which showed considerable weight loss at 72 h postinfection (>10%) (Fig. 4). Overall, our data support that *L. monocytogenes* carrying *inlA* PMSC mutation type 3 shows reduced virulence and that the single nucleotide polymorphism that causes *inlA* PMSC type 3 is specifically responsible for virulence attenuation.

Exposure of guinea pigs to *L. monocytogenes* with an *inlA* PMSC mutation protects against subsequent challenge with fully virulent *L. monocytogenes*. As previous studies showed that *L. monocytogenes* isolates with *inlA* PMSC mutation type 3 represent a considerable proportion of *L. monocytogenes* isolated from ready-to-eat foods in the United States (16, 33), we conducted initial experiments to determine whether oral

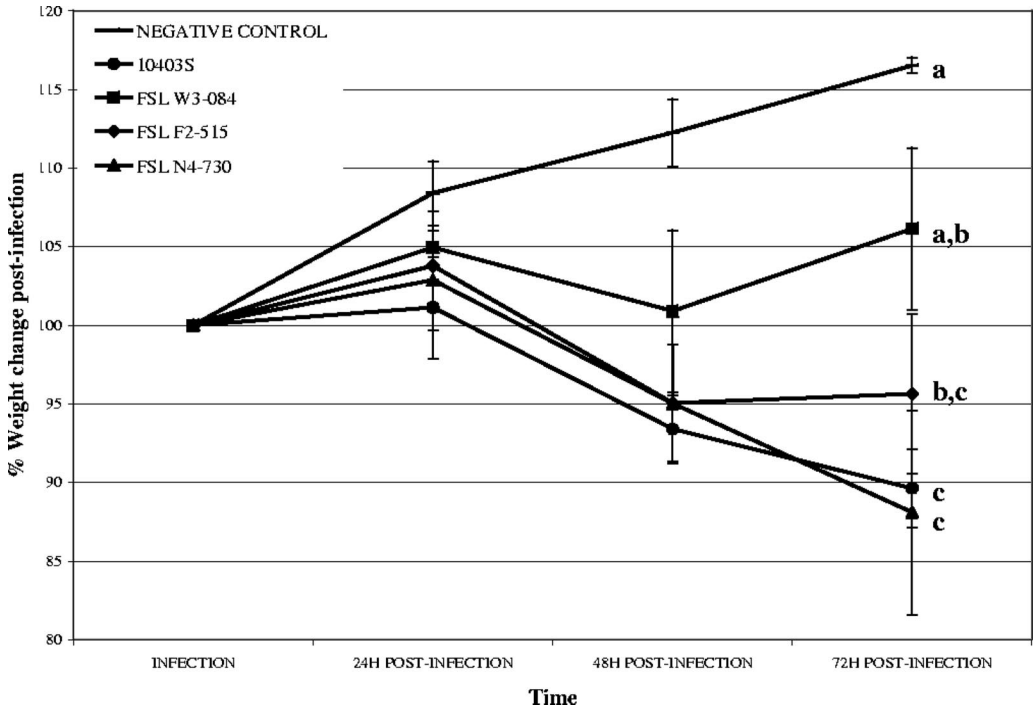

FIG. 4. Postinfection weight changes for guinea pigs orally infected with paired isogenic *L. monocytogenes* strains with and without PMSC mutation type 3 in *inlA*. *L. monocytogenes* strains evaluated included wild-type 10403S encoding a full-length InlA; isogenic mutant FSL W3-083, where *inlA* PMSC mutation type 3 was introduced into a 10403S background; a natural isolate, FSL F2-515, carrying PMSC type 3 in *inlA*; and its paired revertant mutant, FSL N4-730, where PMSC mutation type 3 was reverted in a FSL F2-515 background. Weight change was also monitored for negative control animals, which were only challenged with the carrier solution (phosphate-buffered saline) at the time of infection. The *x* axis indicates time postinfection, and the *y* axis shows the percent weight change postinfection. Lines indicate mean percent weight change for three animals infected with each of the four strains described above, and error bars show the standard deviations around mean weight changes for each time point. Different letters at the right end of the graph indicate significant differences at the $P < 0.05$ level with respect to weight change in animals infected with different strains at 72 h postinfection based on results from a one-way analysis of variance.

exposure to a natural isolate with an *inlA* PMSC can confer protection against subsequent challenge with fully virulent *L. monocytogenes* strains. Briefly, guinea pigs were first infected intragastrically with 1×10^9 CFU of a natural isolate carrying *inlA* PMSC mutation type 3 (vaccinated), while unvaccinated control animals were orally administered only the carrier fluid (phosphate-buffered saline). At 15 days postvaccination, both vaccinated and unvaccinated control animals were orally infected with 1×10^{10} CFU of either FSL J1-116 (serotype 4b; human isolate from the 1988 to 1990 listeriosis outbreak in the United Kingdom [13]) or 10403S (serotype 1/2a [3]). Up to five *L. monocytogenes* colonies recovered from each organ of vaccinated animals after challenge with a virulent *L. monocytogenes* strain (i.e., 10403S or FSL J1-116) were characterized in a PCR-restriction fragment length polymorphism assay to determine whether the *L. monocytogenes* recovered represented the vaccine strain (with the *inlA* PMSC) or the challenge strain (see Table S2 in the supplemental material). Only the vaccine strain (five out of five colonies tested) was recovered from the livers of two vaccinated animals challenged with 10403S; in addition, the mesenteric lymph nodes of one animal contained a mixed culture (see Table S2 in the supplemental material). Among animals challenged with FSL J1-116, only the vaccine strain (five out of five colonies tested) was recovered from the spleen of two animals. All other organs either yielded only the challenge strain or were negative for *L. monocytogenes*. For statistical analyses, organs where only the vaccine strain was recovered were considered negative for the challenge strain, and counts observed for the organ infected with both strains were reported as the challenge strain.

Overall, vaccinated guinea pigs consistently showed lower *L. monocytogenes* numbers in organs compared to unvaccinated animals (Fig. 5). For example, while the *L. monocytogenes* challenge strain was recovered from the spleens of all unvaccinated animals, the challenge strain was not recovered from the spleens of any vaccinated animals. The most striking difference was observed for the small intestine, where five of six vaccinated animals were negative for *L. monocytogenes* and one was only positive after enrichment, while unvaccinated animals showed *L. monocytogenes* numbers between 10^4 and 10^5 CFU/g. An overall Wilcoxon rank sum test showed that vaccination status and challenge strain affected *L. monocytogenes* numbers recovered from organs; only vaccination status significantly affected *L. monocytogenes* numbers recovered, though, when the data for each of the four organs were analyzed separately. For animals challenged with *L. monocytogenes* 10403S, challenge strain numbers recovered from each of the four organs analyzed were significantly lower ($P < 0.05$) in vaccinated animals compared to numbers recovered from unvaccinated animals (Fig. 5). For animals challenged with *L. monocytogenes* FSL J1-116, challenge strain numbers recovered from mesenteric lymph nodes, small intestines, and

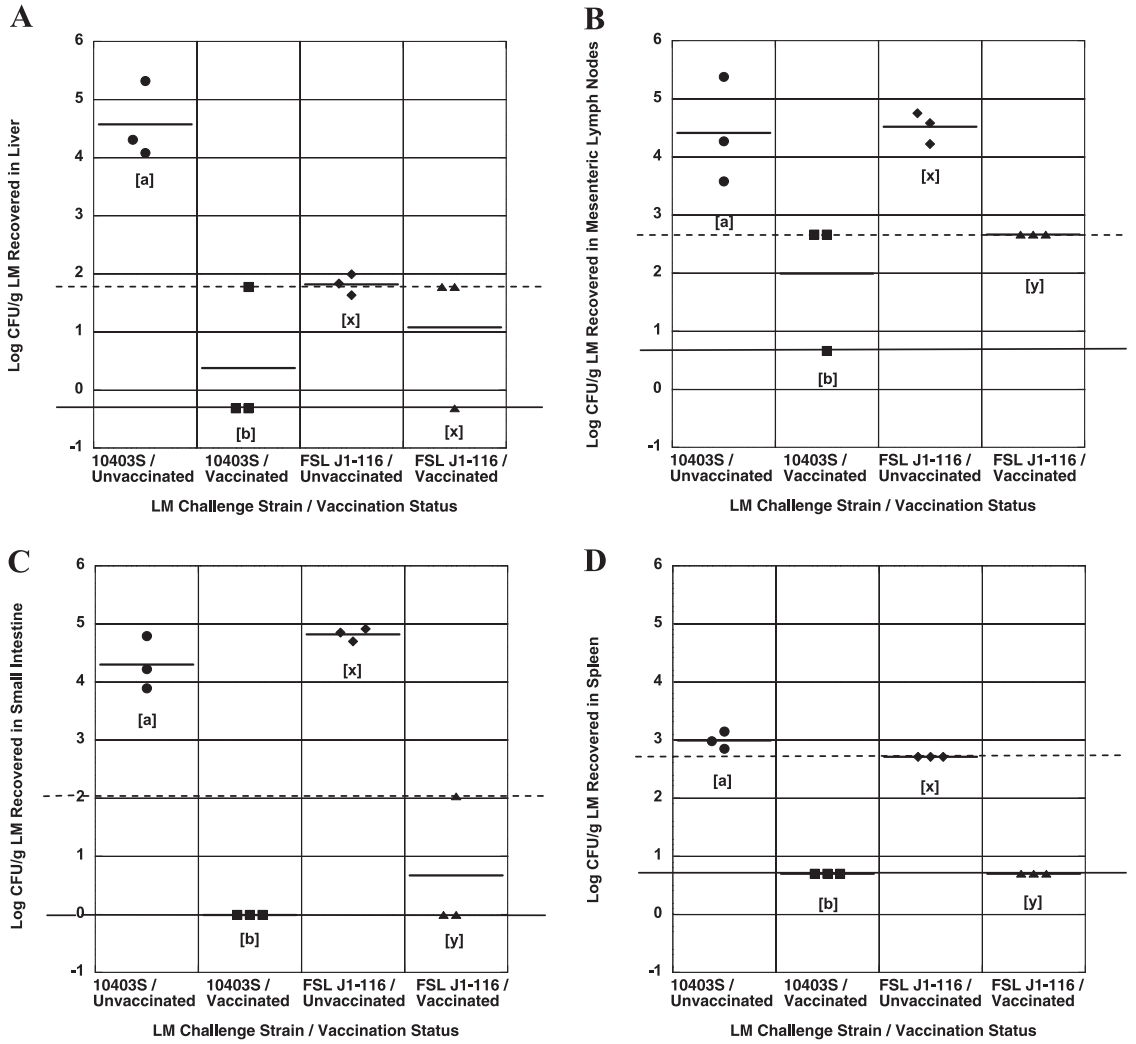

FIG. 5. Dot plot of *L. monocytogenes* recovered from organs of vaccinated and unvaccinated guinea pigs. Vaccinated animals were first orally infected with a serotype 1/2a virulence-attenuated *L. monocytogenes* strain carrying *inlA* premature stop codon mutation type 3 (FSL F2-515), while unvaccinated control animals were infected with only the carrier solution (phosphate-buffered saline) at the time of vaccination. Both vaccinated and unvaccinated animals were subsequently challenged by infection with a fully virulent *L. monocytogenes* strain encoding a full-length InlA 15 days later. The *x* axis indicates treatment groups, including unvaccinated animals challenged by the wild-type serotype 1/2a laboratory control strain 10403S (●), vaccinated animals challenged with 10403S (■), unvaccinated animals challenged with FSL J1-116, a serotype 4b strain from a listeriosis outbreak (◆), and vaccinated animals challenged with FSL J1-116 (▲). The *y* axis shows *L. monocytogenes* challenge strain populations (in log CFU/g) recovered from the liver (A), mesenteric lymph nodes (B), small intestine (C), and spleen (D) of three guinea pigs, representing each of the four treatments. Short solid horizontal bars indicate the mean *L. monocytogenes* population observed for animals representing each treatment. The limit for *L. monocytogenes* detection by direct plating (based on average weights for each organ) is denoted by the dashed horizontal line crossing through each panel. The limit for detection of *L. monocytogenes* by selective enrichment (based on the average weight of each organ used for enrichment) is denoted by the solid horizontal line crossing through each panel. For organs where *L. monocytogenes* was detected by enrichment but not direct plating, data were reported at the detection limit for direct plating. For organs where *L. monocytogenes* was not detected by direct plating or enrichment, data were reported at the detection limit for enrichment. Wilcoxon's rank sum test was used to compare *L. monocytogenes* populations from vaccinated and unvaccinated animals, and animals challenged with 10403S and FSL J1-116 were analyzed separately. Different letters in brackets under the data indicate strains that differed significantly at the $P < 0.05$ level with respect to *L. monocytogenes* populations recovered from each organ.

spleens were significantly lower ($P < 0.05$) in vaccinated animals compared to unvaccinated animals (Fig. 5). Immunohistochemical detection of *L. monocytogenes* antigens (see Table S3 in the supplemental material) also supported that vaccinated animals showed lower *L. monocytogenes* loads in the liver, mesenteric lymph nodes, spleen, small intestine, and brain compared to vaccinated animals. A chi-square test showed that tissues with detectable *L. monocytogenes* antigens were overall significantly more common ($P < 0.01$) among organs from unvaccinated animals than from vaccinated animals.

Both vaccinated and unvaccinated guinea pigs continued to gain weight during the 15 days postvaccination (see Fig. S1 in the supplemental material) and showed similar weights at 15 days postvaccination ($P > 0.05$). While vaccinated guinea pigs showed continued weight gain after oral challenge with either

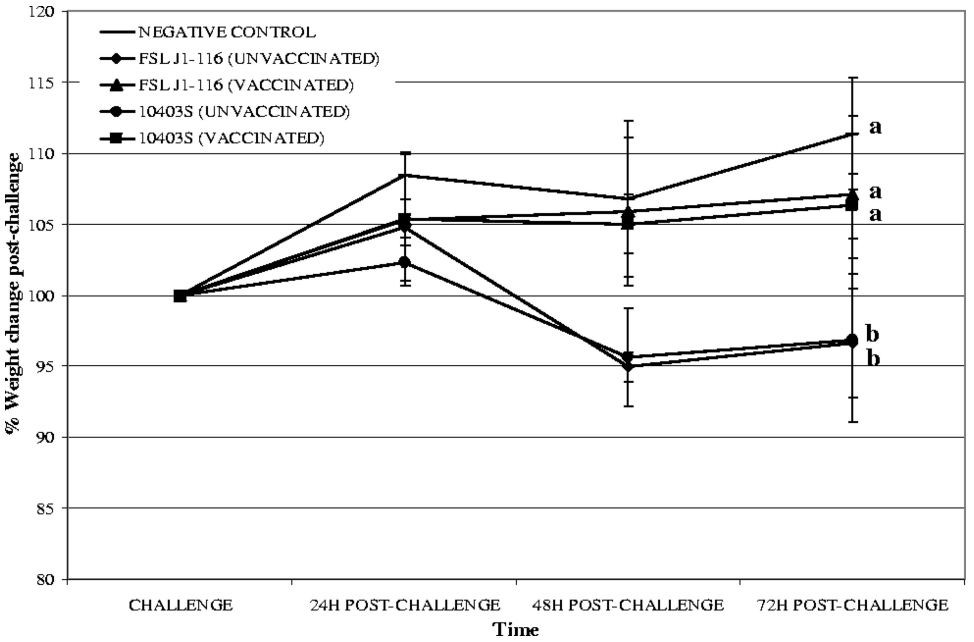

FIG. 6. Postchallenge weight changes for vaccinated and unvaccinated guinea pigs. Vaccinated animals were first orally infected with a serotype 1/2a virulence-attenuated *L. monocytogenes* strain carrying premature *inlA* stop codon mutation type 3 (FSL F2-515) and subsequently challenged with a fully virulent strain 15 days postvaccination, while unvaccinated control animals were only challenged with the carrier solution (phosphate-buffered saline) at the time of vaccination followed by subsequent challenge with a fully virulent strain. Animal treatment groups include unvaccinated animals challenged with serotype 1/2a wild-type laboratory control strain 10403S encoding a full-length InlA, vaccinated animals challenged with 10403S, unvaccinated animals challenged with FSL J1-116, a serotype 4b strain from a listeriosis outbreak, and vaccinated animals challenged with FSL J1-116. Weight change was also monitored for negative control animals, which were gavaged with the carrier solution (phosphate-buffered saline) at the time of vaccination and challenge. The *x* axis shows time postinfection and the *y* axis shows percent weight change postchallenge. Lines indicate mean weight changes for three animals infected with each strain, and error bars show the standard deviations around mean weight changes for each time point. Different letters on the right side of the graph indicate strains that differed significantly at the $P < 0.05$ level with respect to weight change in animals at 72 h postinfection based on results from a one-way analysis of variance.

of the two fully virulent *L. monocytogenes* strains, unvaccinated animals showed clear weight loss. Weight gain (at 72 h after challenge) for vaccinated animals did not differ significantly from uninfected negative control animals ($P > 0.05$), while unvaccinated guinea pigs showed significant weight loss at 72 h postchallenge ($P < 0.05$) compared to negative control and vaccinated animals (Fig. 6), providing further support for a reduced severity of infection in vaccinated animals.

Blinded histopathology data showed a difference in severity of infection signs between vaccinated and unvaccinated guinea pigs challenged with fully virulent *L. monocytogenes*. Quantitative histopathology data on liver, mesenteric lymph node, spleen, small intestine, and brain tissue samples from vaccinated and unvaccinated guinea pigs challenged with fully virulent *L. monocytogenes* (see Table S4 in the supplemental material) showed a significant difference in the severity of infection among vaccinated and unvaccinated guinea pigs ($P = 0.034$; one-sided nonparametric *t* test approximation). Interestingly, all six vaccinated animals evaluated showed signs of inflammation in the small intestine, while only one of the six unvaccinated animals evaluated showed signs of inflammation in the small intestine. This suggests an enhanced local mucosal immune response in vaccinated animals upon challenge with a virulent *L. monocytogenes*.

Guinea pigs exposed to *L. monocytogenes* with an *inlA* PMSC mutation as well as guinea pigs challenged with fully virulent *L. monocytogenes* show no evidence for seroconversion. Antibodies against *L. monocytogenes* were not detected in any of the serum samples analyzed, including (i) those collected from all animals immediately prior to vaccination with a *L. monocytogenes* strain carrying an *inlA* PMSC and (ii) those from all animals immediately following euthanization at 72 h postchallenge. These results are consistent with another previous study that did not detect anti-*Listeria* antibodies in pregnant guinea pigs infected with a fully virulent *L. monocytogenes* strain unless a traumatic event such as a stillbirth occurred (53).

DISCUSSION

To date, several distinct mutations leading to PMSCs in the key virulence gene *inlA* have been observed in *L. monocytogenes* isolates from France (18, 20, 35, 36, 44), the United States (33, 39), Portugal (12), and Japan (17). Furthermore, characterizations of large *L. monocytogenes* collections from the United States (16, 33) and France (18) showed that >30% of *L. monocytogenes* isolates from food products in these countries carry *inlA* PMSCs. To probe the causal relationship between PMSC mutations in *inlA* and virulence, we created and characterized paired isogenic *L. monocytogenes* strains with and without *inlA* PMSCs. As our initial data clearly showed that *L. monocytogenes* isolates with *inlA* PMSC mutations have reduced virulence, we also explored the potential of a naturally occurring *L. monocytogenes* isolate with an *inlA* PMSC to induce protection against subsequent challenge by fully virulent

L. monocytogenes strains. Overall, our data support that (i) mutations resulting in *inlA* PMSC alone are responsible for reduced invasion and attenuated virulence and (ii) exposure to a naturally occurring virulence-attenuated *L. monocytogenes* strain appears to confer protective immunity against subsequent challenge by fully virulent *L. monocytogenes* strains.

Mutations resulting in a PMSC in *inlA* alone are sufficient to cause attenuated virulence. While several different *inlA* PMSC mutations have been identified in *L. monocytogenes* isolates from foods and other sources, some *inlA* PMSC mutations have been found to be particularly common. Specifically, *L. monocytogenes* strains (EcoRI ribotypes) associated with *inlA* PMSC types 1 and 3 represented 30% and 11%, respectively, of >500 *L. monocytogenes* isolates from >30,000 ready-to-eat food samples collected in the United States (16, 33). To probe the causal relationship between these two common *inlA* PMSCs and virulence, we generated two sets of isogenic mutants (one to represent each *inlA* PMSC). Each mutant set contained two mutant pairs, including (i) a wild-type strain encoding a full-length InlA and its derived isogenic mutant, where a given *inlA* PMSC (i.e., PMSC 1 or 3) was introduced, and (ii) a natural isolate carrying a given PMSC and its derived isogenic mutant, where a given *inlA* PMSC was reverted to encode a full-length InlA. Characterization of these isogenic mutant sets clearly showed that introduction of *inlA* PMSC 1 or 3 into wild-type strains that previously encoded a full-length InlA leads to reduced invasion efficiency in human intestinal epithelial and hepatic cells (Fig. 1).

Furthermore, isogenic *L. monocytogenes* mutants and natural strains carrying *inlA* PMSC types 1 and 3 showed similar levels of reduced invasion in cell culture invasion assays. Oral guinea pig infections with the isogenic *L. monocytogenes* strain where *inlA* PMSC type 3 was introduced showed reduced virulence (Fig. 3). Combined with our findings that *inlA* PMSC type 3 did not have a polar effect on *inlB* expression, these data clearly establish that *inlA* PMSC mutations cause attenuated virulence. While experimental evaluation of the causal relationship between *inlA* PMSCs and human virulence is ethically not possible, we and other researchers have accumulated an overwhelming body of evidence supporting that *L. monocytogenes* isolates with *inlA* PMSCs have attenuated human virulence. This evidence includes (i) the reduced ability of *L. monocytogenes* with *inlA* PMSC mutations to invade human intestinal epithelial cells and hepatic cells, as reported here and elsewhere (12, 33, 36, 39, 44), (ii) reduced virulence of *L. monocytogenes* with PMSC mutations in *inlA* demonstrated using an animal model that is appropriate for evaluating interactions between InlA and the E-cadherin isoform present in humans, and (iii) significant underrepresentation of *L. monocytogenes* carrying an *inlA* PMSC among human clinical cases (18, 33). Specifically, *L. monocytogenes* isolates belonging to ribotype DUP-1062A, which exclusively includes isolates carrying *inlA* PMSC type 3 (33), represented about 30% of food isolates but <2.0% of human clinical isolates in the United States (16). Isolates belonging to ribotype DUP-1062A were also estimated to have a 1,000-fold-lower infectious dose compared to epidemic clone I subtypes, which have commonly been linked to sporadic and epidemic human listeriosis cases (8). In addition, a previous study that used an immunoblot assay to investigate InlA expression status (i.e., expression of a full-length or truncated InlA) among a large collection of *L. monocytogenes* isolates from France showed that *L. monocytogenes* strains expressing a truncated InlA are commonplace in foods but only cause human disease on very rare occasions (18). Overall, our findings support that molecular assays (single-nucleotide polymorphism genotyping assays) that detect *L. monocytogenes* with *inlA* PMSCs (11) will allow for specific identification of *L. monocytogenes* strains with attenuated human virulence.

We also found that isogenic *L. monocytogenes* strains where *inlA* PMSC types 1 and 3 were reverted to *inlA* allelic types encoding a full-length InlA showed a fully restored ability to invade human intestinal epithelial and hepatic cells (Fig. 1). In addition, the isogenic *L. monocytogenes* strain where *inlA* PMSC type 3 was reverted to a wild-type *inlA* showed fully restored virulence in guinea pigs after oral infection, which was demonstrated by recovery of significantly higher *L. monocytogenes* numbers in all organs compared to results in animals infected with the corresponding natural strain carrying *inlA* PMSC mutation type 3 (Fig. 3). These findings indicate that *inlA* PMSC type 3 is fully responsible for attenuated virulence and that no other additional mutations, which may have occurred either prior or subsequent to the mutation leading to the *inlA* PMSC, contribute to virulence attenuation in the guinea pig model. Interestingly, Olier et al. (34) previously found that replacement of a different *inlA* PMSC mutation observed among isolates from France in strain LO28 (20) with the *inlA* allele from Scott A (which encodes a full-length InlA) yielded a strain that showed a wild-type Caco-2 invasion phenotype. This revertant strain was not characterized in a guinea pig model and virulence was only partially restored in a chick-embryo infection assay (34), suggesting the possibility of either host- or strain-specific effects of *inlA* premature stop codons. For example, strain LO28 may demonstrate inherently lower virulence in chickens compared to strain Scott A.

Naturally occurring virulence-attenuated *L. monocytogenes* strains appear to confer protective immunity against subsequent challenge by fully virulent *L. monocytogenes* strains. Data from the current *L. monocytogenes* risk assessment by U.S. agencies (48), which suggest frequent human exposure to *L. monocytogenes* through contaminated food, combined with studies that show a high prevalence of *L. monocytogenes* with *inlA* PMSCs in food (16, 18, 33), indicate that humans are commonly exposed to virulence-attenuated *L. monocytogenes* that carry *inlA* PMSC mutations. We thus conducted initial experiments to test the hypothesis that oral exposure to *L. monocytogenes* carrying an *inlA* PMSC mutation may function as a natural vaccination and thus confer protection against infection and disease from subsequent exposure to fully virulent *L. monocytogenes*. Overall, the data from our vaccination studies provide multiple independent lines of evidence that oral exposure to *L. monocytogenes* with an *inlA* PMSC provides protection against subsequent exposure to fully virulent strains, including the following: (i) recovery of significantly lower *L. monocytogenes* challenge strain numbers from vaccinated animals compared to unvaccinated animals; (ii) significantly lower detection of *L. monocytogenes* antigens among vaccinated animals compared to nonvaccinated animals determined by immunohistochemistry at 72 h postchallenge; (iii) significantly less severe signs of disease in vaccinated animals compared to

unvaccinated animals based on histopathological observations at 72 h postchallenge; and (iv) significantly higher postchallenge weight gain in vaccinated animals compared to unvaccinated animals. While a number of previous studies showed that genetically engineered virulence-attenuated *L. monocytogenes* strains can elicit protection against subsequent challenge by virulent *L. monocytogenes* (10, 38, 47), only one previous study (7) evaluated the immunogenicity of a naturally occurring virulence-attenuated *L. monocytogenes* isolate from food. Chakraborty et al. (7) specifically showed that intravenous infection of mice with a natural *L. monocytogenes* isolate that produces a truncated form of the virulence protein ActA conferred protection against challenge by a virulent strain 28 days after vaccination. While the previous study by Chakraborty et al. (7) established that *L. monocytogenes* strains carrying naturally occurring virulence-attenuating mutations in *actA* confer protective immunity, it did not evaluate this ability using a natural exposure route (i.e., live oral vaccine). In addition, strains carrying virulence-attenuating mutations in *actA* appear to be rare among natural *L. monocytogenes* isolates from foods (43); hence, the ability of *L. monocytogenes* strains with *inlA* PMSCs to induce protective immunity is likely to be more relevant and important for the overall human host population.

Our observations that *L. monocytogenes* strains carrying a PMSC mutation in *inlA* still disseminate to the spleen and that vaccinated animals show strikingly reduced recovery of the *L. monocytogenes* challenge strain from the small intestine (Fig. 5c) are consistent with data from a study that measured expression of genes involved in the intestinal immune response in germ-free transgenic mice that expressed the human form of E-cadherin and that were challenged with wild-type *L. monocytogenes* and isogenic Δ*inlA*, Δ*inlAB*, and Δ*hly* mutants (23). Lecuit et al. (23) specifically showed that the intestinal gene expression response was similar in mice infected with the Δ*inlA* mutant and mice infected with the wild-type strain. Importantly, the Δ*inlA* strain was disseminated to the spleen, suggesting *L. monocytogenes* translocation to the Peyer's patches via M-cells by an InlA-independent mechanism (23). These findings support that naturally occurring virulence-attenuated *L. monocytogenes* strains that carry a PMSC in *inlA* may function as particularly good oral vaccine strains due to their ability to elicit a mucosal immune response in the intestine (as supported by low numbers of *L. monocytogenes* in the small intestine of vaccinated animals and inflammation in these tissues) and disseminate to the spleen without causing overt signs of disease.

While our data provide initial evidence that exposure to an *L. monocytogenes* strain carrying an *inlA* PMSC can protect guinea pigs against a subsequent challenge with virulent *L. monocytogenes*, further experiments are needed to characterize the specific immune response induced by *L. monocytogenes* carrying an *inlA* PMSC, including the T-cell-mediated immune response and the duration of protection. While use of a guinea pig model was appropriate to provide an animal model mimicking the InlA–E-cadherin interactions that appear to occur in a human host (24), the lack of immunological methods and reagents for the guinea pig prevents in-depth immunological studies in this model. Future experiments to characterize the immune response after oral challenge with *L. monocytogenes* carrying an *inlA* PMSC should be possible, though, by using either transgenic mice that express the human form of E-cadherin (24) or using *L. monocytogenes* strains that express a rationally designed InlA allele that can bind to the murine E-cadherin (2). Although the current study investigated the effect of *inlA* PMSC mutations on virulence in healthy juvenile guinea pigs, most listeriosis cases occur among neonates and immunocompromised individuals (45), and future experiments are necessary to elucidate the role of *inlA* PMSC mutations in these specific high-risk host populations. In addition, other studies (34) provided initial experimental evidence that the role of a truncated InlA may vary with respect to infection model and additional experiments (e.g., those using pregnant or otherwise-immunocompromised guinea pig infection models) are needed to further probe the relationship between *inlA* PMSC mutations and virulence among the most susceptible host populations.

Our findings that single nucleotide polymorphisms in *inlA* leading to a PMSC cause attenuated virulence and that these strains may represent an effective oral vaccine strain are consistent with findings for other bacterial food-borne pathogens. For example, the live oral typhoid vaccine Ty21a carries a frameshift mutation in *rpoS*, an alternative sigma factor critical for general stress response and murine virulence, and this single nucleotide polymorphism is thought to contribute to the safety of Ty21a as a live oral vaccine strain (41, 42). Interestingly, an enterohemorrhagic *Escherichia coli* strain genetically engineered to contain a single nucleotide deletion in *eae*, which results in production of truncated intimin protein, was also found to induce protective immunity in an oral rabbit model of infection (1). Collectively, data from the *L. monocytogenes* risk assessment (48), previous molecular epidemiology studies (16, 18, 33), and the findings of this study suggest that the average individual is frequently exposed to virulence-attenuated *L. monocytogenes* strains through a natural route and that this exposure may have protective immunizing effects at the population level.

If this is so, it is possible that the reduction of *L. monocytogenes* loads in foods may reduce potentially beneficial exposure to virulence-attenuated *L. monocytogenes* strains that may be responsible for conferring protection on immunocompetent individuals against more virulent strains. While these findings should not be misconstrued as a recommendation to reduce existing efforts for controlling *L. monocytogenes* in the food supply, our data support the "hygiene hypothesis," which, when applied to food safety, proposes that recurrent exposure to low or intermediate doses of pathogens (or to virulence-attenuated subtypes) via ingestion of contaminated food may provide some protection against subsequent exposure to a given pathogen (5). Interestingly, there is also epidemiological evidence that supports this hypothesis. For example, an investigation of a waterborne *E. coli* O157:H7 outbreak revealed a significantly lower attack rate for individuals that had previous long-term exposure to the pathogen and hence appeared to have acquired some immunity (37). There thus appears to be emerging evidence that reduced natural exposure to food-borne pathogens and particularly strains with reduced virulence through contaminated food or water may increase the risk of infection if exposure to fully virulent strains occurs. It may be important to quantify whether this effect is important at a population level through mathematical modeling, which may

also help determine whether there is a need to develop and use novel vaccines against selected food-borne pathogens to maintain population immunity, while reducing pathogen prevalence in food and water.

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