Actin Polymerization Drives Septation of *Listeria monocytogenes* *namA* Hydrolase Mutants, Demonstrating Host Correction of a Bacterial Defect^{∇†}

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The Gram-positive bacterial cell wall presents a structural barrier that requires modification for protein secretion and large-molecule transport as well as for bacterial growth and cell division. The Gram-positive bacterium *Listeria monocytogenes* adjusts cell wall architecture to promote its survival in diverse environments that include soil and the cytosol of mammalian cells. Here we provide evidence for the enzymatic flexibility of the murein hydrolase NamA and demonstrate that bacterial septation defects associated with a loss of NamA are functionally complemented by physical forces associated with actin polymerization within the host cell cytosol. *L. monocytogenes* Δ *namA* mutants formed long bacterial chains during exponential growth in broth culture; however, normal septation could be restored if mutant cells were cocultured with wild-type *L. monocytogenes* bacteria or by the addition of exogenous NamA. Surprisingly, Δ *namA* mutants were not significantly attenuated for virulence in mice despite the pronounced exponential growth septation defect. The physical force of *L. monocytogenes*-mediated actin polymerization within the cytosol was sufficient to sever Δ *namA* mutant intracellular chains and thereby enable the process of bacterial cell-to-cell spread so critical for *L. monocytogenes* virulence. The inhibition of actin polymerization by cytochalasin D resulted in extended intracellular bacterial chains for which septation was restored following drug removal. Thus, despite the requirement for NamA for the normal septation of exponentially growing *L. monocytogenes* cells, the hydrolase is essentially dispensable once *L. monocytogenes* gains access to the host cell cytosol. This phenomenon represents a notable example of eukaryotic host cell complementation of a bacterial defect.

The cell wall of a Gram-positive bacterial pathogen is very often the first structural component of the organism to engage a host cell upon the initiation of bacterial adherence and/or host cell invasion (70). The Gram-positive cell wall is a thick and rigid structure composed of peptidoglycan, which in turn consists of a series of *N*-acetylglucosamine and *N*-acetylmuramic acid residues linked to form a lattice-like meshwork (27, 64). While structurally rigid, the cell wall is a dynamic organelle that must be constantly altered and modified to accommodate bacterial cell division, protein secretion, and the assembly or transport of large molecules such as flagella and DNA (10, 11, 42, 43, 47, 64, 65). Unlike bacterial membranes, the cell wall is a permeable organelle that permits the diffusion of small molecules and regularly adjusts to shifts in environmental conditions (27, 64). Bacteria produce a variety of enzymes dedicated to the synthesis, degradation, and restructuring of peptidoglycan to facilitate bacterial growth and survival in diverse environments. These cell wall-specific enzymes include proteins known as autolysins or peptidoglycan hydrolases, which hydrolyze the peptidoglycan bonds at specific sites to release distinct cell wall cleavage products (28, 58, 65).

The Gram-positive bacterium *Listeria monocytogenes* is an

environmental pathogen that is capable of orchestrating a complex transition from life in the outside environment to life within the cytosol of an infected host (18, 30, 51). As an environmental bacterium, *L. monocytogenes* survives in a number of diverse settings, including soil, water, and silage (51, 60). As an intracellular pathogen, *L. monocytogenes* adheres to and invades host cells and replicates within the cytosol, and individual bacteria are propelled into neighboring cells to spread within host tissues (13, 63). *L. monocytogenes* survival within host cells is dependent on a variety of secreted virulence gene products, whereas bacterial survival in the outside environment depends on the synthesis and assembly of bacterial flagella in addition to the regulated secretion of proteins and factors that aid in nutrient acquisition (12, 62). Thus, the appropriate modulation of the *L. monocytogenes* cell wall for cell division, bacterial organelle assembly, and protein secretion is essential for optimal bacterial fitness under a variety of disparate conditions.

Seven peptidoglycan hydrolases in *L. monocytogenes* have been identified and at least partially characterized. These hydrolases are p60 (also known as CwhA or Iap) (16, 25, 26, 31, 47, 71), p45 (53), Ami (38, 39), Auto (5, 7), NamA (also known as MurA) (10, 33, 36), Imo0327 (49), and IspC (67–69). While most of these proteins contain recognizable domains linked to murein hydrolase activity and cell wall binding and/or associations (7), many display unique characteristics associated with the regulation of catalytic activity (5) or with the anchoring of the proteins to the cell wall (68) as well as distinct activities relating to cell wall structure and function. The hydrolases p60,

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TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Genotype or relevant characteristic(s)	Description	Reference
Strains			
DH5 α	<i>E. coli</i> host strain for recombinant pKSV7 plasmids		29
BH10C	<i>E. coli</i> host strain for recombinant pIMK2 plasmids		
DH5 α I/q	<i>E. coli</i> host strain for recombinant NamA expression plasmid		
NF-L476	<i>L. monocytogenes</i> 10403S <i>actA-gus-plcB</i>	Wild type	
NF-L1001	<i>L. monocytogenes</i> 10403S Δ <i>inlA</i>	Δ <i>inlA</i>	
NF-L1369	10403S <i>actA-gus-neo</i> Δ <i>namA</i>	Δ <i>namA</i>	This work
NF-L1481	NF-L476 + pAM401- <i>gfp3</i>	Wild type + pAM401- <i>gfp3</i>	This work
NF-L1482	NF-L1369 + pAM401- <i>gfp3</i>	Δ <i>namA</i> + pAM401- <i>gfp3</i>	This work
DP-L4092	10403S containing pAM401- <i>gfp3</i>		This work
DP-L3903	10403S::Tn917-LTV3	WT::Tn917-LTV3	2
NF-L1695	NF-L1369 + pIMK2- <i>namA</i>	Δ <i>namA</i> + pIMK2- <i>namA</i>	This work
NF-E1808	<i>E. coli</i> (DH5 α I/q) containing vector pQE30- <i>namA</i>		This work
Plasmids			
pIMK2	pPL2-based integrational vector for single-copy complementation		40
pNF1367	pKSV7 containing 1,068-bp SOEing product for allelic exchange for <i>namA</i> in-frame deletion		This work
pNF1113	pAM401- <i>gfp3</i>		This work
pNF1688	pIMK2 containing a <i>namA</i> open reading frame for complementation		This work

p45, and NamA have been shown to contribute to cell wall peptidoglycan remodeling to enable flagellar motility, cell division, cell elongation, and the secretion of bacterial proteins (10, 33, 36, 47, 53). Ami, Auto, and IspC appear to have more specialized roles related to bacterial virulence (7, 38, 68). Auto is unique in that it is present in *L. monocytogenes* but is not found in the nonpathogenic species *Listeria innocua*. Auto contributes to the bacterial invasion of host cells, and it was suggested previously that it functions to regulate cell wall permeability to facilitate the secretion of virulence factors (7). Similarly, Ami and IspC have been shown to play a role in pathogenesis via their contributions to bacterial adhesion to host cells (38, 68).

NamA was initially identified as a variably expressed immunogenic epitope of *L. monocytogenes* recognized by monoclonal antibodies at the bacterial surface (19, 20, 44). NamA contributes to cell division, as mutants lacking *namA* form bacterial chains during exponential-phase growth in broth culture (10). Despite bacterial chain formation, Lenz et al. reported previously that NamA made only modest contributions to bacterial virulence in a mouse model of infection (33, 34).

We recently identified NamA as one of a number of proteins whose abundance in bacterial culture supernatants was increased in strains containing a mutationally activated form of the central virulence regulator PrfA (PrfA*); this mutation results in the constitutive expression of virulence-associated gene products (50). Given that many of the gene products expressed as a result of PrfA activation have been found to contribute to bacterial virulence (1, 14, 50, 63, 72) and that the loss of NamA has thus far been shown to have only a modest effect on *L. monocytogenes* virulence (33, 34), we sought to further explore the role of NamA in *L. monocytogenes* intracellular and extracellular growth. Here we provide additional evidence that *L. monocytogenes* NamA is a temporally secreted murein hydrolase whose flexible activity is required during the early phases of exponential bacterial growth in broth culture. Interestingly, NamA was found to be dispensable for bacterial

septation for *L. monocytogenes* cells replicating within the cytosol of infected host cells. The physical force provided by actin polymerization disrupted cytosolic bacterial chains and enabled the spread of septation-defective mutants into neighboring cells. Our data thus provide a mechanism to explain how NamA septation-deficient bacteria retain bacterial virulence within an infected eukaryotic host.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All bacterial strains used in this study are listed in Table 1. *L. monocytogenes* containing an *actA-gus* transcriptional fusion (NF-L476) was used as the parent strain for all genetic manipulations (54). *Escherichia coli* alpha select (BioLine, Boston, MA), One Shot TOP10 (Invitrogen Corp., Carlsbad, CA), DH5 α I/q, and BH10C (29) were used as host strains for recombinant plasmids. All *E. coli* strains were grown in Luria-Broth (LB) medium (Invitrogen Corp., Carlsbad, CA), and *L. monocytogenes* strains were grown in brain heart infusion (BHI) medium (Difco Laboratories, Detroit, MI). Where necessary, the medium was supplemented with antibiotics at the following concentrations unless otherwise specified: ampicillin at 100 μ g/ml, carbenicillin at 50 μ g/ml, erythromycin at 0.1 μ g/ml (to induce the expression of the *erm* gene) and 1 μ g/ml (for the selection of erythromycin-resistant bacteria), chloramphenicol at 10 μ g/ml, kanamycin at 50 μ g/ml, and streptomycin at 200 μ g/ml. Vector pAM401 containing a *gfp3* allele (a kind gift of Daniel Portnoy) was used for expressing green fluorescent protein (GFP) in selected strains. The temperature-sensitive shuttle vector pKSV7 (57) was used for generating deletion mutants via allelic exchange, and the integration plasmid pIMK2 (40), a kind gift of Colin Hill, was used for genetic complementation.

Construction of the Δ *namA* in-frame deletion mutant. A 1,755-bp internal in-frame deletion was generated with lmo2691 (*namA*) as follows: two DNA products containing sequences upstream and downstream of *namA* coding sequences were amplified using PCR with primer pairs 2691A1/2691A2 and 2691B1/2691B2 using *L. monocytogenes* 10403S chromosomal DNA as a template (all oligonucleotides are listed in Table 2). The resulting PCR products were purified and used in a splice overlap extension (SOEing) PCR along with primer pair 2691A1/2691B2, generating a 1,068-bp fragment which encompassed the first 9 nucleotides of the lmo2691 coding region along with 519 bp of upstream sequence and the last 9 nucleotides of the same gene with 531 bp of downstream sequence. The 1,068-bp fragment was cloned into the shuttle vector pKSV7 using the BamHI and HindIII restriction sites to generate pNF1367. Plasmid pNF1367 was transformed into *L. monocytogenes* strain NF-L476 (10403S *actA-gus*) (54) using electroporation as previously described (46), and the Δ *namA* mutation was introduced into the bacterial chromosome in a single

TABLE 2. Oligonucleotides used in this work

Oligonucleotide	Sequence (restriction site) ^a
lmo2691 deletion	
2691A1	GGCGGATCCGAACCATTTCCTT TACTTTTT (BamHI)
2691A2	GGCTTTTACATTCACCTAATTT TTTGCATTGGTAAGTCACTT
2691B1.....	AAGTGACTTACCATGCAAAAA ATTAAGTGAATGTAAAA AGCC
2691B2.....	GGCAAGCTTGATTCTTTTGAA CTCATATT (HindIII)
lmo2691 cloned into pIMK2	
namApIMK2f.....	ATATCCATGGCCATGCAAAAA CGAGAAAAG (NcoI)
namApIMK2r.....	ATATGGTACCTTTATCCGCAGT TTCTGACCTATC (KpnI)
lmo2691 cloned into pQE30	
namA-6hisA.....	GTGAGGCCTGACGAAACAGCG CCTGCTG (StuI)
namA-6hisB.....	GTGGGTACCTTAATTGTTAAT TTCTGACC (KpnI)

^a Restriction sites are underlined.

copy by allelic exchange (9) to generate strain NF-L1369. The presence of the in-frame deletion, which encompasses 98.9% of the structural gene, was confirmed by the sequencing of PCR products derived from the bacterial chromosome.

To construct a plasmid vector containing *namA* for complementation, primers namApIMK2f and namApIMK2r were used to amplify the entire open reading frame (ORF) of *namA* (excluding the ATG start codon) from *L. monocytogenes* 10403S genomic DNA using PCR. The product was digested with NcoI and KpnI and subcloned into appropriately digested pIMK2 to generate pNF1688. The insertion of *namA* coding sequences into pIMK2 places the expression of the gene under the control of the Phelp promoter. *namA* was PCR amplified without its native ATG because the NcoI restriction site used overlaps the ATG of Phelp and results in in-frame high-level gene expression (40). Because we encountered difficulty in propagating the complementation vector in our standard *E. coli* hosts (TOP10 and DH5α) due to the apparent toxicity of the expressed gene product, we opted to use host strain BH10C (a kind gift of Nicholas Cianciotto), which results in the efficient restriction of plasmid copy numbers such that each cell contains approximately 1 copy of the recombinant plasmid (29). Recombinant plasmid pNF1688 was then introduced into competent NF-L1369 (*L. monocytogenes* *ΔnamA*) cells by electroporation according to a modified protocol described previously by Monk et al. (40). Following electroporation, bacteria were plated onto selective medium, and the resultant transformants were assessed for the presence of *namA* by PCR amplification of *namA* coding sequences from *L. monocytogenes* chromosomal DNA (pIMK2 is a pPL2 derivative and thus integrates in a single copy into the *L. monocytogenes* phage attachment site located within the tRNA^{Arg} gene following electroporation) (17, 32). The resultant pNF1688-complemented *ΔnamA* strain was designated NF-L1695.

Microscopic examination of bacterial morphology for monocultures and mixed cultures of wild-type and *ΔnamA* strains. To facilitate the microscopic examination and visual assessment of monocultures as well as mixed cultures, plasmid pAM401-*gfp3* (pNF1113) was introduced into NF-L476 (10403S *actA*-*gus*) and NF-L1369 (*ΔnamA*) by an electroporation method described previously by Park and Stewart (46). The GFP-expressing strains were designated NF-L1481 (wild type [WT] with pAM401-*gfp3*) and NF-L1482 (*ΔnamA* with pAM401-*gfp3*). These GFP-expressing strains were used for all subsequent microscopic assessments upon confirmation that plasmid pAM401-*gfp3* did not alter the growth characteristics and septation associated with either the wild type or the *ΔnamA* mutant strain.

For the examination of the cell morphology of monocultures, 100 μl of each culture (taken at the time points designated in the text) was added directly onto individual poly-L-lysine-treated coverslips and allowed to incubate for 5 to 10 min

at 37°C. The edges of the coverslips were then touched to a Kimwipe to remove excess liquid and unbound bacteria. The bacteria were subsequently fixed to the coverslips by the addition of 100 μl of 3.2% formaldehyde in phosphate-buffered saline (PBS) for 5 min at room temperature. Coverslips were then washed with 5 dips in PBS and allowed to air dry. Each coverslip was then mounted onto a glass slide using Vectashield mounting solution (Vector Laboratories, Inc., Burlingame, CA) and allowed to cure in the dark overnight at room temperature. Mixed-culture coverslips were prepared similarly, with a few modifications. In order to distinguish wild-type cells from cells of the *ΔnamA* mutant with pAM401-*gfp3*, bacteria were fixed (as described above), followed by the addition of 100 μl of a 1:320 dilution of Listeria O Antiserum Poly (Becton Dickinson, Sparks, MD) in PBS plus 1 mg/ml bovine serum albumin (BSA) for 30 min. Coverslips were washed by dipping five times in PBS. A 1:5,000 dilution of tetramethyl rhodamine goat anti-rabbit IgG (Molecular Probes, Eugene, OR) in PBS plus 1 mg/ml BSA was added dropwise to the coverslip, followed by incubation for 30 min at room temperature. The coverslip was then washed by dipping in PBS and subsequently mounted onto a glass slide with Vectashield and allowed to cure overnight in the dark. Thus, in mixed cultures, total bacteria appeared red, while *ΔnamA* mutants appeared yellow, due to the colocalization of rhodamine and GFP.

To determine the relative amounts of chain formation due to incomplete bacterial septation in wild-type, *ΔnamA*, *ΔnamA* with pIMK2-*namA*, and mixed wild-type and *ΔnamA* cultures, cells were processed for microscopy as described above, and the total number of cells in chains (greater than three linked cells) was counted for 10 independent fields and compared to the total number of bacteria present in each field. A ratio of the number of cells in chains to the total cell number was then calculated as a measure of chaining frequency. Numbers are derived from at least 3 independent experiments.

Measurement of the ability of bacterial supernatants to complement *ΔnamA* mutant phenotypes. Bacterial supernatants were collected from broth cultures of both wild-type and *ΔnamA* strains grown in BHI broth. Supernatants were obtained from cultures diluted 1 to 100 into 200 ml of fresh BHI broth, followed by shaking at 37°C for up to 8 h. At designated time points (2, 4, 6, and 8 h), 20 ml was removed from each culture, and bacteria were recovered by centrifugation at 12,350 × *g* for 10 min. The supernatant was then filter sterilized using a 0.22-μm vacuum filter (Corning, Corning, NY). These supernatants were then added to fresh cultures of the *ΔnamA* mutant strain to determine if supernatants derived from wild-type strains were capable of complementing the *ΔnamA* chaining phenotype. To roughly balance for the loss of nutrients associated with the use of filter-sterilized supernatants derived from late-exponential- and early-stationary-phase cultures, fresh BHI broth was supplemented in an approximated ratio to provide normal rates of bacterial growth to stationary phase and to comparable final cell densities (optical density at 600 nm [OD₆₀₀] of ~1.8). The calculated ratios were as follows: for supernatants derived from cultures grown for 2 h, no fresh BHI broth was added, since it was observed that essentially no nutrients had been depleted by the dilute starting culture for this time period; for supernatants derived from 4-h cultures, a 4:1 ratio of supernatant to fresh BHI broth was used; for supernatants derived from cultures grown for 6 h, a 3:1 ratio was used; and for supernatants derived from cultures grown for 8 h, a 2:1 ratio was used. Growth curves were done at each of these ratios using wild-type *L. monocytogenes* to confirm that the ratio of supernatant to fresh BHI medium was sufficient to allow comparable bacterial growth rates. Final cell densities obtained beginning from 1/100 culture dilutions incubated for 8 h (early stationary phase) were between OD₆₀₀ values of 1.7 and 1.8 for all cultures. To test for the ability of each supernatant to complement the chaining phenotype associated with a *ΔnamA* mutant, a 1-to-100 dilution of *ΔnamA* cultures was subsequently inoculated into 20 ml of the collected media from cultures grown for 2, 4, 6, and 8 h mixed with fresh BHI medium as indicated above. Cultures were allowed to grow for 4 h at 37°C to an OD₆₀₀ of ~0.5 with shaking, at which point 100-μl aliquots were removed and processed for microscopy to assess the presence and degree of filamentation as described above.

Isolation of bacterial supernatants and whole-cell lysates for protein analysis. Twenty-milliliter cultures of the wild-type and *ΔnamA* strains were grown overnight in BHI medium at 37°C with shaking. The following day, bacterial cells from the cultures grown overnight were washed twice in fresh BHI medium to remove any NamA protein present in the supernatant from cultures grown overnight. Bacteria were then diluted 1/20 into 200 ml of fresh BHI medium. At 3 h postinoculation and every 2 h thereafter, the OD₆₀₀ of each culture was measured, and 20-ml aliquots were removed. The culture aliquots were centrifuged at 12,350 × *g* for 10 min at 4°C to recover supernatant and bacterial cell fractions. Trichloroacetic acid (TCA) was added to the supernatant fractions to a final concentration of 10%, and the fractions were incubated on ice for 30 min. Precipitated protein was recovered by the centrifugation of the fractions at

12,350 $\times$ g for 10 min, and the resulting protein pellets were allowed to air dry. The pellets were subsequently washed with 4 ml of ice-cold acetone followed by centrifugation at 12,350 $\times$ g. The washed pellets were resuspended in 250 μ l of SDS boiling buffer (5% SDS, 0.5% β -mercaptoethanol, 10% glycerol, 60 mM Tris [pH 6.8]). For bacterial whole-cell lysates, the bacterial pellets (upon the removal of the supernatant) were resuspended in 125 μ l of lysis buffer (10% glycerol, 60 mM Tris [pH 6.8]), followed by the addition of lysozyme at a concentration of 10 μ g/ml and incubation for 1 h at room temperature. Samples were then sonicated 10 times on ice (pulse for 30 s followed by 1 min of rest between each pulse) prior to the addition of 125 μ l of 2 $\times$ boiling buffer, boiling for 5 min, and clarification by centrifugation at 11,000 rpm for 30 min at 4°C.

Western blot analysis. TCA-precipitated proteins and whole-cell lysates from both wild-type and Δ *namA* strains were loaded onto 10% SDS-PAGE gels. Loading volumes were altered based on the original bacterial optical density to account for the differences in bacterial CFU at each time point. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes at 1.0 A (constant) for 1 h. All membranes were blocked for 1 h in PBS (Gibco, Carlsbad, CA) containing 0.05% Tween (PBST) and 5% milk. Membranes were incubated with a 1:2,500 dilution of monoclonal antibody C11E9 in PBST (a kind gift of Arun Bhunia), which is known to specifically recognize NamA (19), or a 1:500 dilution of monoclonal antibody against internalin A (Toxin Technologies, Sarasota, FL) at 4°C overnight with shaking, followed by three additional washes with 30 ml of PBST for 10 min each. A 1:2,500 dilution of secondary goat anti-mouse antibody conjugated to alkaline phosphatase (Southern Biotech, Birmingham, AL) was incubated with shaking for 2 h at room temperature, followed by three additional 10-min washes in PBST. Detection was carried out by the addition of the colorimetric substrate BCIP-NBTplus (Southern Biotech, Birmingham, AL) for approximately 10 min. Images were acquired by using an AlphaImager 2200 instrument (Alpha Innotech, San Leandro, CA). NIH ImageJ software (<http://rsbweb.nih.gov/ij/>) was used to perform densitometric analyses on at least three independent blots. The 3-h time point was set to a value of 1.00, to which all other lanes were compared and represented as a deviation from that value. The averages and standard deviations (SD) were then calculated and are indicated in Fig. 3. An *L. monocytogenes* strain with an in-frame deletion of *inlA* (NF-L1004) was used as a negative control for InlA Western blots.

Purification of recombinant NamA and complementation of septation defects with purified protein. The DNA sequence corresponding to the mature form of the NamA enzyme was amplified from *L. monocytogenes* 10403S using primers NamA-6hisA and NamA-6hisB, generating a ~1.6-kb PCR product as previously described (10). The product was digested with StuI and KpnI and subcloned into the appropriately digested pQE30 N-terminal 6 $\times$ -His expression vector (Qiagen, Valencia, CA), followed by transformation into *E. coli* DH5 α I/q cells. The resulting strain was designated NF-E1808.

The expression and purification of recombinant NamA were carried out according to the instructions provided by the supplier of the pQE30 vector (Qiagen, Valencia, CA). Briefly, a 20-ml culture of NF-E1808 grown overnight was inoculated at a 1:50 dilution into 1 liter of LB medium containing ampicillin (100 μ g/ml). The culture was grown with shaking at 37°C until an approximate OD₆₀₀ of 0.5 was reached, at which point isopropyl- β -D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.8 mM. Cultures were allowed to continue growing for another 5 h, followed by centrifugation at 8,500 rpm for 15 min and subsequent freezing of the bacterial pellet at -80°C overnight. Bacterial cell lysates were prepared by resuspending the bacterial pellet in 50 ml of wash buffer (500 mM NaPO₄, 300 mM NaCl, 10 mM imidazole [pH 7.4]), followed by the addition of lysozyme (0.3 mg/ml) (Sigma, St. Louis, MO) and a protease inhibitor cocktail (Pierce, Pittsburgh, PA). The cell suspension was subsequently sonicated on ice 10 times (10 s on and 30 s off), followed by the addition of Triton X-100 (final concentration, 1%) and incubation at room temperature for 30 min. Lysates were clarified by centrifugation at 11,000 rpm for 30 min, followed by passage through a 0.22- μ m sterile filter. The purification of NamA from bacterial lysates was carried out by metal affinity chromatography using Cobalt resin according to the instructions provided by the supplier (Pierce, Pittsburgh, PA). The eluted protein fraction was dialyzed overnight against a final storage buffer (20 mM HEPES, 140 mM NaCl, 10% glycerol, 1 mM dithiothreitol [pH 7.4]), aliquoted, and frozen at -80°C. The final protein concentration was determined by using a bicinchoninic acid assay kit (Pierce, Pittsburgh, PA).

To assess the ability of purified NamA to complement the septation defects associated with a Δ *namA* mutant, 10 ml of BHI broth was supplemented with purified NamA (final concentration, 500 ng/ml), followed by the addition of a culture of the Δ *namA* strain plus pAM401-*gfp* (1:100 dilution) grown overnight. Cultures were allowed to grow at 37°C for 5 h (substantial chaining occurs for Δ *namA* mutants at this time), processed for microscopy as described above, and examined for the complementation of septation defects. Nonsupplemented cul-

tures of the WT and the Δ *namA* mutant with pAM401-*gfp* were grown and processed for microscopy as controls.

Plaque assays. Plaque assays were conducted as previously described (59). Monolayers of L2 fibroblasts were grown and infected at a multiplicity of infection (MOI) of 30:1. After 1 h, gentamicin was added to the medium at a concentration of 20 μ g/ml in an agarose medium overlay. After 3 days, plaque sizes and total numbers of plaques were enumerated. Results were obtained from at least three independent experiments.

Intracellular growth assays and fluorescent microscopy of intracellular bacteria. J774 macrophage-like and PtK2 epithelial cells were maintained as previously described (4, 61), while bone marrow-derived macrophages (BMMs) were cultured from female ND4 Swiss Webster mouse femurs as previously described (21, 59). Briefly, 2 $\times$ 10⁶ cells were placed onto glass coverslips the night before infection. For gamma interferon (IFN- γ)-treated BMMs, the medium was supplemented with 1 ng/ml of IFN- γ (Biosource, Carlsbad, CA) 18 h prior to infection. An MOI of 1:10 was used for all macrophages, and an MOI of 30:1 was used for PtK2 epithelial cells. Gentamicin (20 μ g/ml) was added to the medium of infected cells at 30 min (for macrophages) or 1 h (for PtK2 cells) and at the designated time points, three coverslips were removed, cells were lysed in 5 ml of water, and serial dilutions were plated onto LB agar plates to enumerate bacterial CFU. Fluorescent microscopy of *Listeria*-infected PtK2 cells was carried out as previously described (41). Coverslips were viewed with a DeltaVision microscope (Applied Precision, Issaquah, WA). Images were then captured using Softworx image acquisition software (Applied Precision, Issaquah, WA). For experiments assessing the effects of the inhibition of actin polymerization on bacterial septation, cytochalasin D was added to culture dishes (final concentration, 0.25 μ g/ml) as previously described (56). Because cytochalasin D is dissolved in dimethyl sulfoxide (DMSO), an equivalent volume of DMSO without cytochalasin D was added to all untreated dishes to control for the presence of this solvent in treated dishes. To remove cytochalasin D from infected cells, medium was aspirated, followed by washing 5 times with warm PBS and the subsequent addition of minimal essential medium (MEM) containing gentamicin (20 μ g/ml) only. An additional 2.5 h of infection was allowed to proceed after the removal of cytochalasin D to allow the reestablishment of actin architecture as well as actin-based motility prior to processing for microscopy.

Invasion and adherence assays. To measure the relative invasion efficiency of Δ *namA* mutants, PtK2 epithelial cell monolayers on glass coverslips were infected with the WT, the Δ *namA* mutant strain, or the Δ *namA* mutant plus pAM401-*gfp* at an MOI of 30:1. Infection was allowed to proceed for 1 h, followed by washing three times with warm PBS and the subsequent addition of MEM. At this point, three coverslips were removed, placed into 5 ml of sterile water, lysed by vortexing, and plated onto solid medium to determine the total bacterial burden per coverslip (extracellular and intracellular). Immediately thereafter, gentamicin (20 μ g/ml) was added to culture dishes and incubated for an additional hour. Three coverslips were then removed and used to determine total CFU (now enumerating only intracellular bacteria). The ratio of the number of bacterial CFU at hour 2 to the number of bacterial CFU at hour 1 was determined and is reflected as percent invasion for each strain tested (wild-type invasion was set to 100%).

To measure overall bacterial adherence to PtK2 epithelial cells, monolayers were again infected at an MOI of 30:1. Bacteria were allowed to adhere to and begin invading cells by incubation for 1 h. Cells were subsequently washed five times with warm PBS to remove any unbound bacteria, followed by the addition of warm MEM without antibiotics. Coverslips were immediately removed, and the total number of bacterial CFU per coverslip was determined as described above. The total amounts of bacteria, representing bacteria that have efficiently adhered to and begun invading cells, were compared for each strain tested.

Mouse intravenous infections. Animal procedures were approved by the IACUC and performed in the Biological Resources Laboratory at the University of Illinois at Chicago. Monoculture infections were performed as previously described (1). Briefly, 2 $\times$ 10⁴ CFU (from either exponential- or stationary-phase cultures) of *L. monocytogenes* was inoculated into female 7-week-old ND4 Swiss Webster mice via the tail vein. At 72 h postinfection, livers and spleens were harvested and homogenized. Dilutions of homogenates were plated onto BHI agar plates containing streptomycin (200 μ g/ml). Competitive index experiments were performed as previously described (2, 33). DP-L3903 (10403S::Tn917-LTV3) was used as the reference strain for all competitive index experiments. After 72 h of infection, mice were sacrificed, and the livers and spleens were harvested and homogenized. Prior to plating, erythromycin was added to the homogenates at a final concentration of 0.1 μ g/ml for 30 min to induce erythromycin resistance. The homogenates were then spread onto plates containing either just BHI medium or BHI medium containing erythromycin (1.0

FIG. 1. Construction of a *namA* in-frame deletion mutant and confirmation of its bacterial chaining phenotype. (A) Schematic of the $\Delta namA$ deletion mutant. The black arrow denotes the open reading frame (ORF) of *namA*, while the white and gray arrows denote upstream and downstream genes, respectively. A predicted transcriptional terminator immediately following the *namA* ORF is depicted by a stem-loop structure. The dashed bracket indicates the approximate deletion size of the *namA* ORF. (B) Wild-type and $\Delta namA$ strains containing plasmid pAM401-*gfp3* were fixed onto coverslips after 4 h of growth in broth culture and viewed using fluorescent microscopy. Identical results were also obtained for bacteria lacking plasmid pAM401-*gfp3* when cells were visualized by Gram staining (data not shown). (C) *trans*-Complementation of bacterial chaining in the presence of wild-type *L. monocytogenes*. Wild-type and $\Delta namA$ strains were mixed 1 to 1 and grown in BHI broth for 4 h prior to microscopy. For all microscopic images (B and C), a representative image of a minimum of 10 independently viewed fields is shown. FITC, fluorescein isothiocyanate. (D) The ratio of the total number of cells in chains (>3 cells linked on end) to the total number of cells per field was determined for wild-type, $\Delta namA$, and $\Delta namA$ plus pIMK2-*namA* cultures and for a 1:1 mixed culture of wild-type and $\Delta namA$ bacteria. A calculated ratio approaching 1 indicates that nearly all cells in a field were observed to be part of a chain, while a ratio approaching zero indicates the absence of bacterial chaining. At least 6 to 10 independent fields were observed in 3 independent experiments, and the average values are shown. Statistically significant differences (for D) are indicated and were determined by a one-way analysis of variance with Tukey's multiple-comparison test (***, $P < 0.0001$).

$\mu\text{g/ml}$). The following day, bacterial CFU were enumerated, and the ratio of the test strain to the reference strain was calculated as described above.

RESULTS

The loss of *namA* results in bacterial septation defects that can be complemented in *trans*. *L. monocytogenes* EGDe mutants lacking *namA* (33) (Imo2691, also known as *murA* [10]) (Fig. 1A) were previously reported to exhibit bacterial septation defects leading to the formation of bacterial chains (10). Consistent with these results, the *L. monocytogenes* 10403S-derived *namA* deletion strain also formed bacterial chains of 4 to 12 bacteria when grown in broth culture (Fig. 1B). As NamA is a secreted peptidoglycan hydrolase that can be detected in bacterial culture supernatants (10, 33, 34), we investigated whether the growth of the mutant in the presence of wild-type bacteria could complement the $\Delta namA$ -associated septation defect in *trans*. Equal numbers of *L. monocytogenes* wild-type and $\Delta namA$ (containing a plasmid-harbored copy of the GFP gene) bacteria were mixed and grown in BHI broth for 4 h, and the populations of bacterial cells were examined by using fluorescence microscopy. The growth of the $\Delta namA$ mutant in mixed cultures with wild-type bacteria resulted in the full complementation of the $\Delta namA$ septation defect (Fig. 1C).

A comparison of $\Delta namA$ morphologies in mixed cultures and monocultures indicated that for mutant monocultures, the ratio of the number of cells in chains to the total number of

cells visualized by Gram staining approached 1 after 4 h of growth in BHI broth, indicating that nearly all cells were comprised of chains greater than 3 bacteria in length (Fig. 1D). In contrast, monocultures of the wild type or the complemented $\Delta namA$ mutant with pIMK2-*namA* or mixed wild-type and $\Delta namA$ cultures had relatively small percentages of bacteria in chains compared to the total number of cells in each field (Fig. 1D). Mixed cultures did not become dominated by wild-type bacteria even after extended periods of growth, as evidenced by the maintenance of the 1:1 ratio of GFP-positive mutant bacteria to wild-type cells (Fig. 1C and data not shown). The presence of wild-type *L. monocytogenes* can thus functionally complement in *trans* the septation defect associated with the $\Delta namA$ mutation in broth culture.

NamA is predominantly surface associated during early stages of bacterial growth followed by its release into the supernatant upon entry into stationary phase. Carroll et al. (10) previously reported that a $\Delta namA$ mutant formed chains primarily during logarithmic growth and during entry into stationary phase. In contrast, following growth in broth culture overnight, the bacterial chains appeared to be disrupted, leaving predominantly single rod-shaped bacteria (10) (see Fig. 5A). The transition from chains to single bacteria after overnight incubation suggests that a second *L. monocytogenes* hydrolase expressed during stationary phase resolves the septation defect of the $\Delta namA$ chains formed during exponential

FIG. 2. *L. monocytogenes* secretes abundant NamA during late exponential growth. (A) Bacterial growth curve indicating the optical densities at which the culture was removed for the isolation of protein fractions. (B) Bacterial secreted proteins (TCA-precipitated proteins from supernatants [Sup.]) as well as whole-cell lysates from wild-type and $\Delta namA$ strains were collected at the indicated time points following bacterial inoculation into BHI broth. Prior to loading onto gels, sample volumes were adjusted to reflect equivalent bacterial densities by normalization to the OD₆₀₀. Western blot analysis was conducted using mouse monoclonal antibody C11E9 to detect NamA. The relative amount of protein in each lane was determined by densitometric analysis (with the wild-type 3-h time point lane set to 1.00) from 3 independent experiments. The averages and SD are shown. (C) Western blot analysis of the same samples in B using monoclonal antibody against the peptidoglycan-linked protein internalin A.

growth. Examination of the localization of NamA indicated that during the first 3 h of *L. monocytogenes* growth in broth culture, most of the NamA produced appeared to be associated with the bacterial cell, with a smaller amount released into the supernatant (Fig. 2A and B). As bacterial growth progressed, increasing amounts of NamA appeared to be released into the supernatant, with a peak of accumulation (>65% of total NamA) after 9 h of growth that corresponded with an increasing reduction in the relative amount of cell-associated NamA (Fig. 2B). The accumulation of secreted NamA in the supernatant did not appear to result from a general effect of cell wall turnover, as the abundance of the cell wall-anchored protein internalin A (InlA) remained essentially constant at the surface, with only a modest increase in the supernatant at late time points (Fig. 2C). Overall, these data suggest that NamA remains associated with the bacterial cell during exponential growth, reflecting a period of rapid cell division likely

to require extensive peptidoglycan remodeling. The protein then appears to be increasingly released into the supernatant during entry into stationary phase (Fig. 2B).

Exogenously added NamA is sufficient to resolve $\Delta namA$ septation defects for bacteria in broth culture. It was observed that the septation defects associated with the $\Delta namA$ mutant in broth cultures could be fully resolved by the addition of wild-type culture supernatants (Fig. 3). Consistent with the growth-phase-dependent cell association and release of NamA, the ability of wild-type culture supernatants to complement the $\Delta namA$ septation defect was growth phase dependent. Supernatants derived from wild-type cultures grown for 2 or 4 h did not resolve the $\Delta namA$ chaining defect; however, supernatants derived from cultures grown for 6 h partially restored septation, while supernatants derived from cultures grown for 8 h fully resolved $\Delta namA$ mutant chains (Fig. 3B). Supernatant fractions derived from $\Delta namA$ cultures failed to resolve any $\Delta namA$ -associated chaining defects (data not shown). A 10-fold concentration of the supernatants derived from wild-type cultures grown for 2 and 4 h partially restored septation, suggesting that a minimum amount of enzyme was required for full activity (data not shown).

To confirm that soluble NamA could resolve bacterial septation defects *in trans*, purified recombinant NamA (Fig. 3C) was added at a concentration of 500 ng/ml to $\Delta namA$ cultures in BHI medium. After 5 h of growth, culture sample aliquots were examined by microscopy. The addition of purified NamA completely restored the septation of a $\Delta namA$ mutant in culture (Fig. 3D). These data indicate that the cell wall alterations associated with $\Delta namA$ septation defects are accessible to exogenously added enzymes.

Despite defects in septation during exponential growth, $\Delta namA$ mutants exhibit only a modest attenuation for bacterial virulence when inoculated into mice as single bacterial cells. Lenz et al. (33) previously reported a modest virulence defect associated with a $\Delta namA$ mutant using competitive index assays with mice (a 2- to 7-fold defect in comparison to wild-type strains). However, given the observation that a $\Delta namA$ mutant can be *trans*-complemented for septation defects when mixed with wild-type bacteria in broth culture, we sought to determine whether *trans*-complementation may have occurred during the mixing of cultures for the competitive index assays, thereby potentially masking virulence defects associated with the $\Delta namA$ mutation. When mice were infected with either the wild type or the $\Delta namA$ mutant obtained from exponentially growing cultures, significant 2-log and 4-log reductions in the numbers of bacterial CFU recovered from the spleen and liver, respectively, were observed for $\Delta namA$ bacteria in comparison to wild-type bacteria (Fig. 4A). This difference in numbers of bacterial CFU was not attributable to differences in the bacterial plating efficiency (chains versus individual cells), as the plating method used to enumerate bacteria was determined to disrupt existing chains and enable accurate quantitation (see Fig. S1A in the supplemental material). The virulence defect of the $\Delta namA$ mutant could be partially complemented by the introduction of plasmid-carried *namA*; the partial complementation observed may reflect the expression of *namA* from a nonnative promoter (the Phelp promoter in vector pIMK2) (40) (Fig. 4A). Thus, as might be

FIG. 3. *L. monocytogenes*-secreted NamA is functional and is responsible for bacterial septation during exponential-phase growth. (A) Bacterial growth curve indicating the optical densities at which culture was removed for the isolation of bacterial supernatants. (B) Functional NamA secreted by wild-type bacteria upon entry into early stationary phase complements septation defects of $\Delta namA$ mutants. Wild-type supernatants were collected at the indicated time points after the inoculation of wild-type *L. monocytogenes* into BHI broth, filter sterilized, and assessed for their ability to complement the $\Delta namA$ mutant chaining phenotype. Mutant cultures were grown for 4 h in the presence of the indicated wild-type supernatants, and bacteria were then removed, fixed onto slides, and assessed for bacterial chain formation using fluorescent microscopy. For comparison, a $\Delta namA$ mutant culture (not supplemented with wild-type supernatant) was grown for 4 h and processed for microscopy (rightmost panel). (C) SDS-PAGE gel of purified recombinant NamA (2 μ g and 1 μ g). (D) Purified NamA at a concentration of 500 ng/ml fully resolves septation defects. The panels show bacterial cultures grown for 5 h. Representative images from at least 10 independently viewed fields in 3 independent experiments are shown (B and D).

anticipated, the infection of mice with exponential-phase $\Delta namA$ chains significantly reduces bacterial virulence.

Somewhat surprisingly, $\Delta namA$ attenuation was dramatically reduced when cultures of bacteria grown overnight were used for animal infections (Fig. 4B). The intravenous infection of mice with stationary-phase single-cell cultures of the $\Delta namA$ mutant resulted in modest 5- and 10-fold decreases in numbers of bacterial CFU recovered from the spleens and livers, respectively, in comparison to mice infected with wild-type *L. monocytogenes* (Fig. 4B). These results suggest that the virulence defect observed for exponentially growing cultures of the $\Delta namA$ mutant likely reflects a defect early in infection associated with the presence of bacterial chains. In contrast, infection with $\Delta namA$ stationary-phase single-cell cultures resulted in significantly lower levels of attenuation, suggesting that once infection is established, the loss of *namA* does not significantly impair bacterial growth and survival within the host. Consistent with the results previously reported by Lenz et al. (33), the competitive index observed for mice infected with mixed cultures of wild-type and $\Delta namA$ strains exhibited a modest 2- to 7-fold defect for the $\Delta namA$ mutant (Fig. 4C). Taken together, these results strongly suggest that the loss of NamA attenuates

L. monocytogenes virulence during the initial phases of host infection if the bacteria are inoculated as chains of cells. The restoration of septation in stationary-phase cells or by the presence of NamA supplied by wild-type bacteria in *trans* enables the single-cell *L. monocytogenes* $\Delta namA$ bacteria to efficiently establish infection with only a modest attenuation of virulence.

$\Delta namA$ strains exhibit normal intracellular growth and cell-to-cell spread but are compromised for host cell adhesion. The virulence of stationary-phase $\Delta namA$ cells in mice suggests that either bacterial chain formation within the host does not diminish virulence once an infection is established or, alternatively (and seemingly more likely), bacterial septation is occurring during host infection. Despite the septation defects observed for log-phase $\Delta namA$ cultures, the intracellular growth measured by counting the CFU of the $\Delta namA$ mutant in both J774 macrophages and primary mouse bone marrow-derived macrophages (BMMs) appeared indistinguishable from that of wild-type *L. monocytogenes* (Fig. 5B and C). The treatment of BMMs with IFN- γ restricted the growth of both wild-type and $\Delta namA$ strains, with $\Delta namA$ exhibiting a slightly more apparent and statistically significant

FIG. 4. Single-cell $\Delta namA$ mutants are modestly attenuated *in vivo* in comparison to $\Delta namA$ bacterial chains. (A) $\Delta namA$ mutants are severely attenuated for virulence using a monoculture mouse model of infection with bacteria present as chains. Mice were intravenously infected with 2×10^4 CFU of the wild type, the $\Delta namA$ mutant, or the $\Delta namA$ mutant plus pIMK2- $\Delta namA$ (grown to exponential phase; OD₆₀₀ of ~0.6), and the bacterial burden in livers and spleens was determined at 72 h postinfection. (B) Single-cell stationary-phase (Stat.) $\Delta namA$ mutants are modestly attenuated for virulence using a monoculture mouse model of infection. Mice were intravenously infected with 2×10^4 CFU of the wild type, the $\Delta namA$ mutant, or the $\Delta namA$ mutant plus pIMK2- $\Delta namA$, and the bacterial burden in livers and spleens was determined at 72 h postinfection. (C) *trans*-Complementation of bacterial chaining prior to infection by wild-type *L. monocytogenes* results in a nearly complete masking of the virulence defect associated with exponentially growing $\Delta namA$ mutant cells. Mice were coinoculated with 10403S containing a silent Tn917-LTV3 transposon insertion (contains an erythromycin resistance marker) and the wild type or the Tn917-LTV3-labeled strain along with the $\Delta namA$ mutation. After 72 h a competitive index ratio was calculated by plating organ homogenates onto both BHI agar and BHI agar containing erythromycin (1 μ g/ml). The ratio of the test strain (wild type or

growth restriction (Fig. 5B). In addition to the apparently normal rates of bacterial intracellular replication, $\Delta namA$ mutants exhibited no defects in cell-to-cell spread, as evidenced by the size of infection foci or plaques formed during the infection of L2 mouse fibroblast cell monolayers (Fig. 5D). The mutants did exhibit an approximately 50% reduction in the total number of plaques formed in L2 cells, which is indicative of either a bacterial invasion and/or adhesion defect ($\Delta namA$ mutant cells formed $47.3\% \pm 4.2\%$ of the number of plaques formed by the wild type, whereas the complemented mutant strain formed $116.5\% \pm 5.1\%$ of the total plaques in comparison to the wild type) (Fig. 5D and data not shown).

Given that a number of peptidoglycan hydrolases were previously reported to contribute to bacterial adhesion to nonphagocytic cells (7, 38, 45, 68), *L. monocytogenes* $\Delta namA$ mutant adhesion and invasion were more closely examined in association with another nonprofessional phagocytic cell line, PtK2 epithelial cells. The number of $\Delta namA$ intracellular bacteria recovered from infected PtK2 cells in comparison to wild-type bacteria or the complemented mutant strain was significantly reduced (Fig. 5E). To distinguish between adherence or invasion defects, PtK2 cells were infected with either mutant or wild-type bacteria for 1 h, followed by gentamicin treatment, with total numbers of bacterial CFU determined before and after the addition of gentamicin. Only modest differences in $\Delta namA$ invasion were observed in comparison to that of the wild type, regardless of whether $\Delta namA$ cells were present as single cells (derived from stationary-phase cultures) or as chains (derived from exponential-phase cultures) (Fig. 5F, left). However, when cells were infected for 1 h and washed with PBS prior to the assessment of the total numbers of cell-associated bacteria, a 10-fold decrease in bacterial adherence was observed for the $\Delta namA$ mutant in comparison to the wild-type or the complemented strain (Fig. 5F, right). The difference in bacterial adherence observed was the same regardless of the bacterial growth phase (Fig. 5F). Thus, while $\Delta namA$ strains exhibit an adherence defect for nonprofessional phagocytic cells, the mutants are fully capable of intracellular growth and cell-to-cell spread within infected monolayers.

$\Delta namA$ mutants exhibit intracellular septation defects within the cytosol of infected host cells that are resolved by actin polymerization. The infection of tissue culture cells indicated that $\Delta namA$ mutants were capable of intracellular growth and cell-to-cell spread; however, microscopic examination of infected cells indicated that septation defects were visible for mutant bacteria replicating within the cytosol of epithelial cells (Fig. 6) as well as within macrophages (data not shown). As single-cell stationary-phase bacteria were used for the tissue culture infections (Fig. 5A), chain formation occurred only for the replicating intracellular $\Delta namA$ mutants protected from gentamicin, and the majority of bacterial chains were associated with

$\Delta namA$ mutant) to the reference strain (10403S::Tn917-LTV3) was then determined. Statistical analysis was carried out by using a one-way analysis of variance with Dunnett's posttest. ***, $P < 0.0001$; **, $P < 0.001$; *, $P < 0.05$. ●, wild-type strain; □, $\Delta namA$ strain; ▲, $\Delta namA$ strain plus pIMK2- $\Delta namA$; ▼, 10403S::Tn917-LTV3 and WT mixed; ×, 10403S::Tn917-LTV3 and the $\Delta namA$ strain mixed.

FIG. 5. The $\Delta namA$ mutant exhibits normal intracellular growth in phagocytic cells but is defective for the adherence to and/or invasion of epithelial cells and fibroblasts. (A) Fluorescent microscopy of bacterial cells from cultures grown overnight (O/N) used in tissue culture infection assays. (B, C, and E) Mouse bone marrow-derived macrophages (BMM) (B), J774 macrophage-like cells (C), or PtK2 epithelial cells (E) were infected with the wild type, the $\Delta namA$ mutant, and the $\Delta namA$ mutant plus pIMK2-*namA* (PtK2 cells only). After 30 min (BMM and J774 cells) or 1 h (PtK2 cells), the monolayers were washed, and gentamicin (20 μ g/ml) was added. Intracellular growth was determined following the lysis of host cells and the enumeration of CFU on LB agar plates. (D) Bacterial plaque formation of the wild type and the $\Delta namA$ mutant in L2 fibroblasts. (F) PtK2 epithelial cells were infected with the wild type, the $\Delta namA$ mutant, and the $\Delta namA$ mutant plus pIMK2-*namA* for 1 h, followed by extensive washing and the enumeration of CFU on LB agar plates (adherence) or the addition of medium containing gentamicin (20 μ g/ml) and a comparison of the total numbers of CFU before and after the addition of antibiotic (invasion). The data shown are representative of at least three independent experiments done in triplicate. ●, wild type; □, $\Delta namA$ mutant; ▲, $\Delta namA$ mutant plus pIMK2-*namA*. Solid line, without gamma interferon; dashed line, with the addition of gamma interferon (1 ng/ml) 18 h prior to infection (B). Statistically significant differences (for B, E, and F) are indicated and were determined by a one-way analysis of variance with Tukey's multiple-comparison test. (*, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$).

host cell actin (Fig. 6). More than 90% of all $\Delta namA$ mutant-infected cells contained bacteria in chains throughout the first 7 h of infection (Fig. 6 and data not shown). At 3 and 5 h postinfection, short bacterial chains coated with actin were clearly visible; however, beginning at around 7 h the bacterial cells began to show evidence of separating from the chains, with visible actin filament formation located at the bacterial cell poles (Fig. 6). At approximately 9 h postinfection, numerous single-cell bacteria with actin-associated comet tails were evident in $\Delta namA$ -infected cells (Fig. 6). In contrast, no intracellular chain formation was observed for cells infected with

wild-type *L. monocytogenes* (Fig. 6). Thus, while septation defects were evident for $\Delta namA$ mutants within the host cell cytosol, chain formation appeared to be disrupted by the *L. monocytogenes*-directed process of actin polymerization.

To determine if the force of actin polymerization was indeed capable of separating individual cells from *L. monocytogenes* intracellular chains, cytochalasin D (an inhibitor of actin polymerization) was added at 1 h postinfection, and the infections were continued and monitored by microscopic examinations over time. In the presence of cytochalasin D, $\Delta namA$ mutants formed chains beginning at early time points postinfection (3

FIG. 6. The $\Delta namA$ mutant exhibits intracellular bacterial chains during infection of epithelial cells that are resolved by host cell actin polymerization. PtK2 epithelial cells were infected at an MOI of 30:1 with either the wild type or the $\Delta namA$ mutant. At 1 h postinfection, cells were washed, and gentamicin (20 $\mu\text{g/ml}$) was added. Beginning at 3 h postinfection and at 2-h intervals thereafter, coverslips were removed and processed for fluorescent microscopy. Each image is representative of at least 10 independently viewed fields. Enlarged representative regions from each image are shown as insets. Intracellular $\Delta namA$ chains are visible as long yellow-green filaments, whereas moving bacteria appear as red rods followed by long green actin tails.

and 5 h), with chain formation continuing into later stages of growth (7 and 9 h) (Fig. 7). By 9 h postinfection, $\Delta namA$ mutants were seen to exist as long, uninterrupted bacterial chains. Evidence of actin association was apparent at the poles of individual bacterial cells within the chains; however, the inhibition of actin polymerization prevented movement and tail formation, leaving the chains of $\Delta namA$ mutants intact (Fig. 7). In contrast to what was seen for $\Delta namA$ bacteria, cells infected with wild-type bacteria in the presence of cytochalasin D exhibited normal patterns of growth in the absence of actin-based motility (Fig. 7). If cytochalasin D was removed after 7 h of infection and the incubation was continued with fresh me-

FIG. 7. The $\Delta namA$ mutant forms elongated chains during infection of epithelial cells in which actin polymerization has been inhibited. PtK2 epithelial cells were infected at an MOI of 30:1 with either the wild type or the $\Delta namA$ mutant. At 1 h postinfection, cells were washed, and gentamicin (20 $\mu\text{g/ml}$) as well as cytochalasin D (CytoD) (0.25 $\mu\text{g/ml}$) were added. Beginning at 3 h postinfection and at 2-h intervals thereafter, coverslips were removed and processed for fluorescent microscopy. Each image is representative of at least 10 independently viewed fields. Enlarged representative regions from each image are shown as insets.

dium containing gentamicin, within 2.5 h nearly all $\Delta namA$ bacteria were observed as individual cells associated with host cell actin tails (Fig. 8). These experiments indicate that the force of actin polymerization is sufficient to resolve the septation defects associated with $\Delta namA$ mutants within the cytosol of infected host cells. Consistent with this observation, other physical forces (such as homogenization and shaking of bacterial cultures in the presence of glass beads) also result in the disruption of $\Delta namA$ chains (see Fig. S1 in the supplemental material, and data not shown). The disruption of bacterial chains via the forces exerted by actin polymerization is thus likely to account for the substantial level of virulence observed

FIG. 8. Restoration of actin polymerization disrupts $\Delta namA$ intracellular chains. PtK2 epithelial cells were infected at an MOI of 30:1 with either the wild type or the $\Delta namA$ mutant. At 1 h postinfection, cells were washed, and gentamicin (20 $\mu\text{g/ml}$) as well as cytochalasin D (0.25 $\mu\text{g/ml}$) were added. The infection was allowed to proceed for 7 h, followed by the removal of cytochalasin D, extensive washing with PBS, and further incubation for 2.5 h. Coverslips were processed for microscopy prior to the removal of cytochalasin D (7 h) as well as after the removal of cytochalasin D (9.5 h).

for $\Delta namA$ mutants in animal models of infection despite *in vitro* septation defects.

DISCUSSION

The Gram-positive bacterial cell wall fulfills a variety of functions, serving as a rigid structure that maintains cell shape and counteracts turgor pressure from the cytoplasm. Its cross-linked peptidoglycan has been described to be a single, flexible, three-dimensional macromolecule that must be constantly turned over and remodeled for cell growth, cell division, and protein secretion (5). In this report, we demonstrate that although the peptidoglycan hydrolase NamA is required for bacterial septation during the exponential growth of *L. monocytogenes*, this defect can be readily resolved within infected cells by the physical forces generated via actin polymerization. The disruption of $\Delta namA$ chains by cytosolic actin polymerization enables the spread of single bacteria to adjacent cells. This intriguing example of host compensation for a bacterial defect serves to clarify why $\Delta namA$ mutants are only modestly attenuated for growth and virulence when inoculated as single cells into animal hosts.

The modest virulence defect that remains for $\Delta namA$ mutants when inoculated as single cells may result from the reduction in bacterial adhesion that was observed in association with the infection of nonprofessional phagocytic cells. NamA could potentially contribute to host cell adhesion through the active remodeling of the bacterial cell wall during infection so as to promote the interactions of other bacterial adhesins with host cell receptor molecules. Alternatively, NamA itself could

act as an adhesin and directly contribute to host cell surface binding. As an example, the autolysin Ami functions as an adhesin independent of its catalytic activity and in a manner that is likely to be dependent on its GW repeat modules (38). While NamA does not have GW repeat modules, it does have a series of LysM-containing repeats that could potentially contribute to bacterial adhesion at the host cell surface. Associations of bacterial autolysins with cell adhesion was observed previously for other Gram-positive pathogenic bacteria (22–24). It is possible that these enzymes may recognize and bind host cell surface molecules that resemble bacterial cell wall targets.

The ability of secreted NamA to *trans*-complement *namA*-deficient strains so effectively in broth culture suggests an interesting functional flexibility for this hydrolase, especially given previous reports that initially localized NamA strictly to the bacterial surface. Several previously reported studies classified NamA with primary functions closely associated with the bacterial surface based on the presence of its LysM peptidoglycan binding domains (3, 6, 7, 10), its detection at the bacterial surface (8, 52), and its ability to serve as an immunoreactive epitope in *L. monocytogenes* detection studies (19, 20, 44). Other studies, however, have detected NamA in supernatant fractions derived from *L. monocytogenes* strains containing a mutationally activated form of the virulence regulator PrfA [PrfA(L140F)] as well as in wild-type supernatants in comparison to *secA2* mutants (33, 50), although those studies did not determine if secreted NamA was functionally active. The ability of wild-type bacteria, bacterial supernatants, and purified

NamA to fully complement the phenotypic defects associated with the $\Delta namA$ mutant indicates that secreted NamA is functional and capable of recognizing what must be accessible targets in *trans* to promote septation. It is conceivable that both forms of NamA, the cell-associated and the secreted forms, contribute to normal bacterial growth. The biological rationale for maintaining the full activity of a bacterial hydrolase in culture supernatants is somewhat intriguing. Many peptidoglycan hydrolases have been shown to serve dual purposes, providing a beneficial role for the producer but a toxic role for competitor organisms (15, 35, 55, 65, 66). It will be interesting to determine the relative toxicity of NamA toward common competing bacterial species.

Although *L. monocytogenes* encodes a number of peptidoglycan hydrolases (including p45, p60, lmo0327, Auto, Ami, and IspC) (48, 49, 67), none of these enzymes appears to be capable of compensating for the loss of NamA. This lends support to the hypothesis that hydrolases fulfill distinctly important and specific functions in cell wall physiology, although some of these functions are likely to be complementary. For example, the loss of p60 results in abnormal *L. monocytogenes* septum formation and in the formation of bacterial cell chains that disappear as the bacteria enter stationary phase; however, the length of these chains is shorter than that of chains formed as a result of the loss of *namA* (47). p60 mutants exhibit a cell-to-cell spread defect in infected tissue culture cells, whereas a similar defect was not observed for *namA* mutants (47) (Fig. 5). Host cell actin localizes at bacterial poles in p60 mutants but cannot induce normal tail formation thereafter. As a result, p60 mutants appear to remain in chains, are unable to spread from cell to cell, and are markedly attenuated for virulence (47). The p60 enzyme is predicted to cleave the peptide bond linking the *D*-iso-glutamine and the *meso*-diaminopimelic acid moieties of the peptide side chain in *L. monocytogenes* peptidoglycan (33, 37), while NamA shares homology with enzymes that cleave the *N*-acetylmuramide-*N*-acetylglucosamine linkage (33). It would appear that the strength of the peptidoglycan bonds that connect individual bacteria within chains in the absence of NamA is relatively modest, at least in comparison to p60 mutants.

One of the most surprising findings of this study was the observation that actin polymerization directed by *L. monocytogenes* provided sufficient force to disrupt bacterial chains formed during intracellular growth so as to enable cell-to-cell spread. The finding that the chain disruption was mediated via actin polymerization strongly suggests that ActA is appropriately positioned at the poles of individual bacterial cells during chain formation and must be accessible for the binding and recruitment of actin and actin binding proteins. In addition to chain disruption by actin polymerization, we have observed that the shaking or vortexing of *L. monocytogenes* $\Delta namA$ mutants in the presence of glass beads is also sufficient to break up chains (see Fig. S1 in the supplemental material). The observation that $\Delta namA$ mutants are compromised for cell adhesion but not for cell invasion (Fig. 5) suggests that the forces of bacterial internalization are also sufficient to disrupt chain formation. Taken together, these results provide an interesting example of fundamental host cell processes compensating for a bacterial mutant defect during the process of infection.

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