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Listeria monocytogenes 10403S HtrA Is Necessary for Resistance to Cellular Stress and Virulence

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The HtrA serine protease has been shown to be essential for bacterial virulence and for survival after exposure to many types of environmental and cellular stresses. A *Listeria monocytogenes* 10403S *htrA* mutant was found to be sensitive to oxidative and puromycin-induced stress at high temperatures, showed a reduced ability to form biofilms, and was attenuated for virulence in mice.

The highly conserved family of HtrA (also known as DegP) serine proteases is involved in the stress response of several important gram-negative, as well as gram-positive, pathogens. The proposed function of HtrA is to degrade misfolded or aggregated proteins formed after exposure to harmful environments such as high temperature or oxidative stress (5). Without a mechanism to rid the cell of the aberrant proteins that accumulate after such exposure, survival of the bacteria can be compromised. HtrA has been shown to be essential for the virulence of many pathogens, but it is not essential for the growth of most bacteria under nonadverse conditions. As such, it qualifies as a potential "antipathogenic" drug target. These targets include those that inhibit virulence, as opposed to those that kill bacteria or stop their growth (15). It is assumed that antipathogenic drugs reduce the pressure for the development of resistance to the drug. This would be an important attribute given the rampant spread of resistance to today's antibacterial compounds among both gram-positive and gram-negative organisms.

Recently, HtrA was reported to be necessary for survival of *Listeria monocytogenes* 10403S in elevated NaCl concentrations, for high-temperature growth (44°C), and for resistance to oxidative damage caused by hydrogen peroxide (24). In additional studies, *L. monocytogenes* EGDe HtrA was found to be involved in sensitivity to acid conditions (pH 5) and penicillin G-induced stress and was necessary for efficient colonization of spleens of BALB/c mice (22). In order to gain further understanding of the role of HtrA in *L. monocytogenes* physiology and pathogenesis and to provide support for the hypothesis that HtrA protease is a valid target for a novel class of anti-infectives for gram-positive organisms, further characterization of the phenotype of an *L. monocytogenes* 10403s *htrA* mutant was initiated.

An in-frame deletion of the *L. monocytogenes htrA* gene was constructed. Primers RW9 (5′-CCGCAAGGCTTTTTCAAACGATAGGGC-3′) and RW11 (5′-CGGGGTACCTAAAGTATCCTCGACCTCTCTTTTCGG-3′) and primers RW12 (5′-CCGGGTACCGCAGCAATCAATCCAGGTAAC-3′) and RW10 (5′-CCGGAATTCACCCTCTTTTTCAAGAGAATG-3′) (IDT, Iowa City, IA) were used to PCR amplify the 5′ and 3′ regions of *htrA* by using *L. monocytogenes* 10403S (18) chromosomal DNA (DNeasy Tissue Kit, QIAGEN, Valencia, CA) as a template. The PCR products were introduced into plasmid pCR2.1 (Invitrogen, Carlsbad, CA), forming plasmids pCR-htrA5′ and pCR-htrA3′. pCR-htrA3′ was digested with PstI and EcoRI, and the resulting 524-bp fragment was ligated to PstI-EcoRI-digested pKSV7 (21). A 542-bp BamHI-KpnI product from pCR-htrA-5′ was subsequently introduced into this plasmid, forming plasmid pKSV-htrAΔ. This plasmid created an in-frame deletion of the *htrA* gene that encodes the first 40 N-terminal amino acids and the last 166 C-terminal amino acids of the predicted 542-amino-acid HtrA protein. Integration of temperature-sensitive plasmid pKSV-htrAΔ into the *L. monocytogenes* 10403S chromosome and resolution of the plasmid were performed as previously described (3) to create the *htrA* mutant designated SRL47. Primers RW1 (5′-CGCAAGGCTTTTTCAAACGATAGGGC-3′), specific for the chromosomal region upstream of *htrA* (but not included in pKSV-htrAΔ), and RW8 (5′-CCGCGGATCCGTCACGTAAGGATACACCTAGAG-3′), specific for the 3′ region of *htrA* (included in plasmid pKSV-htrAΔ), were used to confirm by PCR that the *htrA* deletion was located in the chromosomal *htrA* locus. The DNA sequence of the chromosomal *htrA* deletion in SRL47 was determined to be correct by sequencing a PCR fragment generated with primers RW9 and RW10 by using SRL47 chromosomal DNA as a template. The growth rate of the *htrA* mutant at 44°C, but not at 30°C or 37°C, was greatly decreased (not shown), as has been previously described (24).

The antibiotic puromycin interrupts chain elongation during protein synthesis in bacteria, and this leads to the generation of truncated and misfolded proteins. Accumulation of these peptides can lead to cellular stress. *Staphylococcus aureus*, *Brucella melitensis*, and *Lactococcus lactis htrA* mutants show a higher sensitivity to puromycin-induced stress than wild-type strains, suggesting a role for HtrA in the degradation of the truncated proteins (8, 17, 19). To test whether *L. monocytogenes* HtrA played a similar role in the degradation of puromycin-induced peptides, 10-fold dilutions of wild-type and *htrA* mutant cultures (optical density at 600 nm [OD_{600}] = 0.7) were made and

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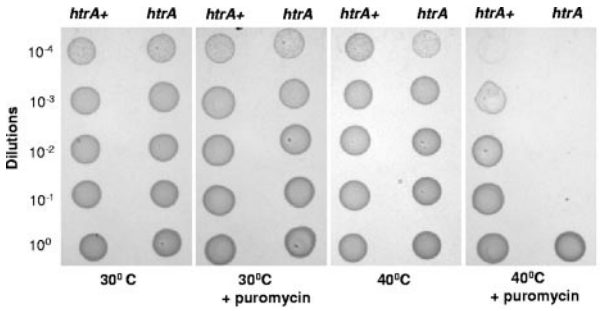

FIG. 1. An *L. monocytogenes htrA* mutant exhibits increased sensitivity to puromycin at high temperatures. *L. monocytogenes htrA*$^+$ and *htrA* mutant strains were grown in BHI broth to an OD_{600} of ~0.7. Tenfold dilutions of the cultures were made, and 10 μl of each dilution was spotted onto BHI agar with or without 8 μg/ml puromycin. Plates were incubated at 30°C or 40°C overnight.

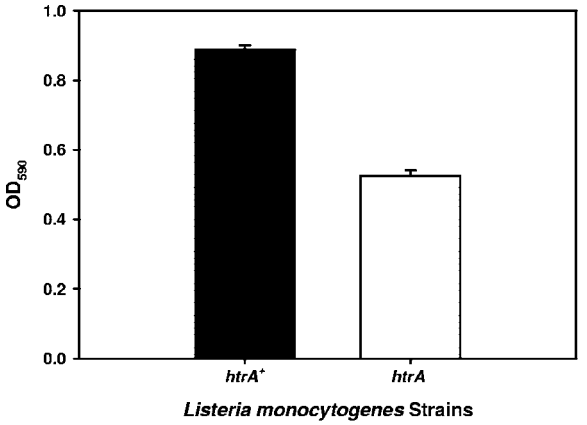

FIG. 2. HtrA is required for efficient *L. monocytogenes* biofilm formation at high temperatures. A microtiter plate crystal violet biofilm assay was performed after 72 h of incubation of wild-type or *htrA* mutant *L. monocytogenes* cultures at 40°C. Data are presented as the average OD_{590} of four wells, normalized to the cell culture densities (at 590 nm). Error bars represent the standard error of the mean. Student's *t* test was used for statistical analysis. The difference in the levels of biofilm formation between wild-type *L. monocytogenes* 10403S and the *htrA* mutant was significant ($P < 0.001$).

spotted onto brain heart infusion (BHI; BD Biosciences, Sparks, MD) agar containing puromycin. A growth defect was noted for most dilutions of the *L. monocytogenes htrA* mutant when grown at 40°C, but not 30°C, in the presence of 8 μg/ml puromycin (Fig. 1). Another *L. monocytogenes* serine protease, ClpP, has been shown to be involved in the degradation of puromycin-induced peptides (9). The results shown in Fig. 1 indicate that HtrA plays an important role, in addition to ClpP, in the elimination of abnormal proteins induced by puromycin treatment.

Recent work reported by S. Ahn et al. (1) indicated that *S. mutans htrA* mutants exhibited altered sucrose-dependent biofilm formation. Formation of biofilms involves many surface proteins, such as adhesins, receptors, and flagella. Because HtrA has also been shown to be necessary for the proper surface expression or secretion of several proteins from *Staphylococcus aureus*, *Streptococcus mutans*, and *Lactococcus lactis* (7, 8, 19), the ability of the *L. monocytogenes htrA* mutant to form biofilms was examined. A crystal violet biofilm assay was performed as previously described (2). Overnight cultures of wild-type and *htrA* mutant *L. monocytogenes* were diluted 1:40 in BHI broth, and 200-μl aliquots were dispersed into four wells of a 96-well Nunc Maxisorp Immunspot plate (VWR, Brisbane, CA). After incubation at either 30°C or 40°C, cells were removed and the OD_{590}s of the cultures were determined. The wells were washed with distilled water three times and stained with 0.1% crystal violet (in 70% ethanol). Residual crystal violet was dissolved with 95% ethanol, and the OD_{590}s of the wells were read. Figure 2 demonstrates that the *htrA* mutant did not form biofilms as efficiently as the wild-type parent at the higher temperature, even though the cultures reached the same ODs. At 30°C, both the wild type and the *htrA* mutant formed similar levels of biofilms (not shown). A recent study identified *L. monocytogenes* proteins that were up-regulated during growth in the biofilm state at 37°C (11). Interestingly, a protein similar to the ClpP protease was found to be induced twofold during growth of *L. monocytogenes* in biofilms. Future studies will address whether the ClpP-like protease and HtrA perform overlapping functions or whether each plays a particular role in biofilm formation by *L. monocytogenes*.

The *S. aureus* hemolysins and *agr*-regulated secreted virulence factors, and the *S. pyogenes* virulence factors SpeB and hemolysin, depend upon HtrA for proper expression (19). The involvement of HtrA in the expression of the secreted *L. monocytogenes* 10403S hemolysin, listeriolysin O, was examined. The *L. monocytogenes htrA* mutant still produced and exported listeriolysin O, as determined by hemolysis of BHI blood agar plates at either 37°C or 40°C (not shown). *L. monocytogenes* expresses flagella at lower temperatures (<30°C) but at higher temperatures (>37°C), production of flagella in several *Listeria* strains (including *L. monocytogenes* 10403S) is down-regulated (10, 16, 23). No differences in motility were noted between wild-type and *htrA* mutant *L. monocytogenes* grown on motility agar (BHI with 0.4% agar) at 25°C or after growth at 25°C and a shift to 37°C or 42°C, indicating that the HtrA protease was not necessary for decreased motility of, or flagellar expression in, *L. monocytogenes* at higher temperatures (data not shown).

The intracellular pathogenic lifestyle of listeriae exposes them to many different types of stresses. After adherence and internalization by macrophages, listeriae are taken into the phagosomal compartment, where most of the bacteria are destroyed (6). Only a small fraction of the bacteria are able to escape into the cytosol. During their time in the phagosome, listeriae encounter several antimicrobial compounds, such as nitric oxide, and the products of the respiratory burst, including superoxide radicals (14, 20). These toxic compounds can cause damage to essential proteins, DNA, and other cellular components. Pathogenic bacteria possess many different genetic loci that contribute to their ability to survive in the presence of these toxic compounds (4, 12, 14). *htrA* is one such locus that, in many gram-negative and gram-positive bacteria, has been shown to be necessary for resistance to oxidative stress. HtrA presumably accomplishes this by ridding the cell of damaged proteins generated as a result of the action of immune cells of the host (12). To determine whether the *L.*

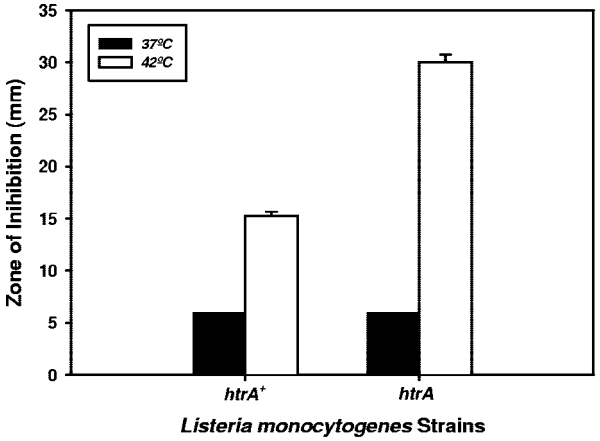

FIG. 3. An *L. monocytogenes htrA* mutant is more susceptible to paraquat than the wild type. Sensitivity of *L. monocytogenes* to paraquat was tested by plating wild-type or *htrA* mutant overnight cultures on BHI agar. Six-millimeter sterile filter disks were placed on the agar plates, paraquat was added to the disks, and the plates were incubated at either 37°C or 42°C overnight. The zones of growth inhibition around the disks were measured. Data represent the average of the zones of inhibition from four different disks ± the standard error of the mean. The Mann-Whitney rank sum test was used for statistical analysis. The difference in the levels of sensitivity to paraquat between wild-type *L. monocytogenes* 10403S and the *htrA* mutant at 42°C was significant ($P < 0.004$).

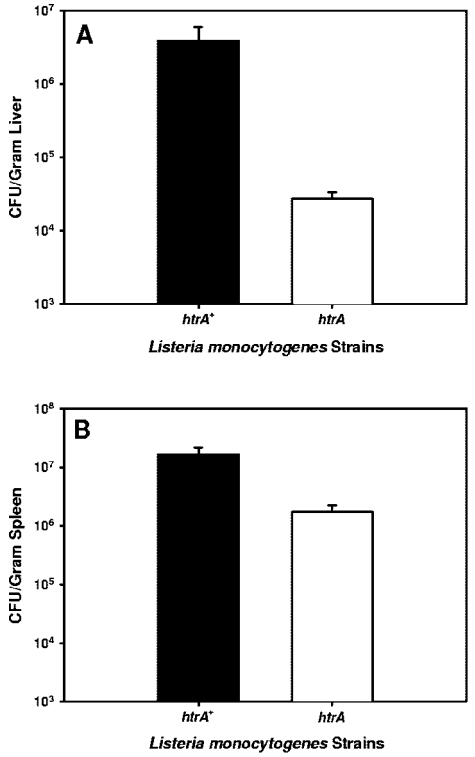

FIG. 4. HtrA is required for full virulence of *L. monocytogenes* 10403S in mice. Eight-week-old female BALB/c mice (groups of five) were injected intravenously with 2×10^4 CFU of wild-type or *htrA* mutant *L. monocytogenes*. Three days postchallenge, the numbers of CFU in their livers (A) and spleens (B) were determined. Data represent the mean (± the standard error of the mean [error bars]) number of CFU per organ from organs of 10 mice pooled from two separate experiments. The Mann-Whitney rank sum test was used for statistical analysis. The numbers of CFU per liver ($P < 0.009$) and per spleen ($P < 0.002$) in *htrA* mutant-infected mice were significantly reduced compared to those in wild-type *L. monocytogenes* 10403S-infected mice.

monocytogenes HtrA protein plays a role in resistance to cellular stress caused by oxidants, a paraquat disk diffusion assay was performed. Overnight cultures were diluted 1:300 in BHI before spreading 50 μl of culture on BHI agar. Sterile 6-mm filter disks were placed on the agar plates, 10 μl of 2 M paraquat was added to the disks, and the plates were incubated at either 37°C or 42°C overnight. As shown in Fig. 3, a wider zone of growth inhibition was measured around the *htrA* mutant. Hence, the HtrA protease is involved in the resistance of *L. monocytogenes* to oxidative stress caused by superoxide radicals generated by redox-cycling agents such as paraquat.

We hypothesized that an increased sensitivity to oxidizing reagents such as paraquat in vitro may affect the ability of an *L. monocytogenes* 10403S *htrA* mutant to survive in vivo. In support of this hypothesis, recent studies by Stack et al. demonstrated that intraperitoneal infection of BALB/c mice with an *L. monocytogenes* EGDe *htrA* mutant resulted in decreased colonization (~1 log) of spleens compared to wild-type strains (22). To examine the virulence of our *L. monocytogenes* 10403S *htrA* mutant, 8-week-old female BALB/c mice (Charles River, five mice per group) were intravenously administered 2×10^4 CFU of wild-type *L. monocytogenes* 10403S or the *htrA* deletion strain. After 3 days, mice infected with the *htrA* mutant looked visibly healthier (i.e., more active, less ruffled) than those infected with wild-type *L. monocytogenes*. Spleens and livers were removed, tissues were homogenized in 0.2% IGEPAL (Sigma, St. Louis, MO), and serial dilutions of the suspensions were plated on BHI agar medium containing streptomycin. Mice inoculated with the *htrA* mutant showed an approximately 2-log reduction in the bacterial load in the liver and an approximately 1-log-decreased level of colonization of spleens compared to mice infected with wild-type *L. monocytogenes* (Fig. 4). Hence, HtrA is required for full virulence of *L. monocytogenes* 10403S in mice.

In conclusion, *L. monocytogenes* 10403S HtrA was found to be necessary for resistance to puromycin-induced and oxidative stress and growth in biofilms at high temperatures. Most importantly, HtrA was essential for full virulence of *L. monocytogenes* 10403S in mice. It will be interesting to determine whether HtrA plays a role in biofilm formation in other clinically important pathogenic gram-positive bacteria, such as *Enterococcus faecalis* (13), *S. aureus*, and *Staphylococcus epidermidis* (25), whose ability to form biofilms is an important pathogenic determinant. If so, it will provide additional support for pursuing HtrA as a potential target for antibacterial therapeutics for gram-positive pathogens.

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