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HtrA Stress Protein Is Involved in Intramacrophagic Replication of Adherent and Invasive *Escherichia coli* Strain LF82 Isolated from a Patient with Crohn's Disease

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Adherent and invasive *Escherichia coli* (AIEC) bacteria isolated from Crohn's disease patients are able to greatly replicate within macrophages without escaping from the phagosome and without inducing macrophage death. In the present study, evidence is provided that in AIEC strain LF82 the *htrA* gene encoding the stress protein HtrA is essential for intracellular replication within J774-A1 macrophages. Deletion of the *htrA* gene in strain LF82 induced increased sensitivity of the isogenic mutant to oxidative stress caused by hydrogen peroxide and a reduced rate of growth in an acid and nutrient-poor medium partly reproducing the microenvironment of the phagosome. In vitro experiments using an LF82 *htrA* gene promoter fusion with the *lacZ* gene revealed a 38-fold activation of the promoter in AIEC LF82 intramacrophagic bacteria. The CpxRA two-component signaling pathway was not involved in this activation. In addition, the activation of the LF82 *htrA* gene promoter was not observed in the nonpathogenic *E. coli* K-12 intramacrophagic bacteria, indicating that the AIEC LF82 genetic background is crucial for induction of *htrA* gene transcription during phagocytosis.

Crohn's disease (CD) is an inflammatory bowel disease of unknown etiology in humans (34). CD has features that might be the result of a microbial process in the gut. Some characteristic pathological elements of CD, including aphthous ulcers of the mucosa, mural abscesses, and macrophage and epithelioid cell granulomas, also occur in well-recognized infectious diseases, such as shigellosis, salmonellosis, and *Yersinia* enterocolitis, in which invasiveness is an essential virulence factor of the bacteria involved (49). Various studies have addressed the hypothesis that virulent bacteria contribute to the pathogenesis of CD (6, 24, 29, 37, 38). The ileal mucosa of a subset of patients with CD is abnormally colonized by pathogenic *Escherichia coli* strains called adherent and invasive *E. coli* (AIEC), which are able to adhere to and to invade intestinal epithelial cells (4, 11, 12). AIEC strains are also able to replicate within macrophages without escaping from the phagosome and without triggering host cell death. AIEC-infected macrophages release large amounts of tumor necrosis factor alpha (19). Given the virulence traits of AIEC strains harbored by CD patients, it has been suggested that the pathogenic bacteria may be able to translocate across the human intestinal barrier, move to deep tissues, continuously activate macrophages, and induce the formation of granulomas, one of the hallmarks of CD lesions (10).

Upon bacterial infection, pathogens are exposed to various stressful conditions, and one of the most survival-threatening conditions encountered by bacteria is the intracellular environment of macrophages. Invasive bacteria have evolved to survive within phagocytic cells and thus have developed various strategies: (i) resistance to phagocytosis, (ii) escape from the phagosome, (iii) inhibition of the fusion of bacteria-containing phagosomes with endosomes and lysosomes, and (iv) resistance to the low-pH and antimicrobial environment of the phagolysosome. Bacteria that are unable to escape from the phagosome, such as *Yersinia enterocolitica* (45), *Salmonella enterica* serovar Typhimurium (5), *Legionella pneumophila* (1), and *Brucella abortus* (27), respond to the hostile environment in macrophages by producing a set of stress proteins, including members of the high-temperature requirement A (HtrA) class described initially in *E. coli* as being essential for protecting bacteria at high temperature (28). HtrA also plays an essential role in the survival of intramacrophagic bacteria. Reports have shown that *htrA* mutants from *S. enterica* serovar Typhimurium (2), *Y. enterocolitica* (26, 46), and *L. pneumophila* (33) have reduced ability to survive within macrophages and/or reduced virulence in mice.

The aim of the present study was to investigate whether stress proteins are involved in the ability of AIEC to resist killing by macrophages and to replicate within phagocytic cells. Genetic screening was performed using a library of random Tn*5phoA* mutants of the AIEC LF82 strain (3). Interestingly, of 12 mutants showing a drastic decrease in the ability to replicate within macrophages, 5 had an insertion of the transposon within the *htrA* gene. We provide evidence that the *htrA* gene, whose transcription is highly up-regulated in intramacrophagic AIEC bacteria, is essential for replication of AIEC within J774-A1 macrophages.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown routinely in Luria-Bertani (LB) broth or on LB agar plates (Institut Pasteur Production)

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
LF82	*E. coli* isolated from an ileal biopsy specimen from a patient with CD	12
LF82-Δ*htrA*	LF82 isogenic mutant with *htrA* gene deleted	This study
LF82-Δ*cpxR*	LF82 isogenic mutant with *cpxR* gene deleted	This study
Plasmids		
pUC18	*E. coli* cloning vector; *ori*ColE1 Am^r	Biolabs[a]
pHSG575	*E. coli* cloning vector; Cm^r	Biolabs
pFPV25.1	Vector for constitutive GFP expression; Am^r	44
pKOBEG	pBAD cloning vector harboring λ phage *red*γβα operon; Cm^r	8
pRS550	*E. coli* multicopy *lacZ* fusion vector; Km^r Am^r	39
pPB105	pHSG575 harboring the 1,812-bp EcoRI fragment with the complete *htrA* gene of LF82	This study
pPB106	pRS550 harboring the 170-bp BamHI-EcoRI fragment containing the *htrA* promoter of LF82	This study

[a] New England Biolabs, Beverly, Mass.

overnight at 37°C. When required, appropriate antibiotics were added to the media at the following final concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 25 μg/ml. Strain LF82 was isolated from a chronic ileal lesion of a patient with CD and belongs to *E. coli* serotype O83:H1. It is sensitive to most antibiotics but not to amoxicillin. It survived and replicated within J774-A1 macrophages until 48 h postinfection (19). *E. coli* strain JM109 was used as the host strain for cloning experiments. *E. coli* strain K-12 C600 was used as a negative control for macrophage survival assays.

The plasmid pFPV25.1, which harbors the green fluorescent protein (GFP), was used to visualize bacteria for confocal microscopy analysis (44). The plasmid vectors pUC18 and pHSG575 were used for cloning and complementation procedures, respectively.

Cell line and cell culture. The murine macrophage-like cell line J774-A1 (American Type Culture Collection no. TIB67) was maintained in an atmosphere containing 5% CO_2 at 37°C in RPMI 1640 medium (Biowhittaker Cambrex Co., Verviers, Belgium) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS; Biowhittaker) and 1% L-glutamine (Life Technologies, Cergy-Pontoise, France). J774-A1 cells were seeded in 24-well tissue culture plates (Polylabo, Strasbourg, France) at a density of 10^5 per cm^2 and were grown for 18 h in an atmosphere containing 5% CO_2 at 37°C.

Transposon mutagenesis and molecular cloning. Random insertional mutations in AIEC strain LF82 were previously generated using the suicide plasmid pUTKm1 carrying the mini-Tn*5phoA* transposon (3).

The single PstI restriction enzyme site, located upstream of the kanamycin resistance gene in the mini-Tn*5phoA*, was exploited for cloning of the genomic region downstream of the transposon insertion. Total DNA from each mutant was extracted and digested with the PstI restriction enzyme (Boehringer Mannheim) and cloned into the pUC18 vector, and the resulting recombinant plasmids were transformed in *E. coli* strain JM109. Transformants harboring the Tn*5phoA* transposon and flanking DNA regions were selected on agar plates for the kanamycin resistance phenotype.

DNA sequencing and sequence analysis. Plasmid DNA templates were prepared using the QIAGEN (Courtaboeuf, France) plasmid minikit. Single-stranded DNA was sequenced by the Euro Sequence Gene Service (Cybergene) by the dideoxynucleotide chain termination method. Cloned insertion regions of the mutants were sequenced using an oligonucleotide primer that anneals to the insertion sequence of the transposon and directs the synthesis of the region immediately downstream of the transposon. DNA sequences were analyzed with BLAST programs available from the National Center for Biotechnology Information at http://www.ncbi.nlm.nhi.gov.

Macrophage survival assay. Bacterial uptake, survival, and replication were measured by gentamicin protection assays. Before infection, cell monolayers were washed twice with phosphate-buffered saline (PBS; pH 7.2), and the medium was replaced by 1 ml of RPMI 1640 supplemented with 10% heat-inactivated FCS. J774-A1 monolayers were infected at a multiplicity of infection of 100 bacteria per macrophage. After 10 min of centrifugation at 1,000 × *g* and a 10-min incubation period at 37°C with 5% CO_2, the infected macrophages were washed twice with PBS, and fresh cell culture medium containing 20 μg of gentamicin/ml was added for a 1- or 24-h period. To determine the number of intracellular bacteria, the cell monolayers were washed once with PBS, and 0.5 ml of 1% triton X-100 (Sigma Chemical Co., St. Louis, Mo.) in deionized water was added to each well for 5 min to lyse eukaryotic cells. This concentration of Triton X-100 had no effect on bacterial viability for at least 30 min. Samples were mixed, diluted, and plated onto LB agar plates to determine the number of CFU recovered from the lysed monolayers. The numbers of intracellular bacteria were determined after 1 and 24 h of gentamicin treatment. Bacterial replication was expressed as the mean percentage of bacteria recovered at 24 h postinfection relative to the number of bacteria recovered after 1 h of gentamicin treatment, defined as 100%.

Construction of isogenic mutants and transcomplementation assay. Isogenic mutants with the *htrA* or *cpxR* gene deleted were generated with a PCR product, using the method described by Datsenko and Wanner (13) as modified by Chaveroche et al. (8). The basic strategy was to replace a chromosomal sequence with a selectable antibiotic resistance gene (kanamycin) generated by PCR. This PCR product was generated using the primers IM *htrA*-1 and IM *htrA*-2 for the LF82-Δ*htrA* isogenic mutant and IM *cpxR*-1 and IM *cpxR*-2 for the LF82-Δ*cpxR* isogenic mutant with 50-nucleotide extensions that are homologous to regions adjacent to the *htrA* or *cpxR* gene and a template *E. coli* strain harboring the kanamycin resistance gene (Table 2). In addition, strain LF82 was transformed with the pKOBEG plasmid, a plasmid encoding λ-Red proteins from phage λ, which protect linear DNA in bacteria, expressed under the control of an inducible promoter in the presence of 1 mM L-arabinose. This plasmid was maintained in bacteria at 30°C with 25 μg of chloramphenicol/ml.

Strain LF82/pKOBEG was grown at 30°C with 1 mM L-arabinose to induce Red expression. When the optical density at 620 nm (OD_{620}) reached 0.5, the bacterial culture was incubated for 10 min at 42°C in order to kill the plasmid. The bacteria were washed three times with 10% glycerol, and the PCR products were electroporated. Isogenic mutants were selected on LB agar containing 50 μg of kanamycin/ml. The replacement of the *htrA* or *cpxR* gene by the kanamycin resistance cassette in the LF82-Δ*htrA* or LF82-Δ*cpxR* isogenic mutant was confirmed by PCR. The locations of the various primers used are shown in Fig. 1.

A 1,812-bp PCR product obtained using primers htrA-3 and htrA-4 and containing the entire *htrA* gene under the control of its own promoter was cloned into the pHSG575 vector and designated pPBI05 (Table 2). This construct was used for transcomplementation experiments with the LF82-Δ*htrA* isogenic mutant.

Sensitivity to oxidative stress. Overnight bacterial cultures were harvested and resuspended in the same volume of minimal medium M9 supplemented with 20% Casamino Acids, 20% glucose, 0.1 M $MgSO_4$, and 1 mg of thiamine/ml. Hydrogen peroxide (H_2O_2) was added at a final concentration of 10 or 20 mM. After a 30- or 60-min incubation period at 37°C with shaking, the bacterial cells were diluted in physiological water (NaCl, 0.9%) and then plated onto LB agar plates. The numbers of CFU were determined after incubation of the plates for 18 h at 37°C. The results were expressed as percentages of viable bacteria after treatment compared to viable bacteria without treatment recovered at the same time and taken as 100%.

Growth ability in acid and nutrient-poor medium. Overnight bacterial cultures were harvested and resuspended in the same volume of the acid and nutrient-poor medium developed by S. Eriksson, I. Hautefort, M. Clements, A. Thompson, J. Hinton, and M. Rhen (submitted for publication) and containing 100 mM Bis-Tris, 0.1% Casamino Acids, 0.16% glycerol, and 10 μM $MgCl_2$. The pH was adjusted to 5.8 with HCl. The cultures were then diluted 1:50 in the medium, bacteria were grown at 37°C with shaking, and the OD_{620} was determined at different times of culture.

Promoter expression assay. To generate the promoter fusion construct LF82 *htrA* gene promoter-*lacZ*, the *htrA* promoter of strain LF82 was amplified by PCR using BamHI-*htrA* and EcoRI-*htrA* primers (Table 2). The resulting 170-bp fragment was ligated into the plasmid vector pRS550 (39) and designated pPBI06.

Strains LF82 and *E. coli* K-12 and the LF82-Δ*cpxR* isogenic mutant harboring the construct pPBI06 or vector pRS550 alone was used to infect J774-A1 macrophages at a multiplicity of infection of 100 for 30, 60, or 120 min, followed by 30 min of incubation in RPMI 1640 culture medium supplemented with 10%

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TABLE 2. Oligonucleotides used and PCR product sizes

Primer name	Oligonucleotide sequence (5′-3′)	PCR product size (bp)	Use
IM *htrA*-1	TCTGAAGAACACAGCAATTTTGCGTTATCTGTTAATCGAGACTGAAATACAAAGCCACGTTGT GTCTCAA	1,057	LF82-Δ*htrA* isogenic mutant construction
IM *htrA*-2	CAGATTGTAAGGAGAACCCCTTCCCGTTTTCAGGAAGGGGTTGAGGGAGATTAGAAAAACTC ATCGAGCA		
htrA-1	GCGAACTGATCGGTATCAAC	168	Allelic replacement of *htrA* in LF82-Δ*htrA* and RT-PCR
htrA-2	GAGTTCAGCTCAGTCCCCA		
htrA-3	GACCGACCTCTATGCGTGGG	1,812	Allelic replacement of *htrA* in LF82-Δ*htrA*
htrA-4	GTGCCACCATATCCTGCGCC		
*Bam*HI-*htrA*	CGGGATCCCGCCAGGCTTTTGTAAAGACG	170	Promoter *htrA-lacZ* fusion construction
*Eco*RI-*htrA*	GGAATTCCTGCTAATGTGGTTTTTTTCATGT		
IM *cpxR*-1	CGCCAGCGTCAGCCAGAAGATGGCGAAGATGCGCGCGGTTAAGCTGCCTATTAGAAAAACT CATCGAGCA	1,057	LF82-Δ*cpxR* isogenic mutant construction
IM *cpxR*-2	GGATTAGCGACGTCTGATGACGTAATTTCTGCCTCGGAGGTATTTAAACAAAAGCCACGTTG TGTCTCAA		
cpxR-1	GCGCAGATGACTATCTCCCG	187	Allelic replacement of *cpxR* in LF82-Δ*cpxR*
cpxR-2	GTTTGCCCGTCGAAGCTGGC		
cpxR-3	CGGCAGCGGTAACTATGCGC	969	Allelic replacement of *cpxR* in LF82-Δ*cpxR*
cpxR-4	TGGCGTGAATCGAGCTTGGG		
IM *rpoE*-1	TTTGTTATCAATAGCTTCCCTCGCTCGGAAGATACGTGAACGCACCGTACTTAGAAAAACTCA TCGAGCA	1,057	LF82-Δ*rpoE* isogenic mutant construction
IM *rpoE*-2	TGGCGTTTCGATAGCGCGTGGAAATTTGGTTTGGGGAGACTTTACCTCGGAAAGCCACGTTG TGTCTCAA		
A2 GBL-3	AAAGCCACGTTGTGTCTCAA	957	Kanamycin resistance cassette amplification
B2 GBLnp-5	TTAGAAAAACTCATCGAGCA		

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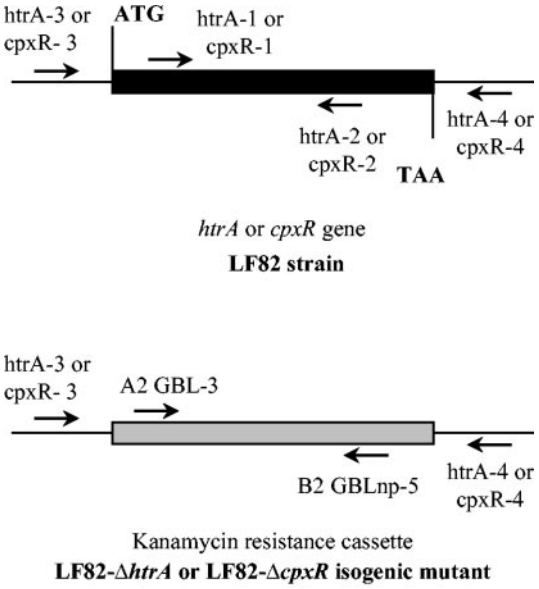


FIG. 1. Allelic replacement of the *htrA* or *cpxR* gene by the kanamycin resistance cassette in the LF82-Δ*htrA* and in the LF82-Δ*cpxR* isogenic mutants. Schematic representation of the locations of various primers on the DNA of strain LF82 and the LF82-Δ*htrA* and LF82-Δ*cpxR* isogenic mutants.

heat-inactivated FCS and containing 20 μg of gentamicin/ml. J774-A1 macrophage monolayers were scraped for each incubation period, and β-galactosidase activity was measured using a β-galactosidase assay kit (QIAGEN). The same experiments were performed with bacteria grown in RPMI 1640 culture medium supplemented with 10% heat-inactivated FCS or in acid and nutrient-poor medium using a quantity of bacteria equivalent to that used to infect macrophages. The β-galactosidase activity of each sample was determined by measuring the OD_{420}. The background of β-galactosidase activity generated by a promoterless *lacZ* construct was subtracted at each time point. The number of bacteria in each sample was determined by plating or OD_{620} determination. The data are presented as the ratio of arbitrary β-galactosidase units per CFU or per OD_{620} unit for intracellular or in vitro-grown bacteria.

Confocal microscopy. J774-A1 macrophages were seeded on glass coverslips in 24-well plates at a density of 0.5×10^5 cells per cm^2 and were grown for 18 h in an atmosphere containing 5% CO_2 at 37°C. After infection with GFP-expressing bacteria as described above, the cells were washed with PBS to eliminate extracellular bacteria and fixed with 3% paraformaldehyde for 10 min. Subsequently, the cells were washed with PBS, incubated for 5 min with 0.1 M glycine, washed with PBS, and permeabilized with 0.1% Triton X-100 for 20 min. After being washed with PBS, the slides were incubated twice for 10 min each time with PBS–0.2% gelatin. The actin cytoskeleton was stained for 15 min using tetramethyl rhodamine isothiocyanate-labeled phalloidin (Sigma). The monolayers were then washed with PBS and distilled water and mounted on glass slides with a Mowiol solution (Calbiochem, Darmstadt, Germany). The slides were examined with an Olympus Fluoview confocal microscope.

RNA manipulations, RT, and real-time RT-PCR. Total RNAs were extracted from bacteria and treated with DNase (Roche Diagnostics, Mannheim, Germany) to remove any contaminating genomic DNA. The RNAs were reverse transcribed and amplified using specific primers to *htrA* mRNAs or 16S rRNA (Table 2). Amplification of a single expected PCR product was confirmed by electrophoresis on a 2% agarose gel. Real-time reverse transcription (RT)-PCR was performed using a Light Cycler (Roche Diagnostics), and quantification of the *htrA* mRNA level or 16S rRNA (as a control) was done using RNA master SYBR Green I (Roche Diagnostics) with 0.5 μg of total RNA.

Statistical analysis. Student's *t* test was used for comparison of the two groups of data. All experiments were repeated at least three times. A *P* value of ≤0.05 was considered statistically significant.

TABLE 3. Abilities of LF82 Tn*5phoA* mutants to replicate within J774-A1 macrophages at 24 h postinfection

Strain or mutant	Tn*5phoA* insertion site	CFU per well (10^5)[a]		% of intracellular bacteria at 24 h postinfection[b]
		1 h postinfection	24 h postinfection	
K-12		0.254 ± 0.025	0.006 ± 0.005	2.1 ± 1.8
LF82		8.1 ± 3.5	17.3 ± 1.6	319.5 ± 110.1
2E12	*dsbA*	8.3 ± 2.0	0.9 ± 0.4	9.2 ± 2.5
3G1		11.2 ± 3.6	1.9 ± 0.6	18.2 ± 2.5
B9D		13.0 ± 4.6	1.6 ± 0.7	11.3 ± 2.4
OA8	*yfgL*	4.3 ± 1.7	1.6 ± 0.9	30.9 ± 6.2
51F8		3.4 ± 1.1	0.9 ± 0.3	28.6 ± 8.6
52E8	*htrA*	8.3 ± 1.9	2.8 ± 0.9	32.3 ± 5.8
54H6		11.8 ± 3.9	4.2 ± 2.2	34.1 ± 8.2
55H8		9.3 ± 3.4	1.3 ± 0.4	15.1 ± 1.1
56A6		7.6 ± 1.9	1.5 ± 0.5	14.6 ± 4.5
57G3		10.1 ± 2.4	2.7 ± 1.0	26.3 ± 7.0
6H3	*slyB*	13.3 ± 5.3	1.6 ± 0.9	9.5 ± 2.8
53H2	*yraP*	11.5 ± 4.1	4.1 ± 1.7	44.9 ± 14.8

[a] Mean number of bacteria surviving gentamicin treatment per well ± standard error of the mean (SEM).
[b] Mean percentage ± SEM of intracellular bacteria at 24 h postinfection relative to the number of intracellular bacteria at 1 h postinfection, taken as 100%.

RESULTS

Screening for the loss of ability by LF82 Tn*5phoA* mutants to replicate within J774-A1 macrophages. To identify the genes involved in the ability of AIEC strain LF82 to resist killing by macrophages and to replicate within phagocytic cells, the LF82 Tn*5phoA* mutant library previously formed (3) was screened with the gentamicin protection assay using J774-A1 macrophages. Of the 767 $PhoA^+$ mutants tested, 12 mutants had reduced ability to replicate within J774-A1 macrophages at 24 h postinfection (Table 3). As a result of the insertion of the transposon, the percentages of intracellular bacteria were significantly reduced ($P < 0.05$) and ranged from 9.2% ± 2.5% to 44.9% ± 14.8% versus 319.5% ± 110.1% for the wild-type strain LF82.

Cloning and characterization of Tn*5phoA* insertion regions. To identify the insertion sites, genomic DNA flanking the mini-Tn*5phoA* transposon was sequenced after molecular cloning of PstI restriction fragments containing both the kanamycin resistance gene and the 3′ DNA genomic region. The nucleotide sequences of the genomic regions were compared with sequences available in the National Center for Biotechnology Information databases. All the sequences identified matched sequences of the *E. coli* K-12 strain MG1566 complete genome (Table 3). The DNA sequence cloned from mutant 6H3 matched the *slyB* gene, which belongs to the Mg^{2+} stimulon in *E. coli* (31). Nucleotide sequence from mutant 53H2 matched that of the *yraP* gene encoding a putative periplasmic protein. The genomic sequences identified for mutants 2E12, 3G1, and B9D matched that of the *dsbA* gene encoding a periplasmic protein required for disulfide bond formation and involved in the maturation of virulence factors (48). Nucleotide sequences from OA8 and 51F8 matched that of the gene *yfgL*, which may be involved in the synthesis and degradation process of peptidoglycan (15). Finally, five mutants (52E8, 54H6, 55H8, 56A6, and 57G3) had an insertion of

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the transposon within the *htrA* gene encoding the stress protein HtrA, also called DegP or Do protease. The HtrA protein, which may have the function of a serine protease and chaperone in the periplasmic space, has been reported to be involved in the virulence of invasive bacteria, such as *S. enterica* serovar Typhimurium (2) and *Y. enterocolitica* (26, 46), so it was decided to further investigate the ability of AIEC to resist macrophage killing.

Phenotype of the LF82-Δ*htrA* isogenic mutant. To confirm that the *htrA* gene plays a role in the ability of strain LF82 to survive and/or replicate within macrophages, an isogenic deletion mutant of the *htrA* gene was constructed as described in Materials and Methods, and double allelic exchange was confirmed by PCR using various sets of primers (Fig. 1). We checked whether deletion of the *htrA* gene in strain LF82 could interfere with bacterial growth. The growth curves for the wild-type strain LF82 and the LF82-Δ*htrA* isogenic mutant in the bacterium-cell incubation medium used for macrophage infection (RPMI 1640 supplemented with 10% heat-inactivated FCS) were similar at all time points tested up to 24 h (data not shown).

The phenotype of the LF82-Δ*htrA* isogenic mutant was analyzed within J774-A1 macrophages at 1 and 24 h postinfection. No significant difference in the numbers of intracellular bacteria was observed at 1 h postinfection between the wild-type strain and the *htrA* null mutant, since the mean numbers of CFU per well were $3.7 \times 10^5 \pm 0.3 \times 10^5$ and $2.8 \times 10^5 \pm 0.6 \times 10^5$ for the wild-type strain LF82 and the LF82-Δ*htrA* isogenic mutant, respectively. As a consequence of deletion of the *htrA* gene, the ability of the LF82-Δ*htrA* isogenic mutant to multiply within macrophages was significantly ($P < 0.05$) impaired (Fig. 2). The percentages of intracellular bacteria at 24 h postinfection compared to those at 1 h postinfection, taken as 100%, were 81.5% ± 7.8% for the LF82-Δ*htrA* isogenic mutant and 388.4% ± 67.5% for the wild-type strain LF82. However, transcomplementation of the LF82-Δ*htrA* isogenic mutant with a cloned wild-type *htrA* gene (plasmid pPBI05) restored intramacrophagic bacterial replication to a level similar to that displayed by the wild-type strain. Transformation of the LF82-Δ*htrA* isogenic mutant with the vector pHSG575 alone did not restore the ability of the mutant to replicate within phagocytic cells (Fig. 2). The absence of bacterial replication of the intramacrophagic LF82-Δ*htrA* isogenic mutant at 24 h postinfection was confirmed by confocal microscopic examination of macrophages infected with the wild-type strain LF82 and the LF82-Δ*htrA* isogenic mutant harboring plasmid pFPV25.1 for constitutive expression of GFP (Fig. 3). At 1 h postinfection, the wild-type strain LF82 and the LF82-Δ*htrA* isogenic mutant showed similar entry into J774-A1 macrophages (Fig. 3A and B). At 24 h postinfection, AIEC LF82-infected macrophages exhibited large vacuoles containing numerous bacteria (Fig. 3C), whereas *htrA*-negative mutant-infected macrophages showed vacuoles similar to those observed at 1 h postinfection containing only a few bacteria (Fig. 3D). Thus, these experiments confirmed that the *htrA* gene is required for replication of the AIEC strain LF82 within J774-A1 macrophages.

Increased sensitivity of the LF82-Δ*htrA* isogenic mutant to oxidative stress. It has been suggested that HtrA is involved in the degradation of aberrant proteins arising under oxidative

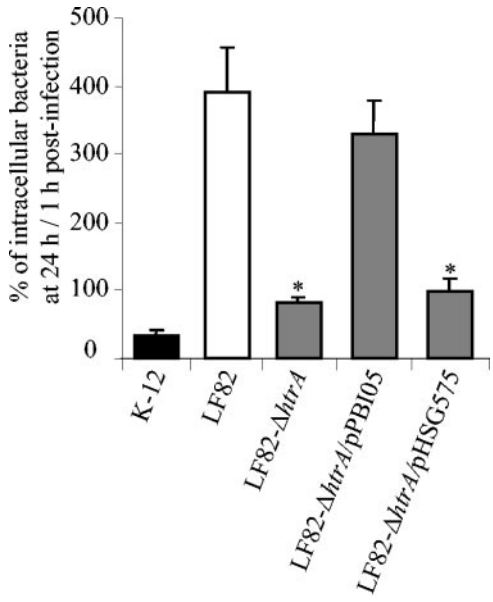


FIG. 2. The *htrA* gene is required for replication of strain LF82 within J774-A1 macrophages. Bacterial replication at 24 h postinfection of the wild-type strain LF82, the LF82-Δ*htrA* isogenic mutant, the LF82-Δ*htrA* isogenic mutant transcomplemented with the plasmid pPBI05 harboring the entire *htrA* gene, and the LF82-Δ*htrA* isogenic mutant transformed with the empty vector pHSG575. The results are expressed as the number of intracellular bacteria at 24 h postinfection relative to that obtained after 1 h postinfection, taken as 100%. Each value is the mean plus standard error of the mean of three independent experiments. *, $P < 0.05$.

conditions encountered in the phagosomal environment, so we followed the survival rates of strain LF82 and the LF82-Δ*htrA* isogenic mutant after exposure to oxidative stress by H_2O_2 (Table 4). After a 30-min H_2O_2 exposure period, the LF82-Δ*htrA* isogenic mutant showed significant 2- and 11-fold decreases in survival rates ($P < 0.05$) compared to the wild type in response to 10 and 20 mM H_2O_2, respectively (Table 4). In addition, after a 60-min exposure to 20 mM H_2O_2, a 56-fold decrease in survival of the LF82-Δ*htrA* isogenic mutant was observed compared to the wild-type strain. Transcomplementation of the LF82-Δ*htrA* isogenic mutant with the cloned *htrA* gene restored resistance to oxidative stress for all the H_2O_2 doses and times of exposure. Interestingly, the transcomplemented LF82-Δ*htrA* isogenic mutant had a greater increase in survival rate after a 30-min exposure to 10 and 20 mM H_2O_2 than the wild-type strain LF82, which is suggestive of a dose effect of HtrA due to differences in the number of *htrA* gene copies.

Role of HtrA in bacterial growth and activation of the LF82 *htrA* gene promoter in acid and nutrient-poor medium. A low-pH (pH 5.8) and nutrient-poor medium containing glycerol and Casamino Acids was used to partly mimic the phagocytic-vacuole content (Eriksson et al., submitted). The growth of the wild-type strain LF82 was compared with that of the LF82-Δ*htrA* isogenic mutant. Growth rates were determined by measuring the OD_{620} over an 8-h period (Fig. 4A). During the first 2 h, the wild-type strain LF82 and the LF82-Δ*htrA* isogenic mutant had similar growth rates. From 3 to 8 h of incubation, growth of the LF82-Δ*htrA* isogenic mutant was

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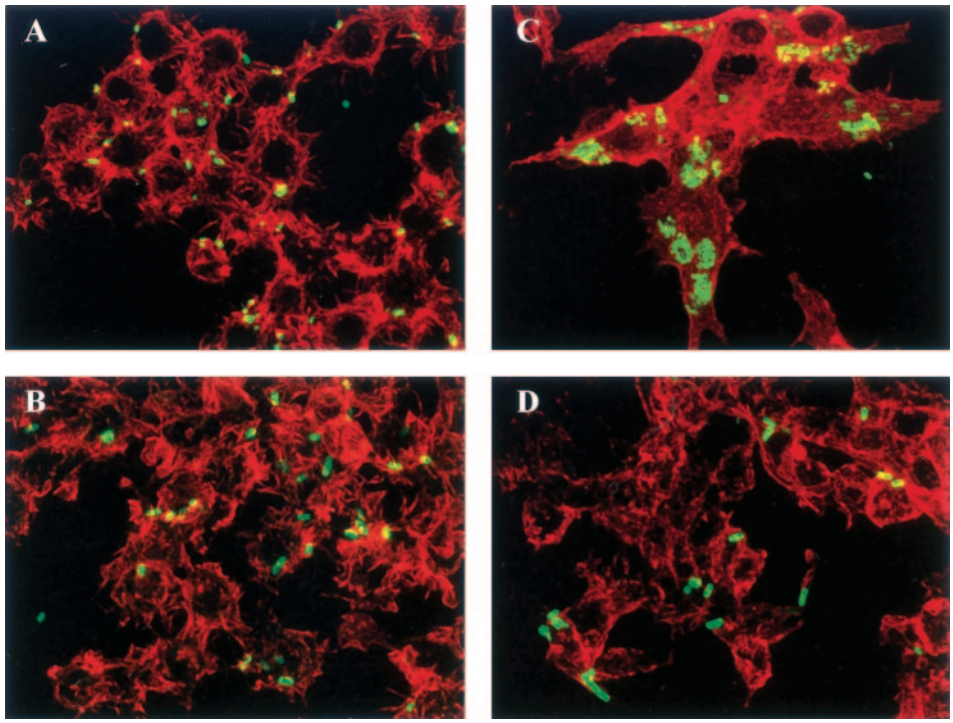


FIG. 3. Confocal microscopic examinations of J774-A1 macrophages infected with wild-type strain LF82 and the LF82-Δ*htrA* isogenic mutant harboring plasmid pFPV25.1 for constitutive expression of GFP. The actin cytoskeletons of J774-A1 cells were stained using tetramethyl rhodamine isothiocyanate-labeled phalloidin. J774-A1 macrophages were infected with the LF82 wild-type strain (A and C) and with the LF82-Δ*htrA* isogenic mutant (B and D) at 1 h postinfection (A and B) and at 24 h postinfection (C and D).

significantly lower than that of the wild-type strain LF82 ($P < 0.05$). The LF82-Δ*htrA* isogenic mutant transcomplemented with the plasmid pPBI05 harboring a cloned *htrA* gene exhibited growth similar to that of the wild-type strain. These results showed that the *htrA* gene is required for growth of strain LF82 in an acid and nutrient-poor medium.

To follow the transcriptional activation of the *htrA* gene when bacteria were grown in acid and nutrient-poor medium, the AIEC LF82 *htrA* gene promoter was cloned upstream of the *lacZ* gene in the vector pRS550 (pPBI06), and the construct was transformed into strains LF82 and K-12. The kinetics of activation of the LF82 *htrA* gene promoter were monitored during the growth of bacteria for a 120-min period in nutrient-poor medium, pH 5.8, or in RPMI culture medium. There was increased activation of the LF82 *htrA* gene promoter in strain LF82 when the bacteria were grown in acid and nutrient-poor medium compared to growth in RPMI medium (Fig. 4B). After 120 min of growth, a significant 4.7- ± 2.3-fold increase in β-galactosidase activity was observed. In contrast, no increased activation of the LF82 *htrA* gene promoter was seen in *E. coli* strain K-12. Thus, the transcription of the *htrA* gene was up-regulated in strain LF82 when the bacteria were grown in vitro under stress conditions that partly mimicked stress encountered within the phagocytic vacuole.

TABLE 4. Survival percentages of strain LF82 and the LF82-Δ*htrA* isogenic mutant after exposure to H_2O_2

Exposure time (min)	H_2O_2 concn (mM)	% Survival[a] (*n*-fold decrease)[b]		
		LF82	LF82-Δ*htrA*	LF82-Δ*htrA*/pPBI05
30	10	24.4 ± 5.0	11.6 ± 3.2[c] (2.1)	53.0 ± 15.8 (0.5)
	20	16.4 ± 4.7	1.5 ± 0.9[c] (10.9)	31.8 ± 5.4 (0.5)
60	10	21.7 ± 5.2	7.6 ± 3.2[c] (2.8)	25.4 ± 5.9 (0.8)
	20	11.2 ± 3.6	0.2 ± 0.1[c] (56.0)	14.9 ± 1.1 (0.7)

[a] Mean percentage of bacteria surviving H_2O_2 exposure compared to bacteria without stress exposure, taken as 100%. Each value is the mean ± standard error of the mean of four independent experiments.

[b] Decrease = percent survival of AIEC LF82/percent survival of the LF82-Δ*htrA* isogenic mutant or the LF82-Δ*htrA* isogenic mutant transcomplemented with pPBI05 harboring the entire *htrA* gene.

[c] $P < 0.05$ for all comparisons between the AIEC strain LF82 and the LF82-Δ*htrA* isogenic mutant and between the LF82-Δ*htrA* isogenic mutant and the LF82-Δ*htrA* isogenic mutant transcomplemented with pPBI05 harboring the complete *htrA* gene.

Activation of the LF82 *htrA* gene promoter in bacteria within macrophages. The transcriptional activation of the *htrA* gene was also studied in intracellular bacteria within infected J774-A1 macrophages (Fig. 5). As early as 30 min after phagocytosis, intracellular LF82 bacteria harboring the fusion construct pPBI06 showed a 7.9- ± 2.9-fold increase in expression of β-galactosidase activity compared to bacteria incubated in RPMI medium. After 120 min of phagocytosis, a 38.3- ± 5.9-fold increase in β-galactosidase expression was observed for intracellular bacteria.

To analyze whether *htrA* gene transcriptional activation could arise in a nonpathogenic *E. coli* strain, the β-galactosidase activity of the fusion construct pPBI06 was also measured in *E. coli* strain K-12 (Fig. 5). When the fusion construct pPBI06 was harbored by *E. coli* strain K-12, low expression of

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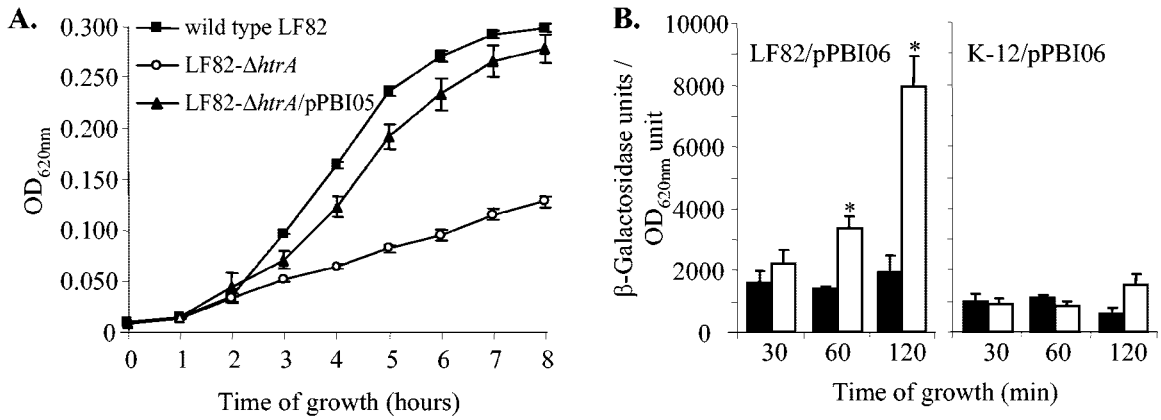


FIG. 4. The LF82 *htrA* gene promoter is activated in acid and nutrient-poor medium that partly mimics the microenvironment encountered by bacteria within phagosomes. (A) Growth of wild-type strain LF82, the LF82-Δ*htrA* isogenic mutant, and the LF82-Δ*htrA* isogenic mutant transcomplemented with plasmid pPBI05 harboring the entire *htrA* gene. (B) Activation of LF82 *htrA* gene promoter. β-Galactosidase activity per OD_{620} unit resulting from the expression of *lacZ* driven by the LF82 *htrA* promoter in bacteria in RPMI 1640 culture medium (black bars) or in acid and nutrient-poor medium (white bars). The LF82 *htrA* gene promoter was cloned upstream of *lacZ* in the recombinant vector pPBI06, and the plasmid was used to transform strain LF82 and strain K-12. The background of β-galactosidase activity generated by a promoterless *lacZ* construct was subtracted at each time point. The data are means plus standard errors of the mean of three independent experiments. *, $P < 0.05$.

β-galactosidase was observed. The difference in LF82 *htrA* promoter activation between AIEC strain LF82 and strain K-12 was not due to the lower number of phagocytized bacteria, since β-galactosidase activity was expressed relative to the number of intracellular bacteria, nor was it due to the macrophage killing of intracellular K-12 bacteria, since our experiments were performed until 120 min postinfection and no decrease in viable K-12 bacteria has ever been observed before 8 h postinfection (19). Thus, the high transcriptional activation of the *htrA* gene in strain LF82 during intracellular trafficking within macrophages is dependent on the genetic background of strain LF82.

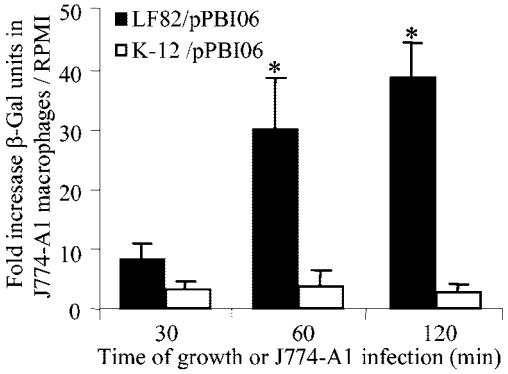


FIG. 5. Expression of *htrA* gene in intracellular bacteria during J774-A1 macrophage infection. β-Galactosidase (β-Gal) activity resulting from the activation of the LF82 *htrA* gene promoter was measured. The LF82 *htrA* gene promoter was cloned upstream of *lacZ*. The resulting recombinant vector pPBI06 was used to transform strain LF82 or strain K-12. The results are expressed as the increase (*n*-fold) of β-galactosidase activity in intracellular bacteria compared to the β-galactosidase activity of bacteria grown in RPMI medium. The background of β-galactosidase activity generated by a promoterless *lacZ* construct was subtracted at each time point. The data are means plus standard errors of the mean of three independent experiments. *, $P < 0.05$.

Regulation of *htrA* expression. Two partially overlapping systems are known to regulate the *htrA* gene in *E. coli* (36): the alternative sigma factor σ^E (RpoE) and the two-component regulatory system CpxRA. Analysis of the DNA sequence corresponding to the *htrA* gene promoter of strain LF82 indicated binding sites for a σ^E consensus region and for phosphorylated CpxR, similar to those of *E. coli* strain MG1655. To analyze the involvement of these regulatory systems, the LF82-Δ*cpxR* isogenic mutant was constructed using primers shown in Table 2. Repeated attempts to isolate the LF82-Δ*rpoE* mutant in strain LF82 failed, suggesting that the *rpoE* gene is essential in AIEC strain LF82 for bacterial viability, as has been previously reported for some strains of *Y. enterocolitica* (21) and for *S. enterica* serovar Typhimurium (42). The *htrA* mRNA levels were measured after growth of the bacteria in acid and nutrient-poor medium by real-time PCR using primers described in Table 2. The levels of *htrA* mRNAs in the LF82-Δ*cpxR* isogenic mutant were not significantly different from those measured in the wild-type strain LF82 (Fig. 6A), indicating that the activation of the *htrA* gene promoter of strain LF82 grown in vitro in acid and nutrient-poor medium does not involve the two-component regulatory system CpxRA. In addition, as shown in Fig. 6B, the percentage of intracellular bacteria at 24 h postinfection for the LF82-Δ*cpxR* isogenic mutant was 716.9% ± 254.1%, not significantly different from that of the wild-type strain LF82, which was 499.0% ± 36.1%. Thus, the CpxRA system is not essential for the multiplication of AIEC strain LF82 within J774-A1 macrophages. The β-galactosidase activity of the fusion construct pPBI06 was also measured in intracellular bacteria within J774-A1 macrophages for the LF82-Δ*cpxR* isogenic mutant (Fig. 6C). An increase in β-galactosidase activity was observed after 60 and 120 min postinfection for the *cpxR* null mutant. It was not significantly different ($P > 0.05$) from that measured for the wild-type strain (Fig. 5). Thus, the *htrA* expression in AIEC strain LF82 is CpxRA independent when the bacteria are cultured in acid

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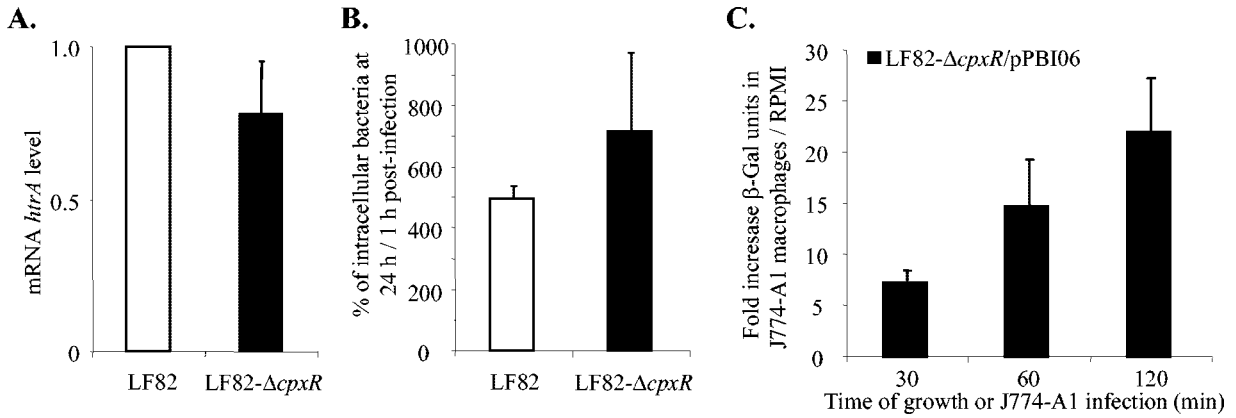


FIG. 6. The CpxRA two-component signaling pathway is not involved in regulation of *htrA* expression and in replication of strain LF82 within J774-A1 macrophages. (A) mRNA levels of the wild-type strain LF82 and the LF82-Δ*cpxR* isogenic mutant were determined by RT-PCR experiments after growth of the bacteria in acid and nutrient-poor medium. Only experiments showing the same levels of 16S rRNA for each sample were taken into account. (B) Intramacrophagic replication at 24 h postinfection. The results are expressed as the number of intracellular bacteria at 24 h postinfection relative to that obtained after 1 h postinfection, taken as 100%. (C) Expression of the *htrA* gene in intracellular bacteria during J774-A1 macrophage infection. β-Galactosidase (β-Gal) activity resulting from the activation of the LF82 *htrA* gene promoter was measured using the *lacZ* recombinant vector pPBI06 transformed in the LF82-Δ*cpxR* isogenic mutant. The results are expressed as the increase (*n*-fold) in β-galactosidase activity in intracellular bacteria compared to the β-galactosidase activity of bacteria grown in RPMI medium. The background of β-galactosidase activity generated by a promoterless *lacZ* construct was subtracted at each time point. Each value is the mean plus standard error of the mean of three independent experiments.

and nutrient-poor medium or internalized within macrophages.

DISCUSSION

The ileal mucosa of patients with CD is abnormally colonized by pathogenic AIEC strains, which have the ability to survive and to greatly replicate within macrophages (19). Pathogenic bacteria have developed two major strategies to counteract macrophage killing after phagocytosis, either by escaping from the endocytic vacuoles and inducing cell death (14, 25, 50) or by resisting the antimicrobial environment of the phagolysosome (32, 41, 43). We previously reported that the reference AIEC strain LF82 did not induce the death of infected macrophages despite the high replication of bacteria observed within large vacuoles (19). Thus, the mechanism developed by AIEC to survive within macrophages was more likely resistance of the bacteria to stress conditions encountered within the phagosomes.

In an attempt to define whether stress proteins are involved in the ability of AIEC to resist killing by macrophages and to multiply within phagocytic cells, we screened a Tn*5phoA* mutant library of the AIEC strain LF82. Five mutants having an insertion of the transposon into the *htrA* gene were attenuated. To confirm the role of HtrA in the ability of strain LF82 to replicate within macrophages, an isogenic mutant with the *htrA* gene deleted was constructed. The LF82-Δ*htrA* isogenic mutant was unable to multiply within macrophages. Transcomplementation of this mutant with a cloned *htrA* gene restored intracellular replication to a level similar to that of the wild-type strain LF82, which confirms the important role of HtrA in the ability of AIEC LF82 to replicate within phagosomes. The HtrA stress protein is thought to play a role in the mechanism through which pathogenic bacteria adapt to and resist the hostile environment of the phagosome, as reported for *S. enterica* serovar Typhimurium (2), *Y. enterocolitica* (26), and *L. pneumophila* (33).

During phagocytosis, there is uptake of bacteria by endocytosis into phagosomes, which mature and finally fuse with lysosomes to form phagolysosomes. This last compartment has oxygen-dependent and -independent mechanisms for killing the invading bacteria. The oxygen-dependent mechanism is consistent with the formation of anion superoxide that can form hydrogen peroxide and reactive oxidants. In the present study, we demonstrated that deletion of the *htrA* gene of strain LF82 induced increased sensitivity to oxidative stress caused by hydrogen peroxide. This may explain why, unlike the wild-type strain, the LF82-Δ*htrA* isogenic mutant was no longer able to replicate within macrophages. Similar increased susceptibility of the *htrA* null mutant bacteria to oxidative stress occurs in some pathogenic bacteria, such as *S. enterica* serovar Typhimurirum (23), *Y. enterocolitica* (26, 46), and *Klebsiella pneumoniae* (9). Surprisingly, this was not observed with nonpathogenic *E. coli*. Indeed, *htrA* mutants of *E. coli* B178 are no more sensitive to oxidative stress caused by hydrogen peroxide than the wild-type strain, since no difference in growth pattern or survival was observed between the *htrA* mutant and the wild-type strain (40).

The HtrA stress protein may play a key role in the adaptation of intracellular AIEC to the hostile environment of the phagocytic vacuoles, since our findings show that *htrA* gene expression is highly up-regulated within macrophages (up to 38-fold) compared to bacterial growth in cell culture medium. Similar findings were observed with *S. enterica* serovar Typhimurirum (16, 17), *L. pneumophila* (33), and *Y. enterocolitica* (47) throughout the intracellular infection of macrophages. In vitro experiments using LF82 *htrA* gene promoter fusion with *lacZ* revealed a fourfold activation of the promoter when the bacteria were grown in an acid and nutrient-poor medium. The

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LF82 *htrA* promoter activation observed under the in vitro stress conditions was much lower than that observed for intramacrophagic bacteria, which indicates that transcriptional activation of the *htrA* gene may also be induced by stresses other than acid pH and a nutrient-poor environment. Interestingly, in intracellular bacteria within macrophages or when bacteria were grown in vitro in the acid and nutrient-poor medium, the promoter of the LF82 *htrA* gene was highly activated in AIEC strain LF82 but not in the nonpathogenic *E. coli* K-12 strain. Hence, the strain LF82 genetic background is crucial for induction of *htrA* gene transcription during phagocytosis. This is consistent with the difference in the regulation of *htrA* gene expression between AIEC strain LF82 and *E. coli* strain K-12. In nonpathogenic *E. coli*, *htrA* gene expression is regulated by two partially overlapping systems, the alternative sigma factor E (RpoE) and the two-component regulatory system CpxRA (36). Both the σ^E and Cpx pathways play roles in the virulence of various pathogenic bacteria. The RpoE factor is sometimes required for bacterial viability (21, 42) and may also be involved in LF82 bacterial viability, since repeated attempts to isolate an LF82-Δ*rpoE* mutant in strain LF82 failed. Thus, the involvement of RpoE in AIEC strain LF82 *htrA* gene regulation was not demonstrated. However, no increased activation of the LF82 *htrA* gene promoter was observed in intracellular *E. coli* K-12 bacteria within macrophages or in K-12 bacteria grown in vitro in the acid and nutrient-poor medium. This was not due to an altered sigma E-based regulation in the *E. coli* K-12 nonpathogenic laboratory strain, since RpoE is essential for bacterial growth at high temperature (22) and the growth rates of the *E. coli* K-12 strain used in the present study were similar at 43 and 30°C (data not shown). The involvement of CpxRA in *htrA* expression has been reported in *Y. enterocolitica*, and *htrA* mutants exhibited a reduction in the ability to survive within macrophages (20, 46). Likewise, in *L. pneumophila*, the CpxR regulator plays a major role in the regulation of the genes *icm* and *dot*, which are required for intracellular multiplication and human macrophage killing (18). A *cpxR* null mutant of AIEC strain LF82 exhibited a rate of replication within macrophages similar to that of the wild-type strain LF82. In addition, under in vitro stress conditions, the levels of *htrA* mRNAs in the LF82-Δ*cpxR* isogenic mutant were not significantly different from those measured in the wild-type strain LF82. Moreover, an induction of *htrA* expression was observed in intracellular bacteria within macrophages for the *cpxR* null mutant that was not significantly different from that measured for the wild-type strain. Thus, the CpxRA pathway is not involved in regulation of virulence genes required for the resistance of intracellular AIEC within macrophages, and *htrA* gene expression in the AIEC strain LF82 is not regulated by CpxRA. These results are intriguing, since nucleotide analysis of the *htrA* promoter in the AIEC strain LF82 indicated that the consensus sequences for CpxR were present and because the Cpx response plays an important role in the resistance of many pathogenic bacteria within macrophages. The role of the CpxRA two-component regulatory system in AIEC strain LF82 should be further investigated, since we cannot exclude the possibility that this regulatory pathway acts differently in AIEC strains and in nonpathogenic *E. coli*.

The function of HtrA in AIEC strain LF82 may not be simply restricted to that of a stress protein degrading misfolded periplasmic proteins. Indeed, like the periplasmic protein DsbA, which is required for disulfide bond formation, HtrA is also involved in the maturation of virulence factors. It has been speculated that HtrA, as a chaperone, is needed for the folding of secreted proteins or that HtrA is involved in the oligomerization and export of virulence factors. For example, in *Shigella flexneri*, HtrA acting as a chaperone facilitated the folding of IcsA in the periplasm or the rapid transit of IcsA to the outer membrane (35). In addition, mutation of the *htrA* gene in *K. pneumoniae* affects its ability to produce capsular polysaccharide (9), and the HtrA protein of *Streptococcus pyogenes* influences the expression of the cysteine protease SpeB and the hemolysin streptolysin S, two virulence factors whose biogenesis requires extensive processing (30). Interestingly, mutants of AIEC LF82 having an insertion of Tn*5phoA* within the *dsbA* gene had highly attenuated ability to replicate within macrophages. Thus, it can be speculated that in AIEC strain LF82 some virulence factors involved in the survival and replication of bacteria within macrophages are folded in the periplasm and that the protein maturation implies HtrA and DsbA acting in concert.

In summary, we have provided evidence that HtrA plays an essential role in the intracellular replication of AIEC strain LF82 within macrophages. It is noteworthy that several pathogenic bacteria depend on HtrA for virulence. The depletion of HtrA decreases the virulence of *Salmonella enterica* serovar Typhimurium (23), *Y. enterocolitica* (26), *K. pneumoniae* (9), and *S. pyogenes* (30) in mouse models. These observations have led some authors to use *htrA* mutants as live attenuated vaccine strains (7, 23). By its function in the replication of intramacrophagic bacteria, HtrA is involved in the virulence of the AIEC strain LF82. Given the activation of the promoter of the *htrA* gene in intramacrophagic bacteria or in bacteria under acid and nutrient-poor stress conditions, which was observed in the AIEC strain LF82 but not in a nonpathogenic K-12 strain, it is likely that the environmental conditions that enhance the expression of HtrA also lead to an increase in the virulence of AIEC.

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