The *Listeria monocytogenes* ChiA Chitinase Enhances Virulence through Suppression of Host Innate Immunity

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ABSTRACT **Environmental pathogens survive and replicate within the outside environment while maintaining the capacity to infect mammalian hosts. For some microorganisms, mammalian infection may be a relatively rare event. Understanding how environmental pathogens retain their ability to cause disease may provide insight into environmental reservoirs of disease and emerging infections. *Listeria monocytogenes* survives as a saprophyte in soil but is capable of causing serious invasive disease in susceptible individuals. The bacterium secretes virulence factors that promote cell invasion, bacterial replication, and cell-to-cell spread. Recently, an *L. monocytogenes* chitinase (ChiA) was shown to enhance bacterial infection in mice. Given that mammals do not synthesize chitin, the function of ChiA within infected animals was not clear. Here we have demonstrated that ChiA enhances *L. monocytogenes* survival *in vivo* through the suppression of host innate immunity. *L. monocytogenes* Δ*chiA* mutants were fully capable of establishing bacterial replication within target organs during the first 48 h of infection. By 72 to 96 h postinfection, however, numbers of Δ*chiA* bacteria diminished, indicative of an effective immune response to contain infection. The Δ*chiA*-associated virulence defect could be complemented in *trans* by wild-type *L. monocytogenes*, suggesting that secreted ChiA altered a target that resulted in a more permissive host environment for bacterial replication. ChiA secretion resulted in a dramatic decrease in inducible nitric oxide synthase (iNOS) expression, and Δ*chiA* mutant virulence was restored in *NOS2*$^{-/-}$ mice lacking iNOS. This work is the first to demonstrate modulation of a specific host innate immune response by a bacterial chitinase.**

IMPORTANCE **Bacterial chitinases have traditionally been viewed as enzymes that either hydrolyze chitin as a food source or serve as a defense mechanism against organisms containing structural chitin (such as fungi). Recent evidence indicates that bacterial chitinases and chitin-binding proteins contribute to pathogenesis, primarily via bacterial adherence to chitin-like molecules present on the surface of mammalian cells. In contrast, mammalian chitinases have been linked to immunity via inflammatory immune responses that occur outside the context of infection, and since mammals do not produce chitin, the targets of these mammalian chitinases have remained elusive. This work demonstrates that a *Listeria monocytogenes*-secreted chitinase has distinct functional roles that include chitin hydrolysis and suppression of host innate immunity. The established link between chitinase and the inhibition of host inducible nitric oxide synthase (iNOS) expression may help clarify the thus far elusive relationship observed between mammalian chitinase enzymes and host inflammatory responses occurring in the absence of infection.**

Received 21 December 2012 **Accepted** 20 February 2013 **Published** 19 March 2013

Citation Chaudhuri S, Gantner BN, Ye RD, Cianciotto NP, Freitag NE. 2013. The *Listeria monocytogenes* ChiA chitinase enhances virulence through suppression of host innate immunity. mBio 4(2):e00617-12. doi:10.1128/mBio.00617-12.

Editor Howard Shuman, University of Chicago **Invited Editor** Denise Monack, Stanford University

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Environmental pathogens are organisms that are capable of survival in the outside environment while maintaining the capacity to cause human disease. These organisms represent reservoirs of infection that are widespread and difficult to eradicate. Defining how environmental pathogens develop and maintain the capacity to infect humans is important for understanding emerging infections and for reducing disease transmission under conditions of environmental change. Selective fitness advantages conferred by gene products expressed during host infection could potentially contribute little to survival in environments outside the host, and thus these coding regions might be anticipated to accrue loss-of-function mutations during bacterial replication in the outside environment. Alternatively, it is possible that gene products that contribute to bacterial virulence are functionally diverse, having been adapted for distinct roles in disparate environments.

Listeria monocytogenes is a Gram-positive facultative intracellular bacterium that survives as a saprophyte in soil but upon ingestion can cause serious disease in susceptible individuals (1, 2). Most outbreaks have been associated with the consumption of *L. monocytogenes*-contaminated food products, and the bacterium is a frequent cause of large and expensive food recalls (3–5). Following ingestion, *L. monocytogenes* crosses the intestinal barrier and replicates within the liver and spleen (6). The bacterium expresses a number of gene products that enable it to invade host cells, escape from cell phagosomes, replicate within the cytosol, and spread to adjacent cells (7). *L. monocytogenes* thus expresses a number of gene products that target distinct aspects of eukaryotic cell physiology.

Recent attention has focused on bacterial chitinases and chitin binding proteins in recognition of their contributions to bacterial virulence within infected mammals (8–13). These proteins have historically been thought to contribute to microbial life outside mammalian hosts based on the absence of chitin in mammals and the abundance of the polymer in fungal cell walls, as well as the exoskeletons of mollusks, arthropods, and crustaceans (14, 15). Chitin is a linear polysaccharide consisting of *N*-acetylglucosamine residues linked by β1,4-glycosidic linkages (16). Hydrolysis of chitin provides an abundant source of carbon and nitrogen for organisms that express chitinases; however, chitinases and chitin binding proteins have now been linked to pathogenesis in mammals for at least four bacterial pathogens, *Vibrio cholerae*, *Serratia marcescens*, *Legionella pneumophila*, and *L. monocytogenes* (8–13). For *V. cholerae*, the GbpA chitin binding protein has been demonstrated to contribute to bacterial colonization of the mouse small intestine via binding to mucin (8, 11, 13). The chitin binding protein CBP21 of *S. marcescens* contributes to bacterial adherence to colonic epithelial cells through its interactions with mammalian chitinase 3-like 1 (CHI3L1) (12). The ChiA chitinase of *L. pneumophila* contributes to bacterial colonization of the lung through an as yet unidentified mechanism (10).

L. monocytogenes has two chitinase-encoding genes, *chiA* and *chiB*, and one gene that encodes a chitin binding protein (*lmo2467*) (17, 18). The gene products of all three genes have been shown to contribute to bacterial virulence *in vivo* (9). The most significant defect in virulence was associated with the loss of *chiA*, which encodes a member of the glycosyl hydrolase family 18 of chitinases (17). ChiA has activity toward chitin as well as the chitin pseudosubstrates *p*-$(GlcNAc)_2$ and *p*-$(GlcNAc)_3$ but not toward a pseudocellulose substrate or toward peptidoglycan (17). The expression of *chiA* is increased in *L. monocytogenes* within macrophages (19) and is regulated by PrfA (20), a transcriptional activator required for the expression of gene products associated with *L. monocytogenes* virulence (21). The role of chitinase in *L. monocytogenes* pathogenesis is not known, nor has it been demonstrated that the chitinase activity of ChiA is required for virulence.

Given that chitin is not produced by mammals, it is intriguing that multiple bacterial chitinases are associated with virulence. Although mammals do not synthesize chitin, the presence of mammalian chitinases has been associated with host inflammatory responses in the apparent absence of microbial infection in diseases such as asthma (22–24). Mammalian chitinases, such as the acidic mammalian chitinase (AMCase) and macrophage differentiation marker YKL-40, both glycosyl hydrolase 18 family members similar to ChiA, may have chitin-like targets that modulate host immunity (25). The direct targets of these chitinases have not yet been identified, but many factors involved in mammalian immune responses are glycosylated with *N*-acetylglucosamine residues linked by β1,4-glycosidic bonds (14) and thus could potentially serve as substrates for an enzyme with chitinase activity. Endogenous carbohydrates, such as heparan sulfate and hyaluronic acid, that share structural similarities with chitin have been proposed as chitinase substrates; however, proof of this interaction has not been demonstrated (25).

In this study, we have further investigated the role of ChiA in *L. monocytogenes* pathogenesis. ChiA chitinase activity was found to be important for the suppression of host innate immune responses that serve to limit bacterial replication within the livers and spleens of infected mice. Mice infected with *L. monocytogenes* strains lacking *chiA* exhibited increased levels of *NOS2* expression, and bacteria were rapidly cleared from target organs. The virulence of Δ*chiA* strains was restored in mice lacking iNOS. These data thus establish the first functional link between a bacterial chitinase and direct modulation of host innate immunity.

RESULTS

Loss of ChiA enhances immune clearance of *L. monocytogenes* from target organs. Our laboratory has previously shown that mice intravenously infected with *L. monocytogenes* mutants lacking *chiA* have an approximately 20-fold reduction in bacterial burdens present in the liver and spleen at 72 h postinfection (9). It was not determined, however, whether ChiA was required at the initiation of infection or whether the protein was required subsequent to *L. monocytogenes* colonization of the liver and spleen. To determine the point during infection at which the requirement for ChiA becomes evident, 7- to 8-week-old Swiss Webster mice were infected with a sublethal dose (2×10^4 CFU) of either wild-type *L. monocytogenes* 10403S or the Δ*chiA* mutant. At 24, 48, 72, and 96 h postinfection, the livers and spleens of infected mice were harvested to enumerate bacterial burdens. The number of *L. monocytogenes* CFU recovered from the livers and spleens of animals infected with either the wild type or the Δ*chiA* mutant strain appeared to be very similar at 24 and 48 h postinfection, indicating that the Δ*chiA* mutant was fully capable of colonizing and replicating within these target organs. However, by 72 h postinfection, the numbers of bacteria recovered from the livers and spleens of animals infected with the Δ*chiA* mutant were 13-fold and 9-fold lower, respectively, than in the organs of mice infected with wild-type *L. monocytogenes* (Fig. 1A). By 96 h postinfection, bacterial burdens in the livers and spleens of animals infected with either bacterium began to drop, reflecting immune clearance of infecting bacteria (Fig. 1A). Mice infected with the Δ*chiA* mutant exhibited significantly lower (>30-fold) numbers of bacteria in target organs, indicating that the mutant strain was more susceptible to immune clearance. ChiA thus does not contribute to the initial establishment of *L. monocytogenes* infection in mice, but the protein is required for enhanced bacterial survival in the face of an effective host innate immune response.

ChiA is not required for bacterial translocation across the host intestinal barrier. Thus far, the most definitive roles reported for the contributions of bacterial chitinases or chitin binding proteins to infection have been the enhancement of bacterial adherence to intestinal or colonic epithelial cells (8, 11–13). Given that the natural route of *L. monocytogenes* infection occurs via bacterial translocation across the intestinal epithelial barrier (26), the potential contributions of ChiA to intragastric infection of mice were examined. Although mice are generally less susceptible to *L. monocytogenes* oral infection due to amino acid variations present in their E-cadherin, the intestinal epithelial cell receptor recognized by the *L. monocytogenes* surface protein InlA (27, 28), Wollert et al. (29) have recently described mutations within *inlA* that increase InlA binding affinity for E-cadherin and facilitate murine oral infection models. The S192N and Y376S mutations encoded by *inlA* (*inlA* S192N and Y376S) were thus introduced via homologous recombination into both the wild-type and Δ*chiA* strains, followed by intragastric inoculation of the strains into

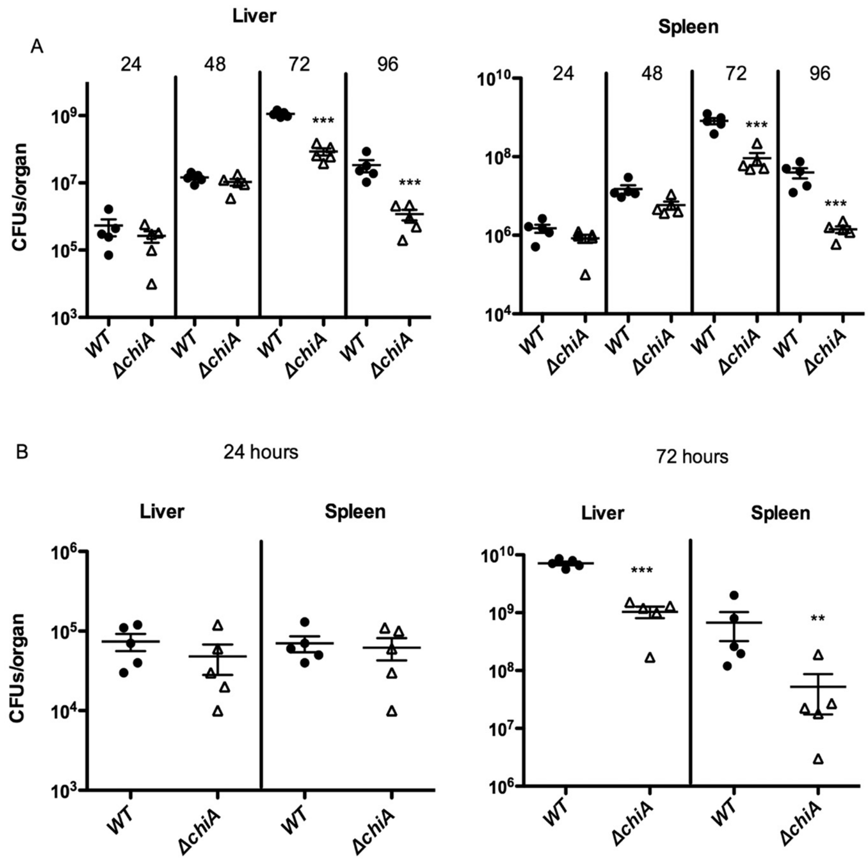

FIG 1 *L. monocytogenes* mutants lacking *chiA* exhibit a virulence defect late in infection. (A) Intravenous infection of mice with the Δ*chiA* strain. Swiss Webster mice were intravenously infected with 2×10^4 CFU *L. monocytogenes* wild type or Δ*chi*A mutant bacteria. The scatter plot shows CFU obtained from the livers and spleens of five individual mice at 24, 38, 72, or 96 h postinfection; the data are representative of two independent experiments. Solid lines and brackets represent the means and standard errors of the means, respectively, for the data points in each group. (B) Intragastric [i.g.] infection of mice with Δ*chiA* bacteria. C57BL6 mice were intragastrically infected with 1×10^8 CFU *L. monocytogenes* wild type or Δ*chiA* bacteria. The scatter plot shows CFU obtained from the livers and spleens of five individual mice at 24 and 72 h postinfection; the data are representative of two independent experiments. Solid lines and brackets represent the means and standard errors of the mean, respectively, for the data points in each group. Statistically significant *P* values (***, $P < 0.0001$; **, $P < 0.001$) for the Δ*chiA* strain in both the liver and the spleen after 72 and 96 h of infection (intravenous [i.v.] infection) or after 72 h (i.g. infection) compared to results for the wild type were determined using a one-way analysis of variance with Dunnett's posttest (GraphPad V.5.0A software program).

mice. At 24 h postinfection, there was no significant difference in the numbers of bacteria recovered from the livers or spleens of mice infected with either the wild type or the Δ*chiA* strain. By 72 h postinfection, mice infected with the Δ*chiA* strain exhibited approximately 10-fold-lower numbers of bacteria in both liver and spleen, (Fig. 1B), a difference similar to the fold reduction in bacterial CFUs observed following intravenous infection (Fig. 1A). ChiA therefore does not appear to significantly influence the ability of *L. monocytogenes* to cross the intestinal epithelium.

The virulence defect of the ΔChiA mutant can be rescued in *trans* during coinfection with wild-type *L. monocytogenes.* ChiA is a secreted protein, and thus we determined if the defect conferred by the absence of ChiA during infection could be rescued by secreted ChiA provided in *trans* by wild-type *L. monocytogenes*. Mice were intravenously injected with a 1:1 ratio of the Δ*chiA* strain and a wild-type derivative of *L. monocytogenes* that contains a chromosomal erythromycin resistance gene cassette that has been shown not to impact bacterial virulence (30). After 72 h of infection, the livers and spleens were harvested and assessed for bacterial burdens on solid medium with and without erythromycin. If the presence of wild-type *L. monocytogenes* was capable of complementing the virulence defect of the Δ*chiA* strain the 1:1 ratio of the wild-type strain to the Δ*chiA* strain should not change significantly during the course of infection. However, if the presence of wild-type *L. monocytogenes* did not restore Δ*chiA* strain virulence, then the wild-type strain should demonstrate a competitive advantage over the Δ*chiA* strain, thereby altering the ratio of the wild type to the Δ*chiA* strain to a value greater than 1. Despite the original 10- to 20-fold decrease in recovery of the Δ*chiA* mutant from livers and spleens of monoculture-infected animals in comparison to results for the wild type at 72 h postinfection (Fig. 1), mice infected with mixtures of the wild type and the Δ*chiA* strain maintained approximately equivalent ratios of the two strains after 72 h (Fig. 2A). The competitive index (CI) value reflecting the numbers of Δ*chiA* bacteria recovered in comparison to the wild type was not significantly different from 1 ($P > 0.1$), indicative of equivalent bacterial fitness levels (Fig. 2A). In addition, the absolute numbers of wild-type bacteria present in the liver and spleen were similar in mixed infections with either the Δ*chiA* strain or the wild-type erythromycin-resistant strain, indicating that the Δ*chiA* mutant did not negatively impact wild-type growth (Fig. 2B). These data indicate that the virulence defect of the Δ*chiA* mutant can be rescued in *trans* by the presence of wild-type *L. monocytogenes*, suggesting that secreted ChiA broadly influences some aspect of host physiology to promote Δ*chiA* replication during the course of infection.

Chitinase activity appears to be important for ChiA-dependent enhancement of *L. monocytogenes* virulence. ChiA contains a highly conserved chitinase catalytic region consisting of 10 amino acids (FDGLDIDDLE); the glutamic acid residue at position 163 within this sequence is required for chitin hydrolysis (31, 32) (Fig. 3A). The substitution of methionine for the catalytic glutamate residue in the related ChiA chitinase of *Vibrio harveyi* eliminates chitinase activity without affecting chitin binding (31). To determine if the chitinase and/or the chitin binding activity of *L. monocytogenes* ChiA was important for bacterial virulence, a similar *chiA* E163M (E163M amino acid change encoded by *chiA*) mutation was introduced into the *chiA* gene carried on the integrative plasmid vector pPL2 (33) (Fig. 3A). The pPL2-*chiA* and

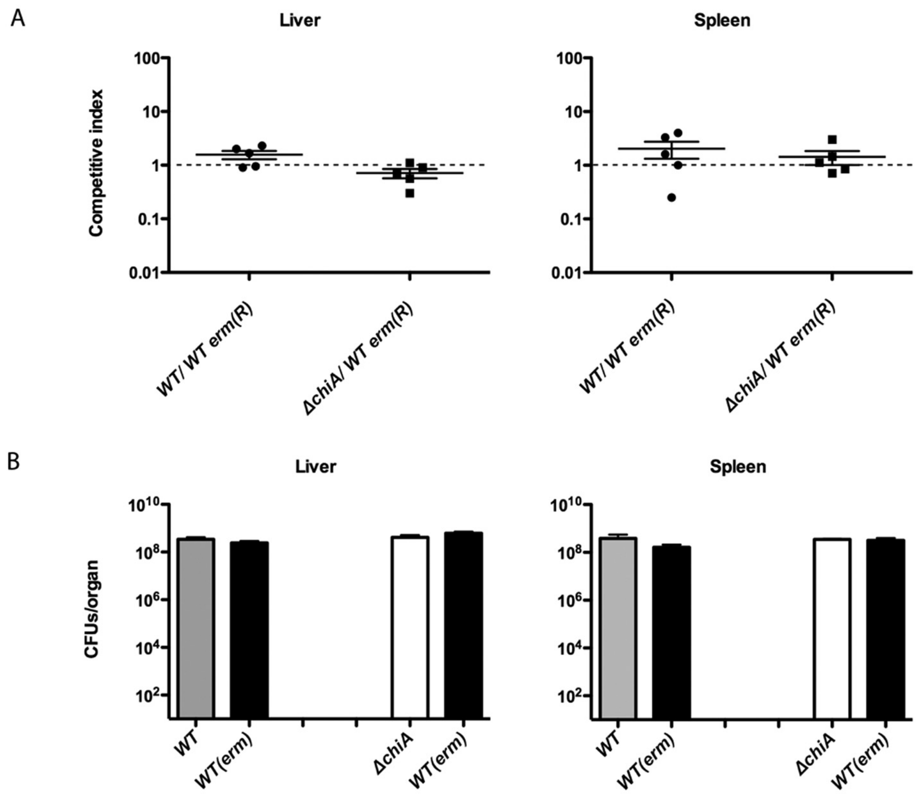

FIG 2 Virulence defects associated with Δ*chiA* are complemented in *trans* by the presence of wild-type *L. monocytogenes*. (A) Wild-type and Δ*chiA* strain mixed infections in mice. Swiss Webster mice were intravenously infected with 2×10^4 total CFUs of a 1:1 mixture of the *L. monocytogenes* DP-L3903 (10403S with a silent Tn*917-lac* insertion conferring erythromycin resistance) and Δ*chiA* strains. After 72 h of infection, livers and spleens of infected mice were harvested and bacteria were enumerated. As a control, groups of mice were infected with a 1:1 mixture of wild-type *L. monocytogenes* strains with the DP-L3903 erythromycin-resistant strain. Each data point represents the competitive index or the ratio of Δ*chiA* CFU recovered to that of wild-type *L. monocytogenes*. Data shown are representative of at least 2 independent experiments. (B) Total CFU of wild-type and Δ*chiA* mutant strains recovered from each organ. Data shown are representative of at least 2 independent experiments.

pPL2-*chiA* E163M plasmids were stably introduced in single copy into the chromosome of *L. monocytogenes* Δ*chiA*, and the resulting strains (the Δ*chiA* + pPL2-*chiA* and Δ*chiA* + pPL2-*chiA* E163M mutants, respectively) were assessed for secreted ChiA, chitin binding, and chitinase activity.

ChiA and ChiA E163M were detected as secreted products in the supernatants of both pPL2-*chiA*- and pPL2-*chiA* E163M-complemented Δ*chiA* strains as determined by Western blot analysis of bacterial supernatants (Fig. 3B). The ChiA E163M protein was observed to migrate as a smaller-molecular-mass species (approximately 1 kDa smaller based on molecular size standards); this appeared to reflect a proteolytic cleavage event, since a similarly sized lower-molecular-mass band could also occasionally be detected migrating below wild-type ChiA (see a faint band detectable in Fig. 3B). The Δ*chiA* + pPL2-*chiA* E163M mutant was deficient in secreted chitinase activity as detected by an *in vitro* chitinase assay of bacterial supernatants (Fig. 3C). However, both wild-type ChiA and ChiA E163M were able to bind chitin, as demonstrated by the binding and retention of the proteins on chitin beads (Fig. 3D). The *chiA* E163M mutation therefore results in the elimination of detectable chitinase activity while not affecting chitin binding.

When the plasmid-complemented Δ*chiA* strains were tested for bacterial virulence in mouse intravenous infection models, the Δ*chiA* + pPL2-*chiA* E163M mutant remained nearly as attenuated as strains lacking *chiA* entirely (Fig. 3E). Mice infected with Δ*chiA* + pPL2-*chiA* E163M strains had a greater than 6-fold reduction in bacterial CFUs recovered from the liver and spleen in comparison to results for animals infected with Δ*chiA* + pPL2-*chiA* strains at 72 h postinfection (Fig. 3E). Although the ChiA E163M protein appears more susceptible to proteolytic cleavage, the undiminished chitin binding activity of the mutant suggests that the chitin binding activity of ChiA alone is not sufficient to enhance *L. monocytogenes* virulence.

ChiA reduces the expression of iNOS, and Δ*chiA* strains are fully virulent in *NOS2*$^{-/-}$ mice. Based on the ability of the Δ*chiA* mutants to be complemented for virulence in *trans* by the presence of wild-type *L. monocytogenes* secreting ChiA, we investigated whether the secretion of ChiA enhanced *L. monocytogenes* replication within host tissues by modulating host immune responses. The expression of the proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin 1β (IL-1β) and of the inducible nitric oxide synthase (iNOS) was assessed in the livers of mice infected with either wild-type or Δ*chiA* strains at 48 h postinfection, prior to the 72-h time point for which Δ*chiA* strain numbers can be observed to decrease in comparison to those for the wild type (Fig. 1). At 48 h postinfection, no significance difference was observed in the levels of either TNF-α or

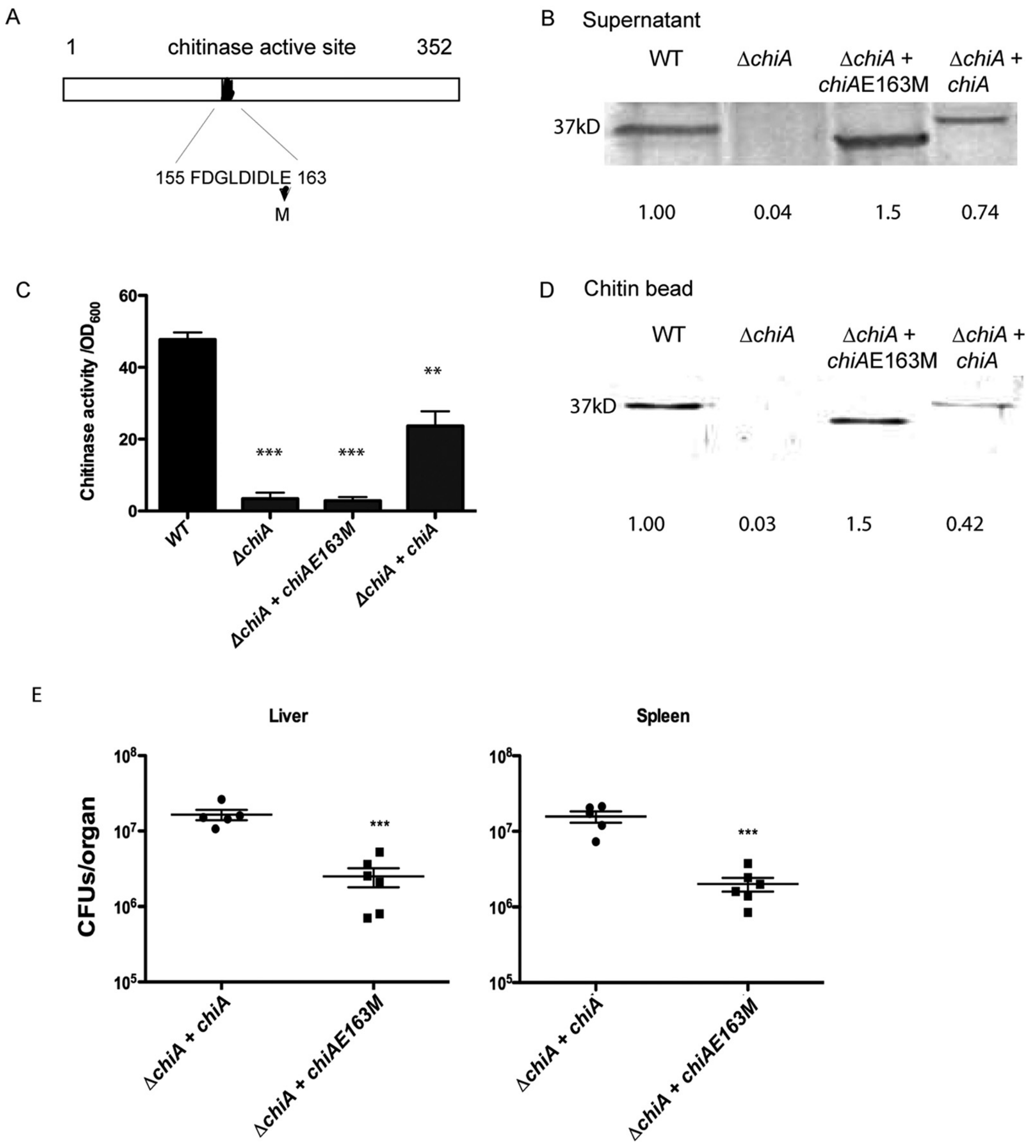

FIG 3 ChiA chitinase activity appears to contribute to *L. monocytogenes* virulence. (A) Schematic diagram of ChiA showing the conserved region of 10 amino acids within the active site of the chitinase. The substitution of methionine (M) 163 for glutamate (E) has been demonstrated to eliminate chitinase activity without affecting chitin binding in other closely related ChiA family members (31). (B) Detection of secreted ChiA by Western blot analysis. Bacterial supernatant proteins from overnight cultures of wild-type, Δ*chiA*, Δ*chiA* + pPL2-*chiA*, and Δ*chiA* + pPL2-*chiA* E163M bacteria were isolated using trichloroacetic acid (TCA) precipitation. Sample volumes were adjusted to reflect equivalent bacterial cell densities. Secreted ChiA was detected using rabbit polyclonal antibody directed against ChiA. The amount of protein detected for each sample in comparison to that in the wild-type lane (set at 1.0) as determined by densitometry is indicated at the bottom of each panel. Data shown are representative of at least 3 independent experiments. (C) Assessment of chitinase activity in bacterial supernatants. Supernatants derived from overnight cultures of wild-type, Δ*chiA*, Δ*chiA* + pPL2-*chiA*, and Δ*chiA* + pPL2-*chiA* E163M were assessed for chitinase activity using the colorimetric substrate nitrophenyl *N*,*N*′-‐diacetyl-*β*-D-chitobioside. Chitinase activity was reflected by substrate hydrolysis as measured by absorbance at an optical density at 405 nm (OD_{405}) as a function of bacterial cell density. Data shown represent the means ± standard errors for three independent experiments. Statistically significant differences as determined by one-way analysis of variance with Tukey's multiple-comparison test are indicated (*, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$) (GraphPad V.5.0). (D) Detection of chitin binding by the ChiA wild-type and mutant proteins. Bacterial supernatants derived from overnight cultures of wild-type, Δ*chiA*, Δ*chiA* + pPL2-*chiA*, and Δ*chiA* + pPL2-*chiA* E163M bacteria were adjusted to control for equivalent cell densities, concentrated 50-fold, and incubated with chitin beads for 1 h at 4°C. Beads were washed with PBS, and bound protein was recovered following boiling of the beads in SDS-PAGE sample buffer. ChiA bound to chitin was detected by Western blot analysis using rabbit polyclonal antibody directed against ChiA. The amount of protein detected for each sample in comparison to that in the wild type lane (set at 1.0) as determined by densitometry is indicated at the bottom of each panel. Data shown are representative of at least 3 independent experiments. (E) Assessment of bacterial virulence in mice. Swiss Webster mice were intravenously infected with 2×10^4 CFU of either Δ*chiA* + pPL2-*chiA* or Δ*chiA* + pPL2-*chiA* E163M bacteria. The scatter plot shows CFU obtained from the livers and spleens of five individual mice at 72 h postinfection; the data are representative of two independent experiments. Solid lines and brackets represent the means and standard errors of the means, respectively, for the data points in each group. ***, statistically significant value ($P < 0.0001$) for Δ*chiA* + pPL2-*chiA* E163M results in both the liver and the spleen after 72 h of infection compared to Δ*chiA* + pPL2-*chiA* results using a one-way analysis of variance with Dunnett's posttest (GraphPad V.5.0A).

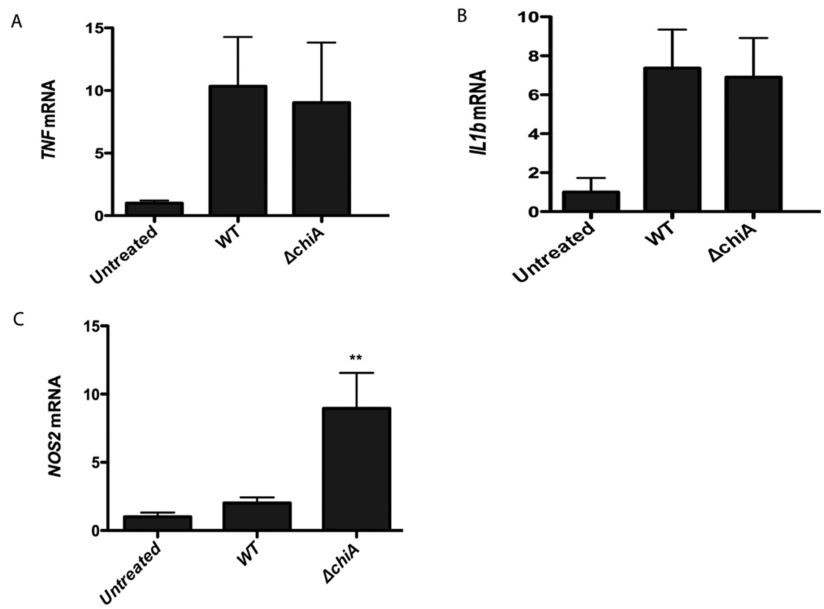

FIG 4 Mice infected with *L. monocytogenes* Δ*chiA* strains exhibit increased levels of iNOS expression. Seven- to eight-week-old Swiss Webster mice were infected via the tail vein with 2×10^4 CFU of wild-type or Δ*chiA L. monocytogenes* for 48 h, followed by analysis of mRNA expression in liver. Data are normalized to *gapdh* expression levels, with the average for untreated samples set at 1. Data shown are from at least two independent experiments. Statistically significant differences as determined by one-way analysis of variance with Tukey's multiple-comparison test are indicated (**, $P < 0.001$) (GraphPad V.5.0).

IL-1β expression for mice infected with either the wild type or the Δ*chiA* mutant (Fig. 4A and B). In contrast, expression of iNOS was approximately 4-fold higher in animals infected with the Δ*chiA* strain than in those infected with the wild-type strain (Fig. 4C).

The significant increase observed in the induction of iNOS in mice infected with the Δ*chiA* mutant strains suggests that a potential *in vivo* function of *L. monocytogenes* ChiA is the suppression of iNOS expression and activity. iNOS activity is an important facet of the host innate immune response that limits *L. monocytogenes* survival and proliferation (34–36). $NOS2^{-/-}$ mice lacking iNOS have been shown to be approximately 100-fold more susceptible to *L. monocytogenes* infection than wild-type mice (36). If ChiA is important for reducing iNOS activity, then the enhanced virulence observed for the wild-type strains versus Δ*chiA* mutants should not be apparent in mice lacking iNOS. C57BL/6 wild-type and $NOS2^{-/-}$ mice were infected with wild-type or Δ*chiA L. monocytogenes*, and bacterial burdens were monitored in the livers and spleens at 72 h postinfection (Fig. 5). Given the 100-fold increase in susceptibility of $NOS2^{-/-}$ mice to *L. monocytogenes* infection (36), these animals were inoculated with 100-fold fewer bacteria (2×10^2 CFU) than wild-type C57BL/6 mice. As shown in Fig. 5A, the virulence defect observed for Δ*chiA* strains in wild-type C57BL/6 mice was similar to that observed for the mutant following the infection of Swiss Webster mice, with the mutant strain exhibiting approximately 10-fold and 7-fold decreases in bacterial CFUs recovered from the liver and spleen, respectively, at 72 h postinfection. Strikingly, this Δ*chiA*-associated virulence defect was not detectable in $NOS2^{-/-}$ animals (Fig. 5B). These results, when combined with the observed increase in iNOS expression in mice infected with Δ*chiA* strains, represent the first example of a pathogen-encoded chitinase functioning to enhance virulence through suppression of iNOS expression, a critical host defense mechanism.

DISCUSSION

Environmental pathogens survive and replicate in disparate habitats that include the outside environment as well as inside human cells. For many of these organisms, humans may be infrequently encountered hosts, and thus to remain pathogens, these organisms must be capable of maintaining a repertoire of virulence factors that allow host colonization. Gene products that function in multiple contexts would provide a fitness advantage to pathogens in diverse habitats while reducing the genetic repertoire that must be maintained. Bacterial chitinases appear to represent such a class of multifunctional virulence factors. These enzymes promote bacterial survival in the environment by facilitating the use of chitin as an energy source and/or by providing a competitive advantage over organisms, such as fungi, that contain chitin as a structural component (37–39). It is now apparent that for some pathogens, chitinases function to enhance bacterial fitness within mammalian hosts either by promoting colonization (8, 10–13) or, as shown in this study, by modulating host innate immune responses. Here we have provided evidence that the secreted ChiA chitinase of *L. monocytogenes* enhances bacterial survival within host tissues through the downregulation of iNOS activity. To our knowledge, this is the first demonstration of a bacterial chitinase influencing a mammalian host innate immune response.

Given that humans and other mammals do not produce chitin, the target of the *L. monocytogenes* ChiA enzyme remains unclear. While it is possible that chitin or organisms producing chitin may be found within the human gastrointestinal tract or within oral and nasal passages, *L. monocytogenes* ChiA must play a role in mammalian pathogenesis that is distinct from the hydrolysis of microbe- or arthropod-synthesized chitin. The virulence defect of the *L. monocytogenes* Δ*chiA* mutant was evident following intravenous inoculation based on bacterial replication within the liver

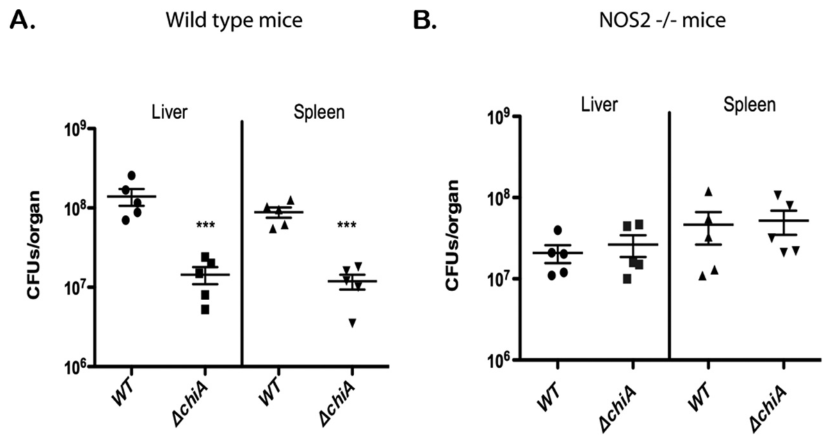

FIG 5 The virulence of *L. monocytogenes* Δ*chiA* mutants is restored in mice lacking iNOS. (A) *L. monocytogenes* intravenous infection. C57BL/6 mice were intravenously infected with 2×10^4 CFU *L. monocytogenes* wild-type or Δ*chi*A mutant bacteria. The scatter plot shows CFU obtained from the livers and spleens of five individual mice at 72 h postinfection; the data are representative of two independent experiments. Solid lines and brackets represent the means and standard errors of the means, respectively, for the data points in each group. ***, statistically significant value ($P < 0.0001$) for Δ*chiA* bacteria in both the liver and the spleen after 72 of infection compared to results for the wild type using a one-way analysis of variance with Dunnett's posttest (GraphPad V.5.0A). (B) Intravenous infection of C57BL/6 *NOS2*$^{-/-}$ mice lacking iNOS. C57BL/6 *NOS2*$^{-/-}$ mice were intravenously infected with 2×10^2 CFU *L. monocytogenes* wild-type or Δ*chi*A mutant bacteria [the infectious dose was adjusted to reflect the ~100-fold increase in susceptibility to *L. monocytogenes* infection for mice lacking iNOS (36)]. The scatter plot shows CFU obtained from the livers and spleens of five individual mice at 72 h postinfection; the data are representative of two independent experiments. Solid lines and brackets represent the means and standard errors of the means, respectively, for the data points in each group. No significant statistical difference was detected between the bacterial burdens of mice infected with Δ*chi*A bacteria for either organ ($P > 0.05$) compared to findings for the wild type using a one-way analysis of variance with Dunnett's posttest (GraphPad V.5.0A).

and spleen, organs not anticipated to harbor exogenous sources of chitin. Molecules similar to chitin can be found associated with glycoproteins and glycolipids present on the cell surfaces of a variety of mammalian cell types (40, 41). Studies have also identified a *Xenopus* chitin oligosaccharide synthase that is conserved in mice and that, when expressed from a plasmid in mouse 3T3 cells, stimulated hyaluronic acid synthesis (42). A number of key molecules involved in mammalian immune responses and signal transduction cascades are glycoproteins containing (β1,4) *N*-acetyl glucosamine residues (43–45), and it is possible that such a target resides within a *NOS2* induction pathway. Elucidation of the specific mammalian target for ChiA is a very challenging task, perhaps best illustrated by the longtime recognition of the association of mammalian chitinases with sterile inflammation and the continuing failure to identify a specific mammalian target for these enzymes. It will thus be interesting to ascertain if similar associations between chitinase activity and reductions in iNOS expression can be established for other bacterial and mammalian chitinase enzymes.

The ability of secreted ChiA to rescue the defect of *L. monocytogenes* Δ*chiA* mutant strains during mixed infections is consistent with ChiA modification of a target that influences host immunity. Δ*chiA* mutants have no detectable defects in host cell adherence or invasion, vacuole escape, intracellular replication, or cell-to-cell spread (9). Δ*chiA* mutants also exhibited robust bacterial replication in *NOS2*$^{-/-}$ mice (Fig. 5B), indicating that the mutants were not metabolically limited as the result of the loss of chitinase-dependent hydrolysis of a substrate needed for growth. It is anticipated that ChiA-dependent suppression of iNOS activity would impact multiple facets of host immunity. iNOS contributes to host resistance to *L. monocytogenes* (34–36, 46, 47), although it has recently been implicated in increasing the susceptibility of activated macrophages to *L. monocytogenes* spread from adjacent cells (48). iNOS catalyzes the synthesis of nitric oxide (NO) from arginine (49), and induced NO plays several important roles in mediating clearance of bacterial infection (34–36, 46, 47). NO is capable of causing significant membrane and protein damage, and it also functions as a mammalian signaling molecule (50–52). Prolific secretion of NO by TNF- and iNOS-producing dendritic cells (TipDCs) at sites of infection has been shown to enhance macrophage activation and bacterial clearance (53, 54). In general, the ability of *L. monocytogenes* ChiA to downregulate *NOS2* expression would appear consistent with enhanced bacterial survival within host tissues.

Mammalian chitinases and chitin binding proteins are expressed in macrophages and in epithelial cells of the lung and digestive tract, where they provide a first line of defense against exogenous agents, including chitin-containing pathogens (55). As mentioned previously, the expression of mammalian chitinases has been associated with a number of chronic inflammatory and tissue-remodeling disorders in the absence of infection, including asthma, chronic obstructive pulmonary disease (COPD), Sjögren's syndrome, and Alzheimer's disease (24, 25, 55–57); however, the specific targets of mammalian chitinases in the context of these diseases have not yet been determined. Mammalian chitinases have been reported to augment Th2 inflammatory responses (25), and thus it is tempting to speculate that one function of *L. monocytogenes* ChiA may be to skew the host immune response from a Th1 to a Th2 inflammatory pathway, leading to the downregulation of iNOS and enhancing *L. monocytogenes* survival within host tissues. It will be interesting to determine if the chitinase enzymes produced by other intracellular bacterial pathogens contribute similar roles in the modulation of host immunity so as to promote bacterial infection.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains are listed in Table 1. The Δ*chiA* and *inlA* S192N Y369S strains were con-

TABLE 1 Bacterial strains used in this study

Strain	Description[a]	Genotype(s)[b]	Reference
NF-L100	Wild type 10403S parent strain		59
NF-L1593	In-frame deletion of *chiA* in NF-L100	Δ*chiA*	9
NF-L1772	10403S with *inlA* S192N Y376S	*inlA* S192N Y376S	This work
NF-L1980	NF-L1593 with *inlA* S192N Y376S	Δ*chiA inlA* S192N Y376S	This work
NF-L1801	NF-L1593 with pPL2-*chiA*	Δ*chiA* + pPL2-*chiA*	9
NFL-1941	NF-L1593 with pPL2-*chiA* E163M	Δ*chiA* + pPL2-*chiA* E163M	This work
DP-L3903	10403S with silent Tn*917* insertion	WT Erm[rc]	30

[a] All strains are derived from *L. monocytogenes* 10403S.
[b] Description of strains as provided within the text.
[c] Erythromycin-resistant strain.

structed using allelic exchange as previously described (9). Bacterial cultures were grown in bovine heart infusion (BHI) medium prior to *in vivo* experiments or in Luria broth (LB) supplemented with 0.1% *N*-acetylglucosamine (20).

Construction of pPL2-*chiA* E163M plasmid. Site-directed mutagenesis was used to introduce a methionine residue in place of glutamate at position 163 of ChiA using plasmid pPL2-*chiA* (9) and the Change-IT multiple-mutation site-directed mutagenesis kit (USB) with primer E163M (5′ GGATTAGACATCGACTTAATGCAAAGTGCGATTACCG CGGGA 3′).

Mouse infection models. Swiss Webster, C57BL/6, and C57BL/6 *NOS2*$^{-/-}$ mice were obtained from Harlan Labs (Swiss Webster) or Jackson Laboratory (C57BL/6 strains). Oral and intravenous infections of 7- to 8-week-old mice were carried out as described elsewhere (58). Mixed infections were carried out as described previously (59) with a 1:1 mixture of the wild-type DP-L3903 (30) and Δ*chiA* strains (total bacterial CFU = 2×10^4) in 200 μl phosphate-buffered saline (PBS) injected via the tail vein.

Chitinase and chitin binding assays. The chitinase and chitin binding assays were carried out using bacterial culture supernatants derived from overnight cultures as described elsewhere (17).

RNA analysis. Mice (7- to 8-week-old Swiss Webster) were intravenously infected as described previously (58) for 48 h. Livers were removed and homogenized in the presence of Trizol (Invitrogen) and processed as directed by the manufacturer. cDNA synthesis was performed with Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen) and oligo(dT) (Invitrogen) as directed. Quantitative PCR (qPCR) was performed with SYBR Advantage mix (Clontech) using oligonucleotides specific for *gapdh*, *nos2*, *IL-1β*, and *tnfa* (IDT).

ACKNOWLEDGMENTS

We thank Omar Jawald for help with the construction of the *chiA* E163M mutation.

This work was supported by Public Health Service grants AI076693 (to N.P.C. and N.E.F.) and AI083241 (to N.E.F.) from NIAID.

The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of the funding sources.

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