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Disruption of the Genes for ClpXP Protease in *Salmonella enterica* Serovar Typhimurium Results in Persistent Infection in Mice, and Development of Persistence Requires Endogenous Gamma Interferon and Tumor Necrosis Factor Alpha

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The enteric pathogen *Salmonella enterica* serovar Typhimurium, similar to other facultative intracellular pathogens, has been shown to respond to the hostile conditions inside macrophages of the host organism by producing a set of stress proteins that are also induced by various environmental stresses. The stress-induced ClpXP protease is a member of the ATP-dependent proteases, which are known to be responsible for more than 90% of all proteolysis in *Escherichia coli*. To investigate the contribution of the ClpXP protease to the virulence of serovar Typhimurium we initially cloned the *clpP* and *clpX* operon from the pathogenic strain serovar Typhimurium χ3306 and then created insertional mutations in the *clpP* and/or *clpX* gene. The Δ*clpP* and Δ*clpX* mutants were used to inoculate BALB/c mice by either the intraperitoneal or the oral route and found to be limited in their ability to colonize organs of the lymphatic system and to cause systemic disease in the host. A variety of experiments were performed to determine the possible reasons for the loss of virulence. An oxygen-dependent killing assay using hydrogen peroxide and paraquat (a superoxide anion generator) and a serum killing assay using murine serum demonstrated that all of the serovar Typhimurium Δ*clpP* and Δ*clpX* mutants were as resistant to these killing mechanisms as the wild-type strain. On the other hand, the macrophage survival assay revealed that all these mutants were more sensitive to the intracellular environment than the wild-type strain and were unable to grow or survive within peritoneal macrophages of BALB/c mice. In addition, it was revealed that the serovar Typhimurium ClpXP-depleted mutant was not completely cleared but found to persist at low levels within spleens and livers of mice. Interferon gamma-deficient mice and tumor necrosis factor alpha-deficient mice failed to survive the attenuated serovar Typhimurium infections, suggesting that both endogenous cytokines are essential for regulation of persistent infection with serovar Typhimurium.

Salmonellae are facultative intracellular parasites responsible for a variety of disease syndromes, ranging from acute gastroenteritis to systemic infections like typhoid fever. *Salmonella enterica* serovar Typhimurium, which generally causes gastroenteritis in humans, can establish systemic infections in mouse that closely resemble typhoid fever in humans. Though many factors required for the virulence of salmonellae have been studied, the molecular mechanisms by which salmonellae cause disease are only beginning to be elucidated. Two major contributors to serovar Typhimurium virulence are encoded within the pathogenicity islands SPI-1 (46) and SPI-2 (26, 53), located at 63 and 30 centisomes on the chromosome, respectively, that code for a type III secretion system. SPI-1 is required for efficient invasion of the intestinal epithelium, suggesting a role in early infection, and SPI-2 is needed for survival and growth within macrophages, indicating a role in systemic infection (25, 26, 53). Besides the genes found within SPI-1 and SPI-2, molecular genetic approaches involving random or gene-targeted mutagenesis have identified many other bacterial genes associated with virulence in various animals and in vitro model systems, such as cultured macrophage cells and epithelial cells from various sources (1, 16, 26, 38). These mutations can be roughly classified according to their assigned gene functions, such as auxotrophy (e.g., *aroA, aroC, aroD, purA*, and *purE*), regulation of gene expression (e.g., *cya, crp, rpoS, rpoE*, and *phoP*), or secretion (*ompR*) (10, 13, 14, 26, 27, 29, 45, 52).

Stress response genes encode a group of proteins collectively referred to as stress proteins (for a review, see reference 47), which are induced in response to hostile environments. They have been studied by focusing on their potential roles in the virulence of various facultative intracellular bacteria, including serovar Typhimurium. During the complex multistage pathogenesis, bacteria are exposed to a variety of environmental stress conditions such as sudden elevated temperature, high osmolarity, oxidative damage, nutrient depletion, and bactericidal mechanisms associated with the host immune system (17). To successfully colonize the host organism and to avoid clearance by the immune system, a large number of general

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stress response systems as well as specific virulence factors would be required. The hypothesis that a global stress response plays an important role in the successful colonization and expression of virulence was initially based on indirect evidence, such as induction of the DnaK and GroEL homologues during intracellular growth of serovar Typhimurium within macrophages (5). DnaK and GroEL are major stress proteins that bind to unfolded polypeptides and function as molecular chaperones in cells (47). These chaperones stabilize the unfolded structure, protecting it from aberrant folding and nonspecific interactions with other proteins. In response to the intracellular stress associated with phagocytosis, induced levels of chaperones would be required to cope with the accumulation of partially unfolded or denatured proteins in the cell. Direct evidence for the essential role of stress proteins in bacterial virulence was originally demonstrated by insertional mutation of the stress protein gene *htrA*, which resulted in the inability of serovar Typhimurium to grow inside macrophages (30). The *htrA* gene encodes a periplasmic serine protease which is necessary for the degradation of abnormally folded proteins transported into the periplasmic space (61). Salmonellae maintain long-term residence in host phagocytic cells with bactericidal mechanisms, suggesting that intracellular bacteria experience a considerable amount of protein misfolding and damage within this compartment. It is proposed that HtrA functions as a stress protein to protect the extracytoplasmic component from damage. The essential role of the *htrA* homologue as a virulence factor has also been demonstrated in other facultative intracellular pathogens, such as *Yersinia enterocolitica* (69) and *Brucella melitensis* (55).

ATP-dependent proteolysis is involved in more than 90% of all cell protein turnover in *Escherichia coli* and appears to be essential for the rapid breakdown of abnormal protein (42). Most ATP-dependent proteolysis in *E. coli* has been attributed to two well-characterized proteases, Lon and Clp, which are also stress-induced proteins (for a review, see reference 17). Two types of Clp protease exist in *E. coli*, ClpP and ClpQ (or HslV). The ClpP proteolytic component associates with either of two ATPases, ClpA or ClpX (21, 43), whereas the ClpQ proteolytic subunit associates with the ClpY (or HslU) ATPase (32, 58). ClpP and ClpQ are not related in either amino acid structure or mode of proteolysis. The ClpP subunits form a cylindrical heptameric particle possessing the catalytic core of a serine protease (36, 43). Substrate specificity is determined by either ClpA or ClpX as a regulatory ATPase.

Though the disruption of *clpP* in *E. coli* creates no obvious phenotype and the bacteria appear to grow normally (43), the ClpP protease has a more important and diverse role in gram-positive bacteria. The disruption of the *clpP* gene in *Bacillus subtilis* causes pleiotropic effects. The *B. subtilis clpP* deletion mutant is highly filamentous and nonmotile (48) and cannot grow under several stress conditions, being most severely affected by starvation (67) and high temperature (48). ClpP is also required for sporulation in *B. subtilis* (48). The inactivation of the *clpP* gene in *Lactococcus lactis* results in significant loss of cell viability (18), indicating a major role for ClpP in basic cell metabolism. *Streptomyces coelicolor* contains at least two *clp* genes, *clpP1* and *clpP2*. Disruption of the *clpP1* gene in *S. coelicolor* blocks differentiation at the substrate mycelium step (12). The importance of ClpP has been also demonstrated in connection with bacterial pathogenesis. In *Yersinia enterocolitica*, a gastrointestinal pathogen in humans and animals, ClpP proteolysis modulates *ail* gene expression (54). Ail is a 17-kDa cell surface protein that confers resistance to serum killing and the ability to attach and invade cells in vitro (4). In *Listeria monocytogenes*, a gram-positive facultative intracellular pathogen responsible for infrequent but often serious opportunistic infections in humans and animals, ClpP plays a crucial role in intracellular parasitism and virulence (19). In *S. enterica* serovar Typhimurium, the *clpP* gene was detected in a pool of transposon-tagged mutants with attenuated virulence (26), but the mutant has not been precisely characterized.

Recently, we cloned the *clpP clpX* operon of *S. enterica* serovar Typhimurium pathogenic strain χ3306, constructed insertional mutations in the operon, assayed their pathogenicities in an animal system, and found that disruption of the *clpP* and *clpX* genes results in persistent infection with serovar Typhimurium rather than loss of virulence in BALB/c mice. In this report, we demonstrate that the depletion of ClpXP protease in serovar Typhimurium results in inability to survive and multiply within peritoneal macrophages, inability to cause systemic infection, and ability to cause persistent infection in BALB/c mice. In addition, we show evidence that endogenous gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) are required for development of a persistent infection with serovar Typhimurium in mouse.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. Bacteria were routinely grown in L-broth and L-agar (Difco Laboratories, Detroit, Mich.). When necessary, the media were supplemented with ampicillin (50 μg/ml), kanamycin (25 μg/ml), chloramphenicol (25 μg/ml), and/or nalidixic acid (25 μg/ml).

Animals. The mice used in this study were all 5 to 8 weeks of age and included BALB/c, C57BL/6, IFN-γ-deficient (IFN-$\gamma^{-/-}$) mice from a C57BL/6 × Sv129 cross (64), and TNF-α-deficient (TNF-$\alpha^{-/-}$) mice from a C57BL/6 × Sv129 cross (65).

DNA isolation and manipulation, and PCR amplification and sequencing. DNA purification, ligation, restriction analysis, and gel electrophoresis were carried out as described by Sambrook et al. (59). Restriction enzymes, T4 DNA ligase, and Klenow enzyme were products of Takara Shuzo (Ohtsu, Japan). The DNA fragments of the *clpP* region were amplified using genomic DNA of *S. enterica* serovar Typhimurium strains χ3306, CS2007, CS2016, and CS2018 as a template by PCR using primers S66 (5′TAAGCGTCGTGTAGTTGTCG), S529 (5′CCGTCCATCAGGTTACAATC), S589 (5′ATGTCATACAGCGGAGAA CG), A1110 (5′AGATTGACCCGTATGATGCG), A1211 (5′CAATTACGAT GGGTCAAAAT), and A2828 (5′TTTCCCACACATTCAACGGC). Southern blotting was done basically as described before (69), and hybridizations using the ECL direct nucleic acid labeling and detection system (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) were performed according to the manufacturer's instructions. Sequencing was carried out with Sequenase (USB, Cleveland, Ohio) and synthetic primers.

Insertional inactivation of *clpP* and *clpX* in strain χ3306. Plasmid pTKY320 was cleaved at the *Eco*RV sites situated at nucleotide (nt) 682 and nt 802 (see Fig. 1) and ligated to the chloramphenicol (Cm) resistance gene block generated from the *Bam*HI-digested and filled-in pNK2884. The resultant plasmid, pTKY323, was cleaved at the *Mlu*I site situated at nt 138 and *Hin*dIII in the vector plasmid, and the overhanging ends were filled in with Klenow enzyme. The generated *clpP*:Cm fragment was ligated to the filled-in *Eco*RI site of pTKY229, which is a transferable suicide vector previously constructed by us (70). A suicide feature of pTKY229 is based on one of the replication origins of R6K, *ori*γ, which is functional only in a host when the π protein is encoded by the *pir* gene. The resulting mutator plasmid, pTKY349, carrying *clpP*:Cm was introduced into strain SM10λ*pir*, which can mobilize the plasmid by the conjugative function provided in *trans* from the RP4 integrated chromosome. Conjugative crosses with serovar Typhimurium χ3306 were carried out as previously reported

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
S. enterica serovar Typhimurium		
χ3306	Nx resistance derivative from SR-11	Provided by R. Curtiss III
CS2007	*clpP*:Cm in χ3306	This study
CS2016	*clpP*:Km in χ3306	This study
CS2018	*clpX*:Cm in χ3306	This study
CS2022	*lon*:Cm in χ3306	This study
χ3306 *rpoE*	*rpoE*:Km in χ3306	This study
E. coli		
SM10 λ*pir*	*thi thr leu tonA lacY supE recA*:RP4-2Tc::MuKm λ*pir*	70
JM109	*recA endA gyrA thi hsdR supE recA* Δ*(lac-proAB)*/F′ *(traD proAB*$^+$ *lacI*^{q}*) lacZΔM15*	Our collection
Plasmids		
pTKY229	Carrying *ori*γ of R6K and *oriT* of RP4	69
pTKY320[b]	pTW229 with 1.3-kb *Fsp*I-*Pst*I fragment containing *clpP* region	This study
pYKY323	pTKY320 carrying *clpP*:Cm	This study
pTKY349	pTKY229 with 2.4-kb *Mlu*I-*Pst*I fragment containing *clpP*:Cm	This study
pTKY366	pTKY320 carrying *clpP*:Km	This study
pTKY367	pTW229 with 0.9-kb *Nru*I-*Eco*RI fragment containing part of *clpX*	This study
pTKY368	pTKY229 with 2.0-kb *Mlu*I-*Pst*I fragment containing *clpP*:Km	This study
pTKY369	pTKY367 carrying *clpX*:Cm	This study
pCLP01	Carrying *E. coli-clpP*	Provided by M. Kitagawa
pNK2884	Carrying Cm resistance gene cassette	33
pUC18K	Carrying Km resistance gene cassette	44
pTW229	Cloning vector	Our collection
pHSG422	Ts for replication, Ap, Km, Cm resistance	24

[a] Abbreviations: Nx, nalidixic acid; Cm, chloramphenicol; Km, kanamycin; Ap, ampicillin; Ts, temperature sensitive.
[b] The structure of the *clpP* region is shown in Fig. 1.

(70). The chromosomal *clpP* was replaced by the *clpP*:Cm construct by double recombination. The *clpP* mutant was selected by resistance to chloramphenicol and nalidixic acid. Allelic exchange was checked by Southern blot analysis and direct sequencing of the *clpP*:*cat* region in the resultant strain (CS2007) mutant amplified by PCR.

To generate a nonpolar mutation in the *clpP* gene, *Eco*RV-cleaved pTKY320 was ligated to the kanamycin (Km) resistance gene block generated from *Sma*I-digested pUC18K. The resultant plasmid, pTKY366, was digested with *Mlu*I and *Hin*dIII, and the overhanging ends were filled in with Klenow enzyme. The generated *clpP*:Km fragment was ligated to the filled *Eco*RI site of suicide vector pTKY229. Strain SM10λ*pir*, harboring the resulting mutator plasmid pTKY368 with *clpP*:Km, was mobilized into serovar Typhimurium χ3306 by conjugation. The chromosomal *clpP* was replaced by the *clpP*:Km construct by double recombination. The *clpP* mutant was selected by resistance to kanamycin and nalidixic acid. Allelic exchange was checked by Southern blot analysis and direct sequencing of the *clpP*:Km region in the resultant strain (CS2016) amplified by PCR.

To insert a mutation into the *clpX* gene, pTKY367 was cleaved at *Cla*I site situated at nt 2020 and then ligated to the *cat* gene block prepared from pNK2884. The resulting plasmid, pTKY369, was partially digested with *Eco*RI and at the generated *clpX*:Cm of the *Eco*RI site of suicide vector pTKY229. The chromosomal *clpX* was replaced in the same way used to construct the *clpP*:Cm mutant. Allelic exchange was checked by Southern blot analysis and direct sequencing of the *clpP*:Cm region in the resulting strain (CS2018) amplified by PCR.

Immunoblot analysis. Equivalent numbers of bacterial cells were suspended in sample buffer (35), boiled for 5 min, and subjected to sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis. Separated proteins on the gels were transferred onto Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, Mass.) as suggested by the manufacturer. Proteins were reacted with rabbit anti-*E. coli* ClpX (1:25,000) antibody or anti-*E. coli* Lon antibody (1:25,000), followed by alkaline phosphatase-conjugated anti-rabbit immunoglobulin G as the secondary antibody. The enzymatic reactions were performed in the presence of nitro blue tetrazolium (30 mg/ml) (Dojindo, Kumamoto, Japan) and bromochloroindolylphosphate (Amresco, Solon, Ohio) (15 mg/ml).

Survival and growth of serovar Typhimurium strains in macrophage cells. The ability of the different strains of serovar Typhimurium to survive and grow in macrophage cells was assessed by using resident peritoneal macrophages prepared from BALB/c mice. The macrophages were harvested from peritoneal lavage using cold phosphate-buffered saline (PBS), washed with Hanks' balanced salt solution (HBSS; Sigma, Saint Louis, Mo.) suspended in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, N.Y.) containing 10% fetal calf serum (FCS) and 50 μM β-mercaptoethanol, allowed to adhere to each well of 24-well plates, and then incubated at 37°C before infection. Bacterial cells were grown in L-broth at 37°C to the exponential growth phase (optical density at 600 nm of approximately 0.5), and a portion was opsonized with 50% fresh normal mouse serum for 15 min at 37°C and diluted in DMEM. Opsonized bacteria were added to each well at a multiplicity of infection of 5. The plates were centrifuged for 5 min at 500 × *g* to enhance and synchronize infection. The cells were incubated for 30 min at 37°C to permit phagocytosis, and the free bacteria were removed by three washes with HBSS warmed at 37°C. DMEM containing 10% FCS and 100 μg of gentamicin per ml was added, and the cells were incubated for 1.5 h at 37°C. The cells were washed with the warmed HBSS three times, followed by incubation with DMEM containing 10% FCS and 10 μg of gentamicin per ml at 37°C. Wells were sampled at various times by aspirating the medium, three washes with HBSS, and lysing each well with PBS containing 0.1% sodium deoxycholate. The triplicate samples were plated individually after appropriate dilutions.

Determination of viable bacteria in organs of mice after infection with serovar Typhimurium. Serovar Typhimurium strains grown in L-broth at 37°C to the late exponential growth phase were diluted in sterile PBS. The actual number of bacteria present was determined by counting viable cells. Mice were challenged either orally or intraperitoneally by injection. The spleens and livers were aseptically removed at indicated times after infection and homogenized in PBS. The numbers of viable bacteria in the organs of infected mice were established by plating serial 10-fold dilutions of organ homogenates on L-agar plates. Colonies were routinely counted 18 to 24 h later.

Nucleotide sequence accession number. The sequence data of the complete operon reported here will appear in the DDBJ/EMBL/GenBank nucleotide sequence database under accession number AB033628.

RESULTS

Genetic organization of the strain χ3306 *clpP* locus. To determine the nucleotide sequence of the *clpP* locus of *S. enterica* serovar Typhimurium χ3306, we initially cloned the

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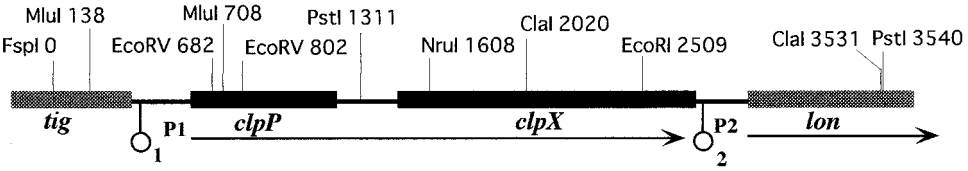

FIG. 1. Genomic organization and restriction site of the serovar Typhimurium χ3306 *clpP clpX* locus. The DNA sequence established in the present work extends from the *Fsp*I site at position 0 to the *Pst*I site at position 3540 (DDBJ/EMBL/GenBank database accession number AB033628). The horizontal arrows indicate the direction of transcription. The promoters P1 and P2 were deduced from the sequences. Potential stem-loop structures numbered 1 and 2 are indicated (circles).

16-kb *Bam*HI fragment prepared from chromosomal DNA which hybridized to the DNA fragment containing the *E. coli clpP* gene from pCLP01 (data not shown). By using subclones and synthetic oligonucleotides, the sequence of the *clpP* locus was determined. A total of 3,540 bp were sequenced, revealing the presence of four open reading frames (the region is shown schematically in Fig. 1). The ClpP protein predicted from the sequence was 99.0% identical to ClpP of *E. coli*. Downstream was an open reading frame encoding a protein 97.6% identical to ClpX of *E. coli*. Immediately upstream of the ATG start codon for *clpP*, a consensus ribosome-binding site and putative −10 and −35 sequences were located. There is an intergenic region of 252 bp between the *clpP* and *clpX* genes. No apparent promoter elements for *clpX* were found in this region. A consensus ribosome-binding site was identified 9 bp upstream of *clpX*. A potential stem-loop structure is located downstream of *clpX*, indicating a rho-independent transcriptional terminator. Downstream of *clpX*, the sequence is assigned to a homologue of the *E. coli lon* gene, which encodes an ATP-dependent serine protease (8), with a consensus ribosome-binding site. A consensus heat shock promoter sequence (9) was also identified upstream of the *lon* gene. Upstream of *clpP*, the sequence is predicted to be a homologue of the *E. coli tig* gene, whose product is known to be a ribosome-associated chaperone, trigger factor (23).

Construction of *clpP* and *clpX* disruption mutants of strain χ3306. Since sequence analysis revealed that the *clpP* gene exists in an operon with *clpX*, we constructed a *clpP clpX* double mutant, a *clpP* mutant, and a *clpX* mutant. The *clpP* gene was insertionally inactivated in vitro by using a Cm resistance cassette, and this construct was used to create the serovar Typhimurium χ3306 *clpP*:Cm mutant (strain CS2007) by allelic exchange. The disruption of the *clpP* gene in CS2007 was confirmed by PCR and Southern blotting (Fig. 2). To examine whether the insertion affected the expression of *clpX*, which is downstream of *clpP*, lysates from the wild-type parent and strain CS2007 were subjected to immunoblot analysis with anti-ClpX antiserum (Fig. 3A). The absence of the band corresponding to ClpX suggests that insertion of the cassette in *clpP* resulted in a polar mutation in the *clpX* gene. Since a *lon* gene which is downstream of *clpX* is preceded by a consensus promoter sequence, it is unlikely that the insertion of a polar mutation in the *clpP* gene blocks the expression of the *lon* gene. This was confirmed by immunoblot analysis with anti-Lon antiserum (Fig. 3B). The result suggests that the insertion of the Cm cassette in the *clpP* gene does not affect the expression of the *lon* gene.

To create a nonpolar mutation in *clpP*, a Km resistance cassette that allows natural downstream transcription (44) was used. This cassette has no promoter or transcription terminator. In addition, the Km resistance gene is preceded by a translation stop codon and immediately followed by a consen-

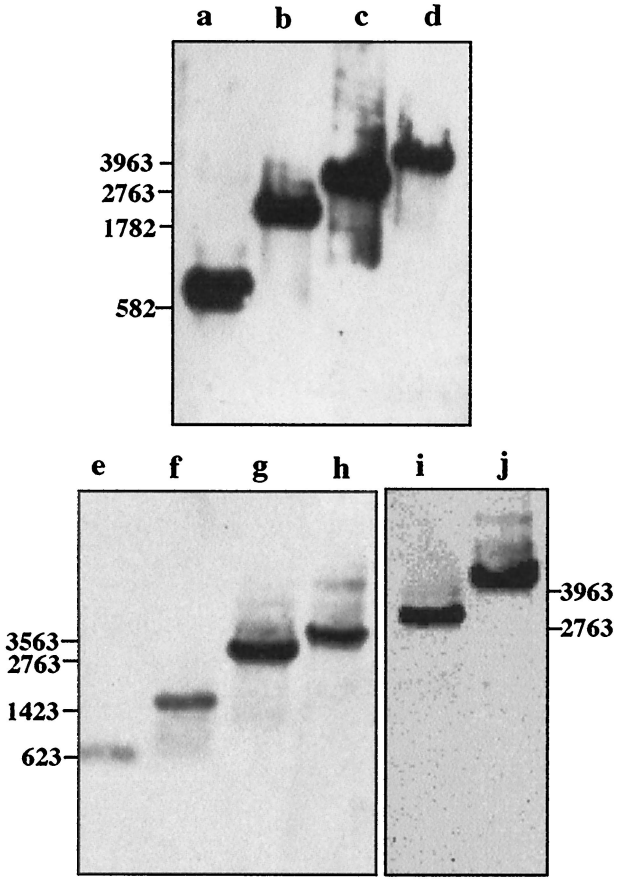

FIG. 2. Southern blot analysis showing insertional inactivation of *clpP* and *clpX* in mutant derivatives of serovar Typhimurium χ3306. The *clpP* or *clpX* region was amplified by PCR, using genomic DNA prepared from strains χ3306 (lanes a, c, e, g, and i), CS2007 (*clpP*:Cm; lanes b and d), CS2016 (*clpP*:Km; lanes f and h), and CS2018 (*clpX*:Cm; lane j) as templates and three sets of oligonucleotide primers, S529-A1110 (a and b), S589-A1211 (e and f), and S66-A2828 (c, d, g, h, i, and j). The nucleotide sequences of these primers are shown in Materials and Methods. The PCR products were separated on a 1% agarose gel by electrophoresis and subjected to Southern hybridization using the DNA fragments between nt 529 and 1110, covering the entire *clpP* open reading frame (a to h), and between nt 1743 and 2391, carrying a part of the *clpX* gene (i to j), as probes. Sizes are shown in nt.

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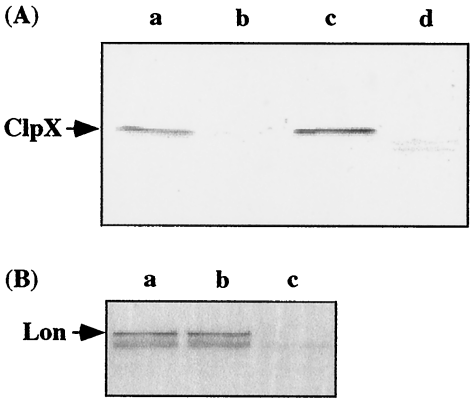

FIG. 3. (A) Immunoblot analysis of proteins from serovar Typhimurium strains χ3306 (lane a), CS2007 (*clpP*:Cm; lane b), CS2016 (*clpP*:Km; lane c), and CS2018 (*clpX*:Cm; lane d) with an antiserum against *E. coli* ClpX. (B) Immunoblot analysis of proteins from strains χ3306 (a), CS2007 (*clpP*:Cm; b), and CS2022 (*lon*:Cm; c) with an antiserum against *E. coli* Lon.

sus ribosome-binding site. The disruption of the *clpP* gene in the resulting strain, the *S. enterica* serovar Typhimurium χ3306 *clpP*:Km mutant (strain CS2016), was confirmed by PCR and Southern blotting (Fig. 2). The immunoblot analysis with anti-ClpX antiserum (Fig. 3A) suggests that the insertion of the cassette in the *clpP* gene does not affect the expression of the *clpX* gene.

The *clpX* gene was insertionally inactivated in vitro by using a Cm resistance cassette, and this construct was used to create the serovar Typhimurium χ3306 *clpX*:*cat* mutant (strain CS2018) by in vivo homologous recombination. The disruption of the *clpX* gene was confirmed by PCR and Southern analysis of DNA prepared from the mutant (Fig. 2) and by immunoblot analysis using anti-ClpX antiserum (Fig. 3A).

Analysis of the role of the ClpXP protease genes in virulence in mouse. One estimation of the virulence of serovar Typhimurium is the ability of the bacteria to establish a lethal systemic infection in mice. To measure the contribution of the ClpXP protease in serovar Typhimurium virulence, we determined the abilities of serovar Typhimurium *clpP*:Cm, *clpP*:Km, and *clpX*:Cm mutants to grow in the organs of BALB/c mice. As shown in Fig. 4A, mice that were infected by intraperitoneal administration with 10^2 CFU of χ3306 had more than 10^6 bacteria in both the spleen and liver on day 5 after infection. All five mice infected with χ3306 died at day 5. In contrast, mice infected with mutant strain CS2007 (*clpP*:Cm), CS2016 (*clpP*:Km), or CS2018 (*clpX*:Cm) appeared to be much more capable of controlling infection. Mice infected with each mutant strain had approximately 10^2 bacteria in the spleen and 10^3 bacteria in the liver. Unlike mice infected with the wild-type strain χ3306, which colonizes the spleen and liver in large numbers, resulting in death 5 days after infection, all mice challenged with mutant strain CS2007, CS2016, or CS2018 survived beyond day 5 after infection, indicating that these strains had lost the ability to cause a systemic infection in mice.

BALB/c mice were inoculated orally with 2×10^8 cells of strain χ3306 or CS2007 (*clpP*:Cm). The number of bacteria in spleens and livers was assessed on day 5 after challenge (Fig. 4B). The wild-type strain χ3306 colonized the spleen and liver in large numbers and resulted in death 5 days after infection. Again, the ClpXP-depleted mutant strain, CS2007, exhibited an impaired ability to cause a systemic infection in mice.

Depletion of the ClpXP protease in strain χ3306 impairs survival in macrophages. One of the most probable factors contributing to the reduced virulence of serovar Typhimurium

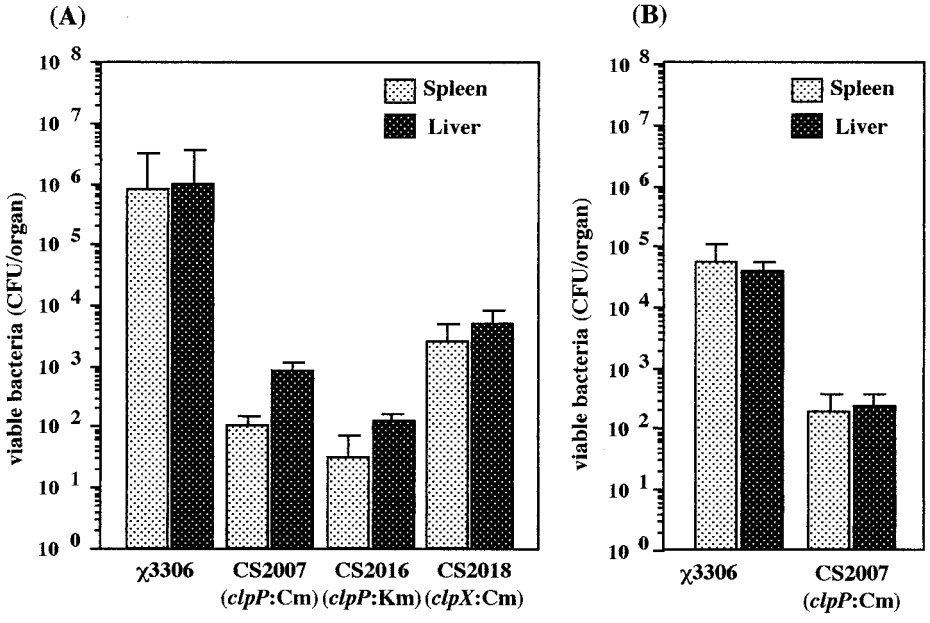

FIG. 4. Colonization of the organs of BALB/c mice following intraperitoneal (A) and oral (B) administration of serovar Typhimurium strains χ3306, CS2007 (*clpP*:Cm), CS2016 (*clpP*:Km), and CS2018 (*clpX*:Cm). On day 5 after infection, the numbers of bacteria recovered from the spleens and livers of five mice were determined. The error bars indicate the standard deviations of the means of these counts.

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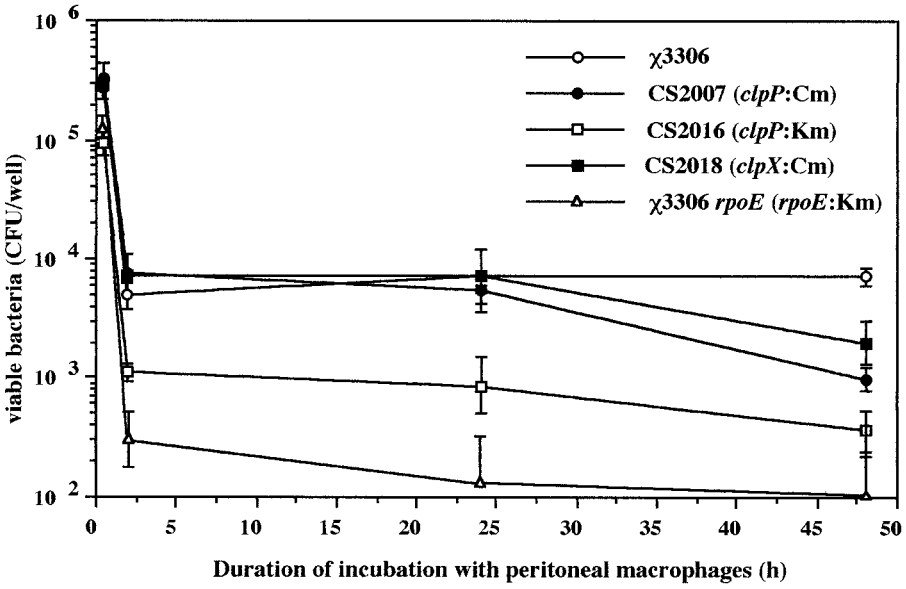

FIG. 5. Fate of serovar Typhimurium strain χ3306 and mutant derivatives within peritoneal macrophages prepared from BALB/c mice. The error bars indicate the standard deviations of the means of triplicate samples assayed individually.

Δ*clpP* and Δ*clpX* mutants is the reduced capacity to survive interactions with professional killing cells such as macrophages in mouse. To address this possibility, we examined the capacity of mutant strains CS2007 (*clpP*:Cm), CS2016 (*clpP*:Km) and CS2018 (*clpX*:Cm) to survive and grow in macrophages. It is known that serovar Typhimurium grown under conditions such as hyperosmolarity (0.3 M NaCl) or a change in the pH of the medium from 6.0 to 8.0 that allow expression of the type III secretion system encoded by SPI-1 readily kills cultured macrophages (7, 11). To avoid the cytotoxic effect of expression of the type III secretion system, bacterial cells were grown in L-broth (pH 7.0) to infect the macrophages. The resident peritoneal macrophages from BALB/c mice were cultured and exposed to the different strains for 30 min at a multiplicity of infection of 5 bacteria per macrophage cell. Bacterial growth was then monitored for 48 h (Fig. 5). Previous studies with serovar Typhimurium showed that the *rpoE* gene encoding an alternative σ factor, σ^E, is critically involved in intracellular survival within macrophages (29). To test the validity of the resident peritoneal macrophages of mice prepared in the present study, the macrophage cells were challenged with the opsonized serovar Typhimurium *rpoE*:Km strain. The number of viable bacteria was drastically decreased during the first 2 h of incubation. Between 2 and 48 h, the number of *rpoE*:Km bacteria present intracellularly had decreased 10-fold, whereas the number of the wild-type strain, χ3306, had increased 4-fold, suggesting a valid *Salmonella*-macrophage interaction system. When macrophages were challenged with strains CS2007, CS2016, and CS2018, none of the mutants seemed to grow intracellularly. Between 2 and 48 h after infection, the number of mutant bacteria present within macrophages had decreased ~9-fold. These results suggest that the ClpXP protease is required for the intracellular survival and growth of serovar Typhimurium within macrophages.

In addition, the sensitivity of these mutants to hydrogen peroxide and superoxide, which mimic the oxidative killing mechanisms by respiratory burst in macrophages and phagosomes, was assessed by determining their sensitivity to 3% hydrogen peroxide and 2% paraquat (a superoxide anion generator) using the disk diffusion assay. All of the serovar Typhimurium Δ*clpP* and Δ*clpX* mutants were as resistant to these killing mechanisms as the wild-type strain (data not shown). Salmonellae are typically resistant to the killing activity of complement that is present in serum. Since serum resistance is known to be an important factor for full expression of virulence in serovar Typhimurium, we examined the effect of Δ*clpP* and Δ*clpX* mutations on serum sensitivity. Mutant strains CS2007, CS2016, and CS2018 were exposed to normal and heat-inactivated BALB/c mouse serum, and their viability was determined by counting the CFU for each assay. None of the mutants was sensitive to the killing action of mouse serum (data not shown).

Depletion of ClpXP protease in strain χ3306 results in persistent infection of BALB/c mice. To determine whether the loss of virulence was due to the inability of the mutants to grow in the host, the number of bacteria in spleens and livers of a group of mice infected with strain CS2007 (*clpP*:Cm) was monitored for up to 35 days after infection (Fig. 6). At 3 days postinfection, a few bacteria were recovered from both organs of mice infected with the *clpP*:Cm mutant compared with the parental strain, whose number increased to approximately 2×10^3 CFU in the spleens and 3×10^4 CFU in the livers of the infected mice. Unlike mice infected with the wild type, which resulted in death by 5 days after infection, mice challenged with the same number (10^2 CFU) of the *clpP*:Cm mutant survived. Beginning at 7 days and continuing through 35 days postinfection, however, the *clpP*:Cm mutant bacteria were not cleared from the mice, but a similar number of the bacteria were recovered from the spleens and livers even on day 35 after initial infection. The *clpP*:Cm mutant also persisted in

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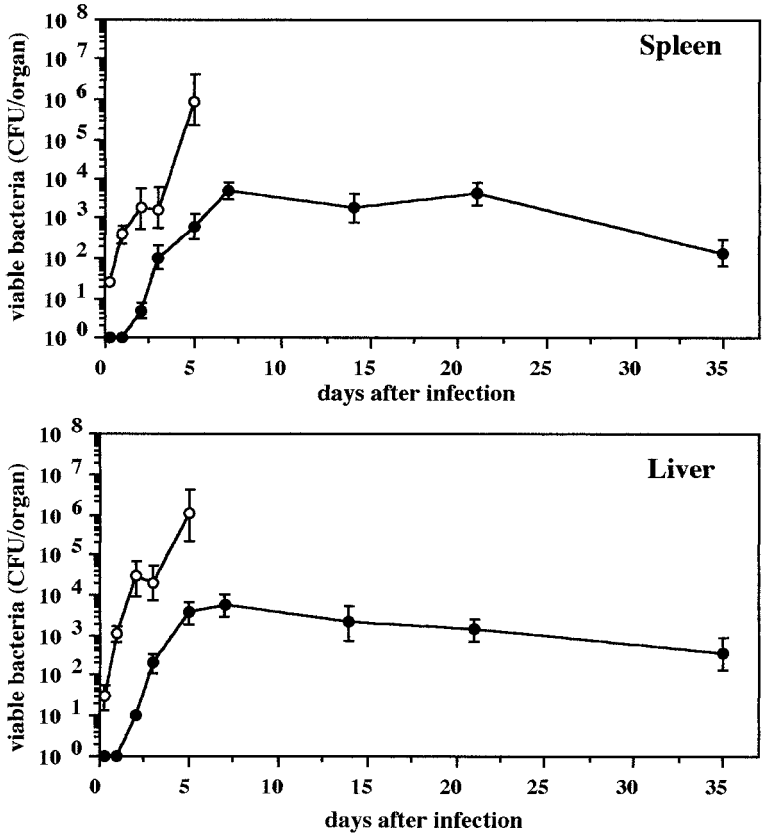

FIG. 6. Kinetics of bacterial growth in the organs of BALB/c mice after intraperitoneal administration of 10^2 CFU of serovar Typhimurium strains χ3306 (open circles) and CS2007 (*clpP*:Cm, solid circles). Each time point indicates the mean ± standard deviation for a group of five mice.

mice for several weeks following oral challenge with 10^8 cells (result not shown).

To examine whether the reduced virulence of the ClpXP-depleted mutant is due to its inherently poor growth, the growth rate of strain CS2007 (*clpP*:Cm) was compared to that of wild-type strain χ3306 using bacteria carrying a few copies of plasmid pHSG422. This plasmid exhibits defective replication above 37°C and is diluted out during bacterial growth (24). The use of this plasmid to differentiate bacterial growth rate has been described previously (3, 22). Bacterial growth at 37°C was monitored for 8 h. As shown in Fig. 7, the proportions of the population carrying pHGS422 were indistinguishable between strains χ3306 and CS2007 up to 8 h, indicating no significant differences in growth rate of these strains. Therefore, it is unlikely that the reduced virulence of the ClpXP-depleted mutant is attributable to impaired bacterial growth.

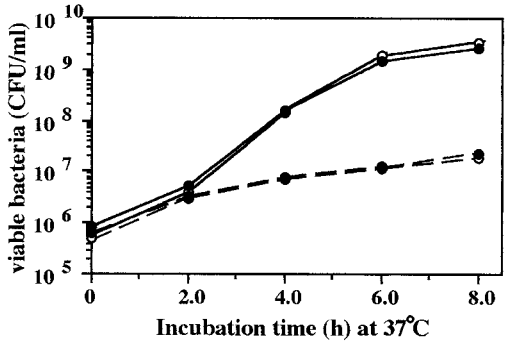

FIG. 7. Growth curves of serovar Typhimurium strains carrying pHGS422 at 37°C. Bacterial cells of strains χ3306 (open circles) and CS2007 (*clpP*:Cm, solid circles) were grown overnight at 30°C to obtain uniform plasmid copy number, diluted 1:500 into fresh medium, and then incubated for 8 h at 37°C. Bacterial cells were diluted at the indicated time points and then plated to determine the numbers of total bacteria (solid lines) and ampicillin-resistant (pHSG422-containing) bacteria (dotted lines).

These results indicate that the ClpXP protease is essential for systemic infection by serovar Typhimurium and the depletion of its function results in persistent infection with serovar Typhimurium in BALB/c mice.

Effect of endogenous cytokines on persistence of ClpXP-depleted mutant in mice. A previous report demonstrated that IFN-γ and TNF-α play an essential role in acquired resistance during the early phase of serovar Typhimurium infection (49). Therefore, we assessed the relevance of endogenous cytokines in the establishment and maintenance of the persistent infection developed by the ClpXP-depleted mutant by monitoring bacterial growth in IFN-γ- and TNF-α-deficient mice following infection. C57BL/6 mice, IFN-$\gamma^{-/-}$ mice, and TNF-$\alpha^{-/-}$ mice were infected with strain χ3306 or mutant strain CS2007 (*clpP*:Cm), and the number of bacterial cells in the spleens and livers was determined on day 3 after infection (Fig. 8). The number of wild-type cells in both spleens and livers of the IFN-γ- and

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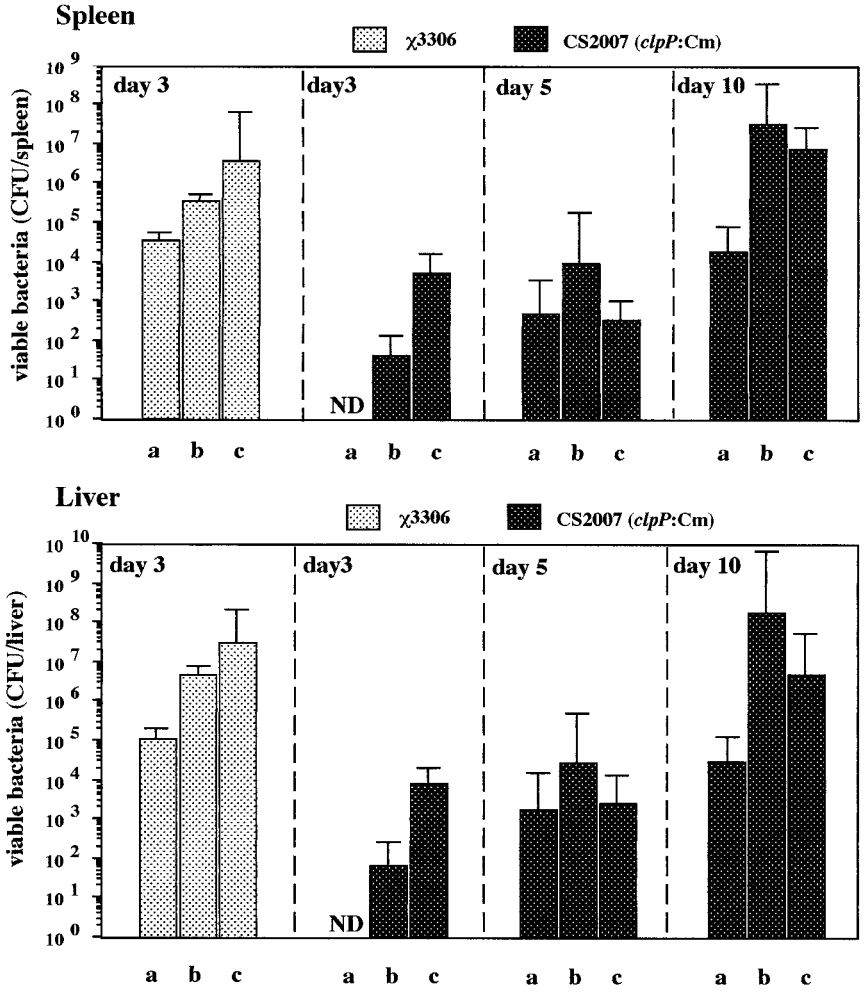

FIG. 8. Bacterial growth in the organs of IFN-γ-deficient and TNF-α-deficient mice during infection with different strains of serovar Typhimurium. C57BL/6 mice (a) and IFN-γ- (b) and TNF-α- (c) deficient mice were infected intraperitoneally with 10^2 CFU of serovar Typhimurium strains χ3306 and CS2007 (*clpP*:Cm). The numbers of bacterial cells in the spleens and livers were determined on days 3, 5, and 10 of infection. ND, not detected.

TNF-α-deficient mice was higher than in C57BL/6 mice. All cytokine-deficient mice died following infection with the wild-type strain. While no *clpP*:Cm bacterial cells were detected in either the spleens or livers of C57BL/6 mice, significant numbers were detected in the organs of IFN-γ- and TNF-α-deficient mice. The number of bacteria in the cytokine-deficient mice infected with the *clpP*:Cm mutant was also examined on days 5 and 10 after infection (Fig. 8). In these mice, *clpP*:Cm bacteria progressively increased to 10^8 and 10^7 per organ in the IFN-γ- and TNF-α-deficient mice, respectively, by day 10 postinfection. After infection with the *clpP*:Cm mutant, all the cytokine-deficient mice died on day 10 after infection. Though no *clpP*:Cm bacterial cells were detected in either the spleens or livers of C57BL/6 mice on day 3 postinoculation, significant numbers were detected in the organs of the mice 5 and 10 days postinoculation (Fig. 8). Furthermore, we confirmed that the *clpP*:Cm mutant persisted in the C57BL/6 mice by monitoring the numbers of bacteria in the spleens and livers for several weeks (data not shown). These results suggest that both endogenous IFN-γ and TNF-α are necessary for developing in vivo persistence after infection with the *clpP*:Cm mutant.

DISCUSSION

ClpP proteases have been identified not only in a wide range of bacteria but also in plants and animals (56). In *E. coli*, the ClpP protein is a cylindrical heptameric particle, forming the catalytic core of the protease, which associates with one of two ATPases, ClpA or ClpX, thus determining the substrate specificity (21, 36, 43). A hexamer of the Clp ATPase is located on the ClpP rings. The sequence analysis of the serovar Typhimurium *clpP-clpX* operon cloned in the present study revealed that these homologues show high identity with the equivalent *E. coli* proteins. The functional regions in both proteins are also conserved between serovar Typhimurium and *E. coli*. In ClpP, these include Ser-111 and His-136, which are required for proteolytic activity, and the 14-amino-acid precursor peptide, which is processed to produce the active form of ClpP

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(43). An ATP-binding motif, two tail motifs, and a zinc finger motif (21) are also found in the serovar Typhimurium ClpX homologue. The genetic organization of the *clpP* region in serovar Typhimurium, *tig-clpP-clpX-lon*, is also identical to that of *E. coli* (21).

The inactivation of *clpP* in *E. coli* results in no obvious phenotype, and *clpP* mutants appear to grow normally (43). However, as *clpP* genes from other organisms have been identified, it has become increasingly apparent that the ClpP protease performs more important and diverse roles in other bacteria. The inactivation of the *clpP* gene in *L. lactis* results in significant loss of cell viability, suggesting a major role for ClpP proteolysis in basic cell metabolism (18). Similarly, depletion of ClpP in *B. subtilis* causes pleiotropic effects such as filamentation, nonmotility, and impaired growth under certain stress conditions, starvation and high temperature (48). In the green alga *Chlamydomonas reinhardtii*, ClpP is essential for cell growth (28).

Here we have extended the study of the Δ*clpP* and Δ*clpX* mutants by comparing their virulence to that of the parental strain, serovar Typhimurium χ3306. The virulence assay in the mouse model demonstrated that the ability of the Δ*clpP* and Δ*clpX* mutants to cause systemic infection was apparently decreased (Fig. 4). During the course of infection in mice, serovar Typhimurium, which colonizes many different organs, including the Peyer's patches of the small intestine, mesenteric lymph nodes, spleen, and liver, is found in both extracellular and intracellular locations (16, 57). The ability of serovar Typhimurium to multiply inside professional phagocytic cells has been linked to virulence in mice (11, 37). Pleiotropic regulators of *Salmonella* virulence have been identified and characterized in the mouse model and in the cultured macrophage cell model as well. Mutations in any of these regulators render salmonellae avirulent. These include mutations in two-component regulator systems (*phoP*/*phoQ* and *ompR*/*envZ*) (13, 45), the heat shock protein *htrA* (30), and sigma factors (*rpoS* and *rpoE*) (14, 29). The *rpoS* product, σ^S, which regulates gene expression in response to nutrient deprivation during stationary phase, is known to also regulate the *spv* genes carried on a plasmid essential for *Salmonella* virulence (34). Though serovar Typhimurium *rpoS* mutants are avirulent, they replicate normally inside macrophages (50). On the contrary, the mutant of *rpoE* encoding σ^E, which is involved in the gene expression for several extracytoplasmic proteins, was severely defective in its ability to survive in macrophages and highly attenuated in mice (29).

To gain a better understanding of why the Δ*clpP* and Δ*clpX* mutants have lost the ability to cause systemic disease, we examined the ability of these mutants to replicate inside macrophages using an in vitro assay with resident peritoneal macrophages from BALB/c mice and found that none of these mutants could survive or grow within the peritoneal macrophages of mice (Fig. 5). While the rates of survival of the Δ*clpP* and Δ*clpX* mutants were only four- to ninefold lower than that of the wild-type strain over a 48-h period, the disparity could explain the reduced growth rate of the mutants in the spleen and liver over the entire mouse infection period. We cannot explain definitively why the Δ*clpP* and Δ*clpX* mutants are unable to replicate intracellularly and are attenuated in mice at present. This is probably due to the combination of defects generated from the depletion of the ClpXP protease. The activity would be required to cope with the accumulation of partially unfolded or denatured protein in bacteria exposed to various killing mechanisms associated with the host defense system during infection. Furthermore, the ClpXP protease could be involved in the expression of virulence genes and/or the turnover of virulence factors. One virulence gene known to be regulated by ClpP, *rpoS*, is also modulated by DksA (68) and MviA (2) in connection with serovar Typhimurium virulence. DksA appears to positively regulate the expression of *rpoS* at the level of translation. The decreased virulence of a *dksA* mutant can be explained, at least partially, by the effect of DksA on the expression of *rpoS*, which is required for virulence (14, 50). However, it is probable that σ^S is overproduced in serovar Typhimurium Δ*clpP* and Δ*clpX* mutants, because the ClpXP protease rapidly degrades σ^S in exponentially growing *E. coli* (60). MviA is also known to affect σ^S production post-translationally via proteolysis (2). Therefore, at present, it is not clear why mutations (*clpP*/*clpX* and *mviA*) that cause increases in σ^S levels also attenuate *Salmonella* virulence. It would appear that the bacteria need to modulate σ^S activity as they encounter areas of high and low stress within the host during pathogenesis. Alternatively, it is possible that ClpXP directly modulates the levels of the major contributors for virulence specified by the SPI-1 and SPI-2 regions on the serovar Typhimurium chromosome.

Of further interest is the finding that the ClpXP-depleted mutant persists in the mouse for long periods of time without causing an overwhelming systemic infection. The ability of the ClpXP-depleted mutant to survive and grow within the lymphatic environment of the mouse was examined by monitoring colonization in the spleens and livers of mice for up to 35 days. The monitoring revealed that there was persistence and net growth of serovar Typhimurium with a moderate growth rate in both organs for 35 days. It is clear that the mice, while not able to eliminate the ClpXP-depleted mutant organisms, were able to control growth of the bacterial strain in the lymphatic organs and survive. Most salmonellae in the spleens and livers of the infected mice are localized within the phagocytes present in the focal lesions (57). TNF-α, IFN-γ, and nitric acid derivatives appear to be required for the suppression of *Salmonella* growth in the reticuloendothelial system (39, 49, 66). TNF-α is required for the recruitment of mononuclear cells in the tissues and for granuloma formation (41) and IFN-γ activates macrophages to kill salmonellae (31). We therefore examined whether the endogenous cytokines TNF-α and IFN-γ are necessary for the development of a persistent infection by the serovar Typhimurium ClpXP-depleted mutant by monitoring bacterial growth in TNF-α- and IFN-γ-deficient mice following infection. In the organs of these mice, the ClpXP-depleted mutant colonized and progressively grew, resulting in bacterial counts in the spleens and livers of the cytokine-deficient mice that were 100- to 1,000-fold higher than normally observed in C57BL/6 mice. These mice did not survive beyond 10 days postinfection (Fig. 8).

Mice infected with salmonellae become hyper-susceptible to endotoxin. A previous study reported that interleukin-12 neutralization prevented the death of infected mice following subcutaneous injection of lipopolysaccharide (40). It is therefore possible that interleukin-12 is also required to control the

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persistence of the serovar Typhimurium ClpXP-depleted mutant in mice. The abilities to control growth and persist in the lymphatic organs of the host are considered important in the development of a live vaccine strain. Attenuated *Salmonella* mutants present potential live vaccine candidates to protect against infection or to deliver heterologous antigen to the mammalian immune system. The present results show that such an attenuated strain may cause severe infections, but only in animals with serious and persistent immunological defects.

Several other serovar Typhimurium strains that cause persistent infections in mice have been described. These include a *purE* derivative (52), an *ompR* mutant (13), an *aroA* mutant (6), an *htrA* mutant (62), an *agfA* mutant (62), and an *surA* mutant (63). Whereas these mutants have been well characterized for their potential usefulness as vaccines against virulent *Salmonella* infection and carriers expressing foreign protein antigens derived from unrelated pathogens, the specific involvement of these genes in persistent infection has not been demonstrated. Persistent infection is the result of balance between virulence and host immunity. Though the inability of the Δ*clpP* and Δ*clpX* mutants to cause systemic infection could be explained by the loss of ability to survive or grow inside macrophages, the conclusion that the mutation somehow specifically associates with the persistent infection in mice cannot be definitively reached on the present results.

Systemic infection by serovar Typhimurium in mice closely resembles typhoid fever in humans caused by infection with *Salmonella enterica* serovar Typhi. It is well known that, in certain persons, *S. enterica* serovar Typhi persists in the gall bladder and that these persons can shed bacteria in their feces for years as chronic carriers. The ClpXP-depleted mutant in the present study will be useful for resolving the mechanisms by which chronic infection with salmonellae is established and developed, in combination with further studies on the fundamental mechanisms of immunity to salmonellosis.

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