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Construction and Characterization of a *Yersinia enterocolitica* O:8 High-Temperature Requirement (*htrA*) Isogenic Mutant

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The high-temperature requirement (HtrA) family of stress response proteins are induced by different environmental stress conditions in a variety of bacteria and have been shown to contribute to the pathogenicity of some of these species. In this study, the *htrA* gene from *Yersinia enterocolitica* O:8 was amplified, cloned, and sequenced. Analysis of the deduced amino acid sequence predicted that the putative HtrA homolog contains a serine protease active site and a catalytic triad characteristic of trypsin-like serine proteases, structural features characteristic of previously described HtrA proteins. In order to evaluate the biological function(s) of *Y. enterocolitica* HtrA, an isogenic mutant was constructed by a reverse-genetics PCR-based approach. Characterization of the mutant provided evidence supporting a stress response function for the *Y. enterocolitica htrA* gene product. In contrast to the parent strain, the mutant showed increased sensitivity to killing by H_2O_2, O_2^- and temperature stress (50°C). The mutant was avirulent in the murine yersiniosis infection model and offered partial protection to mice challenged with the parent strain. Further studies with the *Y. enterocolitica htrA* mutant should increase our knowledge of the host-pathogen interactions which occur during *Yersinia* infections.

Yersinia enterocolitica is an invasive enteropathogen that causes a spectrum of diseases in humans ranging from self-limiting gastroenteritis to fatal septicemic disease (4, 13). After oral infection of a mammalian host, the bacteria cross the epithelium overlying the Peyer's patches of the gut and multiply in the lymphoid follicles. Eventually, the bacteria can spread through the lymphatics to disseminate to the liver and spleen (12, 44). Serotypes O:3 and O:9, commonly isolated in Europe, usually produce self-limiting gastroenteritis, whereas O:8 strains, usually isolated in America, are more likely to cause systemic infections (7, 12, 13). *Y. enterocolitica* O:8 strains are virulent for mice, which represent a convenient model with which to study the sequential steps of the infection (7, 8, 23, 35).

Y. enterocolitica is subjected to a range of stress conditions in the transition from its free-living state in the environment to colonization and then invasion of the mammalian host. *Y. enterocolitica* is particularly adept at counteracting oxidative stress conditions, as evidenced by its ability to resist phagocytosis (12, 18, 44). Inhibition of phagocytosis and other virulence properties has been linked to the expression of a set of plasmid-encoded *Yersinia* outer proteins (Yops) (10, 11, 18–20, 22, 24, 38). However, conserved chromosomal determinants are also likely to contribute to the organism's survival in hostile environments (40). Bacteria often upregulate the expression of specific groups of proteins which maintain the integrity of the cell when exposed to stress. One such family of stress response proteins includes the HtrA (high-temperature requirement) proteins (14, 15, 25, 26, 28–30, 37, 42, 45, 46). HtrA production in different bacterial species appears to be induced by different environmental stress conditions. For example, in *Escherichia coli*, in which it was first identified, the *htrA* gene is essential for bacterial survival at high temperatures (28, 30, 42, 46), whereas *Salmonella typhimurium htrA* mutants have been shown to be temperature insensitive (26). Further characterization of *S. typhimurium htrA* mutants in vitro revealed that they were more susceptible to oxidative stress than the parent, suggesting that they may be less able to withstand oxidative killing within macrophages (3, 17). By contrast, a *Brucella abortus htrA* mutant was sensitive to both temperature and oxidative stress (15). Evidence for a direct role of HtrA in virulence has been shown in *S. typhimurium* and *B. abortus* (15, 26). *S. typhimurium htrA* mutants show a >10,000-fold increase in the 50% lethal dose in BALB/c mice compared with that of the parental strain (26). Furthermore, the *S. typhimurium* mutants were able to confer protection against a lethal challenge with virulent organisms and are excellent live attenuated vaccine strains (9, 26, 43). More recently, HtrA homologs have been identified in phylogenetically distinct bacterial pathogens, including *Campylobacter jejuni*, *Helicobacter pylori*, and *Rochalimaea henselea*, suggesting a fundamental role for HtrA in response to environmental stress and possibly in the pathogenesis of disease (27, 53).

This report describes the amplification, cloning, and sequence analysis of a *Y. enterocolitica* O:8 *htrA* homolog. To determine if *htrA* encodes a stress response protein, an isogenic *Y. enterocolitica htrA* mutant was constructed by a PCR-based strategy. The *Y. enterocolitica htrA* mutant appears to be sensitive to oxidative and temperature stress. Results indicating that the *Y. enterocolitica htrA* gene product contributes to virulence in the murine yersiniosis model are also presented.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Y. enterocolitica* strains were stored at −80°C in Luria broth (LB) containing 25% (vol/vol) glycerol and were grown on *Yersinia* selective agar (CIN agar; Oxoid, Basingstoke, England). At 28°C, *Y. enterocolitica* was cultured in LB, and at 37°C, 5 mM $CaCl_2$ was added to the growth medium. The presence of the *Yersinia* virulence plasmid was confirmed by determining the low-calcium response phenotype. This was achieved by plating the bacteria on Columbia agar base (Oxoid) supplemented

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
Y. enterocolitica		
8081v	Serotype O:8; r^- m^+ derivative of 8081	55
YHT61	Cm^r Kn^r *Y. enterocolitica* 8081 *htrA* merodiploid	This study
YHT67	Kn^r *Y. enterocolitica* 8081 *htrA* double crossover	This study
E. coli		
XL2-Blue MRF′	Cloning strain	Stratagene
CC118-λ*pir*	Donor strain in tri-cross mating experiments	31
S17::pNJ5000	Tri-cross mating strain with Tet^r helper plasmid	21
Plasmids		
pUC19	Ap^r	Pharmacia
pUC4K	Kn^r; source of Kn^r *Bam*HI cassette	Pharmacia
pACYC184	Cm^r Tet^r; source of Cm^r for suicide vector	New England Biolabs
pGP704	Derivative of pJM703.1; *oriR6K mobRP4* Ap^r	31
pSC1	Cm^r suicide vector; 838-bp *Bsa*BI-*Bsa*AI pACYC184 fragment in *Pvu*II-*Dra*I pGP704	This study
pNJ5000	Tet^r, for triple mating	21
pYHT38	pUC19 plus 1.3 kb of *htrA*	This study
pYHT43	pYHT38 with 53-bp deletion in *htrA*	This study
pYHT46	pYHT43 plus Kn^r	This study
pYHT58	Donor plasmid for conjugation; Cm^r Kn^r pYHT46 *Pvu*II-*Xba*I fragment containing the mutated *htrA* gene cloned into *Eco*RV-*Xba*I pSC1	This study

[a] Abbreviations: Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Kn^r, kanamycin resistant; Tet^r, tetracycline resistant.

with 20 mM sodium oxalate and 20 mM $MgCl_2$ (CR-MOX agar) (36). *E. coli* strains were routinely grown in LB or on LB agar. The antibiotics used for selection purposes were ampicillin (100 μg/ml), chloramphenicol (40 μg/ml), kanamycin (50 μg/ml), and tetracycline (20 μg/ml).

PCR and cloning procedures. The oligonucleotide primers used for PCRs are summarized in Table 2. The *Y. enterocolitica htrA* gene fragment was amplified by PCR with degenerate oligonucleotide primers (PCRDOP) (51). Primers H8 and H10, based on known HtrA sequence data for *E. coli* (29) and *S. typhimurium* (26), were used to amplify the *Y. enterocolitica htrA* homolog. Chromosomal DNA isolated from wild-type strain 8081v in a PCRDOP reaction mixture was subjected to 40 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 40°C, and 2 min of extension at 72°C in an Omnigene thermal cycler (Hybaid, Teddington, United Kingdom). The amplified product of the predicted size was digested with *Hin*dIII and *Pst*I, ligated into similarly digested pUC19, and transformed into *E. coli* XL2-Blue MRF′ cells (Stratagene, Cambridge, United Kingdom). A clone (pYHT38) containing the appropriate-size insert was sequenced by the dideoxynucleotide chain termination method with an Applied Biosystems (Warrington, United Kingdom) PRISM sequencing kit, and the data were compared with known HtrA sequences by using BLASTX software (1).

A 53-bp deletion and unique *Bgl*II site were engineered into the *htrA* gene fragment in clone pYHT38 by the inverse-PCR mutagenesis (IPCRM) procedure (52) using the primer pair H13 and H14. For IPCRM, 2 to 20 ng of template plasmid was added to a PCR mixture which was then subjected to 40 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and 4 min of extension at 72°C in an Omnigene thermal cycler. PCR products were digested with *Bgl*II, self-ligated, and transformed into *E. coli* XL2-Blue MRF′ cells. *Y. enterocolitica htrA*-specific primers (H17 and H18) that flank the deletion site were used to screen for mutants by PCR, using 1 μl of boiled cells added to a standard reaction mixture which was subjected to 40 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and 1 min of extension at 72°C in an Omnigene thermal cycler.

The complete *htrA* sequence was determined by using inverse PCR directly on a ligation mix (ligation anchor PCR) consisting of 100 ng of *Eco*RV-digested 8081v DNA and 100 ng of *Sma*I-digested pUC19. One microliter of the ligation sample was added to a standard reaction mixture with primers H28 (specific for *Y. enterocolitica htrA*) and V2 (reverse primer from pUC19) and subjected to thermal cycling conditions as described for specific-primer PCR. The 1.9-kb amplified product was cloned into the T-tailed vector pTAg as described by the manufacturers (R & D Systems, Abingdon, United Kingdom) and sequenced.

Plasmid and genomic DNA extractions, restriction enzyme digests, DNA ligations, and transformations into *E. coli* were performed by standard procedures (41) using enzymes supplied by Promega Ltd., Southampton, United Kingdom.

Construction of *Y. enterocolitica htrA* mutant. Plasmid DNA from pYHT43 was digested with *Bgl*II and ligated with a *Bam*HI fragment containing a kanamycin resistance cassette (Pharmacia, St. Albans, United Kingdom) to form pYHT46. Plasmid DNA from pYHT46 was digested with *Xba*I and *Pvu*II, and the 2.6-kb fragment containing the mutated *htrA* gene was ligated with similarly digested pSC1 (a pGP704-based suicide vector containing the chloramphenicol-resistant derivative from pACYC184) and electroporated into *E. coli* CC118-λ*pir* cells to form pYHT58. *E. coli* CC118-λ*pir*::pYHT58 was introduced into the wild-type *Y. enterocolitica* 8081v recipient by conjugation using a tri-cross mating procedure with *E. coli* S17::pNJ5000 as a helper strain to increase the efficiency of conju-

TABLE 2. Oligonucleotides used for PCR

Oligonucleotide	PCR method	Strand	Sequence (5′→3′)[a]
H10	PCRDOP	+	GAACTGCAGTATATGAARAARACNAC
H8	PCRDOP	−	TGAAGCTTNGCNCCNATDATNACRTCNCC
H13	IPCRM	−	TGAGATCTCGATAGCCTKACGGTATAATC
H14	IPCRM	+	TGAGATCTAGTGGTTTGAATGTGGAAA
H17	HtrA specific	+	GCCGTAGTTATGAAGCAAAAGTGATT
H18	HtrA specific	−	ACTAATGCACCACCCGAGTTACCAC
H28	Ligation anchor PCR	+	AAGCCATCAATAGCTTCGCT
V2	Ligation anchor PCR	−	ATGTTGTGTGGAATTGTG
O57	pYV specific	+	TCATGGCAGAACAGCAGTCAG
O58	pYV specific	−	CTCATCTTACCATTAAGAAG

[a] Underlined nucleotides represent *Hin*dIII (H8), *Pst*I (H10), and *Bgl*II (H13 and H14) restriction endonuclease sites. Degenerate oligonucleotides are based on amino acids 1 to 6 of *S. typhimurium* HtrA (H10) and 450 to 456 (H8). R = A or G; D = A, G, or T; and N = A, C, G, or T.

gation (21). Plasmid pYHT58 cannot replicate in the parent strain; therefore, chloramphenicol-resistant transconjugants (e.g., YHT61) which represent the merodiploid state at the *htrA* locus, because of integration of pYHT58 in the chromosome, were selected. Cycloserine was used to select for bacteria that had undergone a second recombination event as described by Pepe et al. (34). Cycloserine kills only growing bacteria, while chloramphenicol is a bacteriostatic antibiotic. Hence, bacteria that segregate the plasmid are chloramphenicol sensitive and will not grow, while nonsegregates will grow and will be killed by cycloserine. The cycloserine-enriched culture was plated on CIN agar containing kanamycin. A single colony (YHT67), in which the mutated gene had replaced the wild-type gene by a double-recombination event, was identified as chloramphenicol sensitive by replica plating.

Oxidative and temperature stress experiments. To determine the effect of high-temperature stress on *Y. enterocolitica*, overnight stationary-phase cells grown at 37°C were transferred to prewarmed 50°C tubes and incubated for 2 min. Similarly, for oxidative-stress experiments, stationary-phase cells were incubated at 37°C in the presence of H_2O_2 (10 and 40 mM) for 30 min or menadione (15 and 50 mM) for 30 min as described previously (26). To determine the number of viable bacteria after exposure to the appropriate stresses, the cells were pelleted and resuspended in LB, and appropriate dilutions were plated on CIN agar for 8081v cells and CIN agar containing kanamycin for YHT67 cells and incubated at 37°C.

Experimental infection of BALB/c mice. *Y. enterocolitica* organisms were grown overnight without aeration at 37°C, washed with phosphate-buffered saline (PBS), and diluted to the appropriate infectious dose. Six- to eight-week-old BALB/c mice (Harlan Ltd., Bicester, Oxon, United Kingdom) were challenged orally with 10^8 bacteria by using a gavage needle. Gastric acidity was neutralized before inoculations by oral administration of 0.2 ml of 7.5% (wt/vol) sodium bicarbonate solution. The livers and spleens of infected mice were removed at appropriate times and homogenized in sterile PBS in stomacher bags (Colworth Ltd., Colworth, United Kingdom) to grind the tissue as described by Mukkur et al. (32). Two groups of 10 mice were orally given 10^8 bacteria of either strain 8081v or YHT67, and the time to death was recorded. Viable counts of bacteria in liver and spleen tissues were made up to day 4 for mice infected with 8081v and up to day 21 for mice infected with YHT67. The ability of the *htrA* mutant to protect against wild-type infection was assessed by challenging the mice 28 days after the initial dosing with YHT67. Deaths and survival were subsequently recorded, and in simultaneous experiments, a control group of unprotected mice was infected with 10^8 8081v bacteria.

Nucleotide sequence accession number. The nucleotide sequence of the *Y. enterocolitica htrA* gene has been submitted to the EMBL database under accession no. X94153.

RESULTS

***Y. enterocolitica* HtrA.** PCRDOP experiments consistently amplified a single band of 1,375 bp, which represents 95% of the *htrA* sequence. The nucleotide sequences of *htrA* clones from two independent PCRDOP experiments were determined and found to be identical. Southern blot analysis with the 1,375-bp *htrA* gene fragment used as a probe against *Y. enterocolitica* chromosomal DNA digested with *Eco*RV, *Hin*dIII, *Eco*RI, and *Bam*HI revealed single hybridization bands of 4.0, 5.2, 6.5, and 7.5 kb, respectively (data not shown). However, repeated attempts to clone the complete gene sequence, even by using low-copy-number vectors, were unsuccessful, possibly because the *Y. enterocolitica htrA* gene product may be lethal when expressed in *E. coli*. The *C. jejuni htrA* sequence also appears refractory to cloning in *E. coli* (53). The remaining 5% of the 3′ end of the *htrA* gene sequence was obtained by ligation anchor PCR as described in Materials and Methods. The deduced amino acid sequence of the complete *Y. enterocolitica htrA* gene was aligned with published HtrA sequences by using Clustal V multiple sequence alignment software (Fig. 1). The *Y. enterocolitica* HtrA homolog revealed extensive amino acid identity with *S. typhimurium* (74.5%), *E. coli* (69.7%), *C. jejuni* (40.1%), *H. pylori* (37.1%), *B. abortus* (36.9%), and *R. henselae* (36.6%).

Construction and characterization of a *Y. enterocolitica htrA* mutant. We constructed a *Y. enterocolitica htrA* mutant by allelic replacement as summarized in Fig. 2A. The *Y. enterocolitica htrA* cloned gene fragment was mutated by IPCRM with a 53-bp deletion, resulting in the inclusion of stop codons and the insertion of a kanamycin cassette (52). Typically, tri-cross mating experiments resulted in 60 kanamycin-resistant *Y. enterocolitica* exconjugants per ml of donor strain *E. coli* CC118-λ*pir*. These colonies were also resistant to chloramphenicol, suggesting that they were merodiploid strains. This was verified by PCR using primers H17 and H18, in which the amplified products corresponding to the wild-type *htrA* gene of 286 bp and the mutated *htrA* gene of 1,515 bp (including 1,282 bp from the size of the kanamycin resistance cassette with a 53-bp deletion) were always present (Fig. 2B). One merodiploid strain, YHT61, was subjected to cycloserine enrichment. After 2,000 colonies were replica plated on CIN agar containing kanamycin, a single colony, YHT67, was identified as being chloramphenicol sensitive. PCR analysis using primers H17 and H18 confirmed that a double-recombination event had occurred (Fig. 2B). YHT67 grew as small colonies on CR-MOX medium, and the presence of the *Yersinia* virulence plasmid was confirmed by PCR using primers O57 and O58 (54). Southern blot analysis of YHT67 DNA using a 1,375-bp *Y. enterocolitica htrA* probe confirmed that the mutated *htrA* gene had undergone a double-recombination event (data not shown).

Initial characterization of YHT67 revealed that in contrast to the parent strain, the mutant failed to grow at 42°C. However, comparable growth rates were observed for YHT67 and 8081v at 28 and 37°C (data not shown). When YHT67 was grown at 37°C and subjected to high-temperature stress at 50°C, survival of the mutant was reduced more than twofold compared with that of 8081v (Table 3). In experiments designed to compare the sensitivities of YHT67 and 8081v to oxidative killing, survival of the mutant was reduced significantly on exposure to H_2O_2 (10 and 40 mM) and menadione (15 and 50 mM) (Table 3).

Experimental infection of BALB/c mice. At an infectious oral dose of 10^8 bacteria, all 10 mice challenged with 8081v died. By comparison, the 10 mice challenged with an equivalent dose of the *htrA* mutant YHT67 survived and appeared healthy. Infection in these mice was cleared from the liver and spleen tissues around day 21 (Fig. 3B). The counts in both organs steadily declined over the period observed. In contrast, 8081v killed all animals by day 5, with counts steadily increasing in both liver and spleen tissues until death (Fig. 3A). Immunization of a group of 10 mice with YHT67 and subsequent challenge with 10^8 8081v bacteria resulted in the survival of 7 mice of the challenge group, whereas all of the unimmunized mice died upon challenge with 8081v. Thus, BALB/c mice orally immunized with a single dose of YHT67 were partially protected against virulent oral challenge with the *Y. enterocolitica* parent strain.

DISCUSSION

During bacterial infection, pathogen-host interactions expose the bacterium to multiple physiological and biological stresses. Members of the HtrA class of stress response proteins are serine proteases which apparently function by degrading (oxidatively or otherwise) damaged proteins before they can accumulate to toxic levels in cells (14, 45, 46). In this study, a *Y. enterocolitica htrA* gene fragment was amplified, cloned, and sequenced. Alignment of the deduced amino acid sequence of the *Y. enterocolitica* HtrA homolog with HtrA sequences from other bacterial species revealed extensive amino acid identity (Fig. 1). A significant proportion of this identity is in regions that have been demonstrated to be important for the biological activity of *E. coli* HtrA (14, 28, 30, 42, 45, 46). The conserved GNSGG motif closely resembles the core sequence GDSGG that usually surrounds the active serine residue of the catalytic

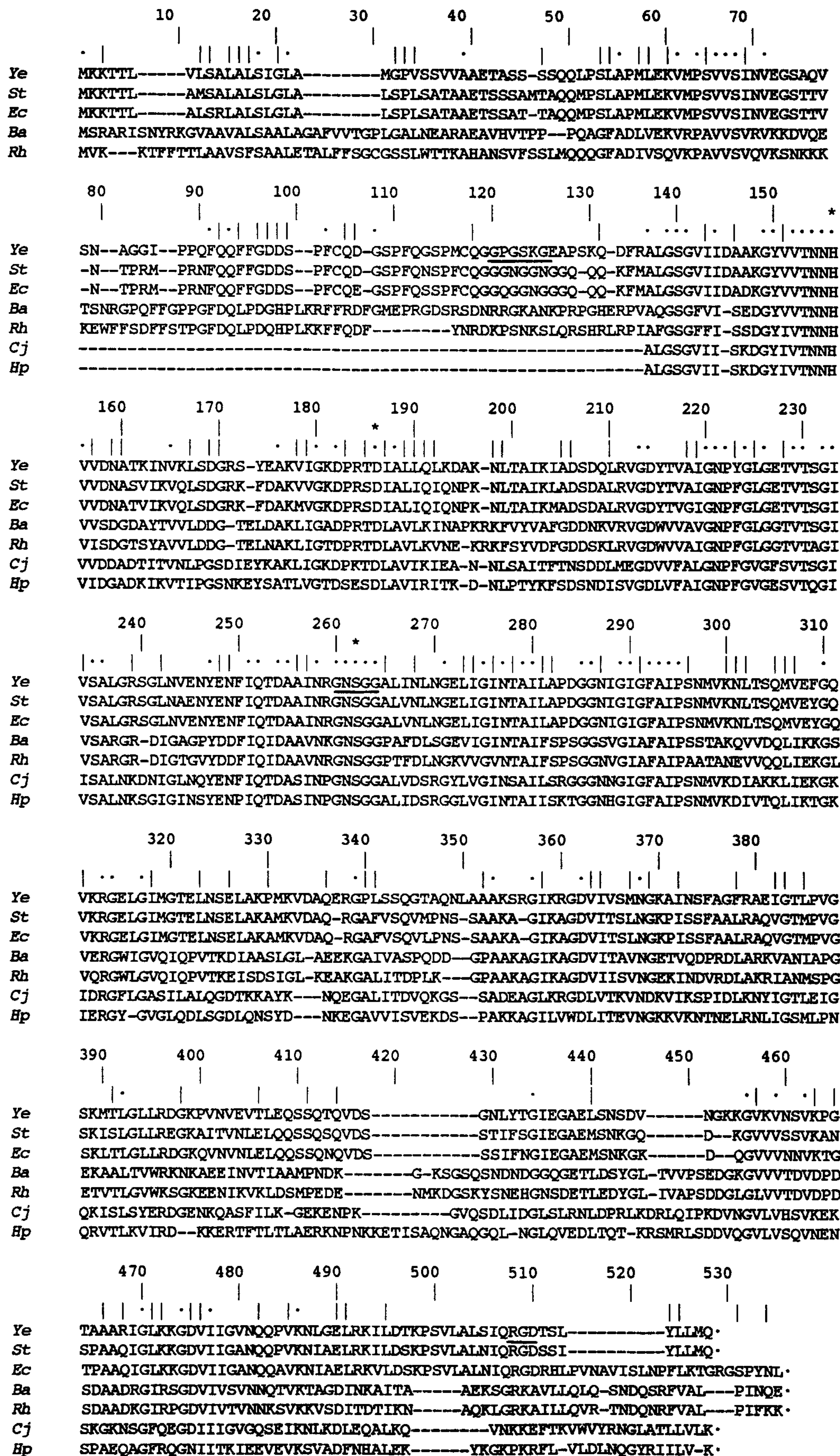

FIG. 1. Comparison of the deduced HtrA amino acid sequences of *S. typhimurium* (*St*) (26), *E. coli* (*Ec*) (29), *B. abortus* (*Ba*) (15), *R. henselae* (*Rh*) (GenBank accession no. L20127), *H. pylori* (*Hp*) (27), *C. jejuni* (*Cj*) (GenBank accession no. U27271), and *Y. enterocolitica* (*Ye*), aligned by using ClustalV multiple sequence alignment software. Identical amino acids (dots), conserved changes (vertical lines), the putative GNSGG serine protease catalytic domain, GXGXXG dinucleotide binding pocket, and RGD motif (underlined), and the serine, histidine, and aspartic acid residues, which are part of the catalytic triad characteristic of a trypsin-like serine protease (asterisks) are indicated.

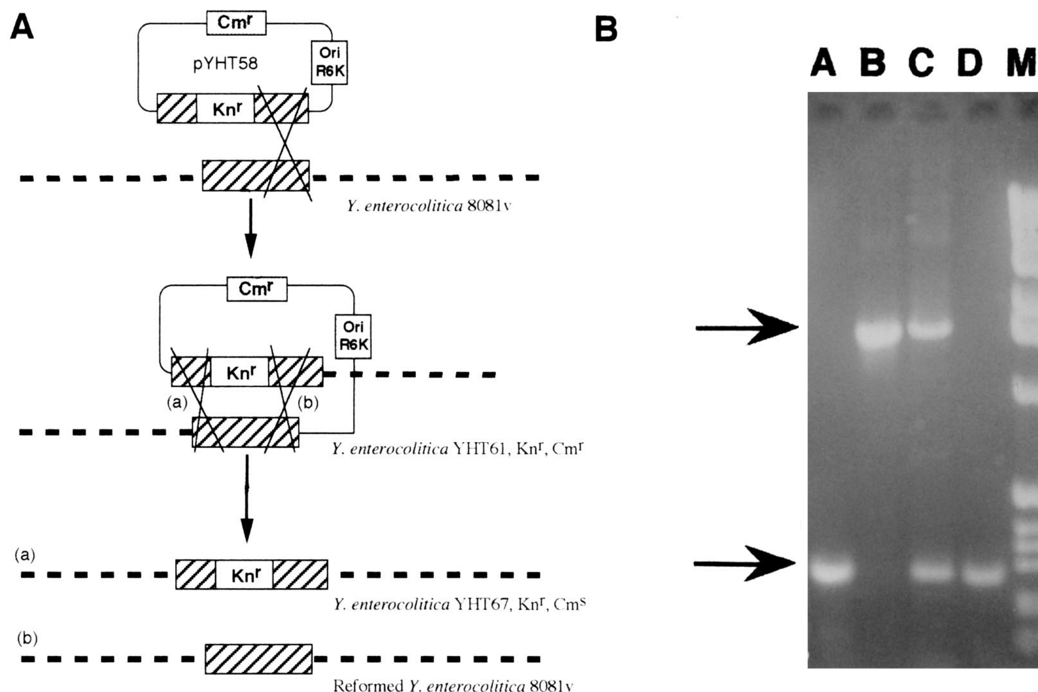

FIG. 2. (A) Generation of the *htrA* mutant by double recombination with pYHT58. (B) PCR analysis with primers H17 and H18 of the wild-type strain 8081v (lane A), the double-crossover *htrA* mutant YHT67 (lane B), the merodiploid strain YHT61 (lane C), and 8081v after the introduction and loss of suicide vector pYHT58 (lane D). Lane M contains as a marker the 1-kb molecular size ladder (Gibco/BRL Ltd., Paisley, United Kingdom). The 286- and 1,515-bp fragments (arrows) are indicated.

domain of known serine proteases (6). Three further regions of homology surround the histidine, aspartic acid, and serine residues (Fig. 1), which are part of the catalytic triad characteristic of trypsin-like serine proteases (6). In common with the *S. typhimurium* and *E. coli* HtrA sequences, the *Y. enterocolitica* HtrA homolog has a GXGXXG motif, a sequence that has been identified in some proteins as being involved in dinucleotide binding (50), and an RGD motif, which has been characterized as being important for cell binding of bacterial adhesion molecules (39) (Fig. 1). The finding of putative dinucleotide binding and cell-binding motifs within the deduced protein sequence of HtrA has not been correlated with any biological properties of the molecule.

Studies of HtrA proteins among several bacterial species have shown that these proteins play varied roles in the survival of the organism under conditions of oxidative and high-temperature stress. In contrast to *E. coli htrA* mutants, *S. typhimurium htrA* mutants are insensitive to temperature stress but sensitive to oxidative stress (26, 28, 30, 42, 45, 46), whereas a *C. jejuni htrA* mutant appears to be insensitive to temperature and oxidative stress (53). Conversely, Elzer et al. (15) have reported a *B. abortus htrA* mutant that is sensitive to temperature and oxidative stress, although Tatum et al. (47) found *B. abortus htrA* mutants to be insensitive to oxidative stress. The varied phenotypes of *htrA* mutants in different bacteria may reflect different environmental stresses encountered by the bacteria during infection and/or the free-living state. For example, *S. typhimurium* and *B. abortus* depend on survival and intracellular growth in macrophages as part of their infection strategy and are able to withstand oxidative killing within macrophages, which is thought to be the primary mechanism by which host macrophages kill bacteria. Experimental evidence from studies in which *S. typhimurium htrA* mutants failed to replicate in cultures of macrophages further support this hypothesis (3, 17). In contrast to *S. typhimurium* and *B. abortus*, *Y. enterocolitica* replicates extracellularly in lymphatic tissues (12, 44). However, the organism has been observed multiplying within mononuclear cells of infected rabbits (48, 49) and has been observed in the cytoplasm of large mononuclear cells in the Peyer's patches of mice (35). Irrespective of the extracellular or intracellular location of *Y. enterocolitica* during infection, an ability to counteract host phagocytes, including their oxidative burst, is required. Virulent *Yersinia* organisms achieve this, at least in part, through plasmid-encoded Yops acting on phagocytes (10, 11, 18–20, 22, 24, 38). However, Ruiz-Bravo et al. (40), studying the survival of *Y. enterocolitica* within mouse macrophages, have suggested that the observed resistance to intracellular killing by mouse macrophages was due to a chromosomally located determinant.

In this study, the *Y. enterocolitica htrA* mutant (YHT67) was shown to be avirulent at an infectious dose of 10^8 bacteria, despite the presence of the virulence plasmid and hence pre-

TABLE 3. Percent survival upon exposure to oxidative and high-temperature stress

Stress	% Survival[a]	
	8081v	YHT67
H_2O_2		
10 mM	91.2 ± 2.4	29.8 ± 8.9
40 mM	61.7 ± 12.8	2.8 ± 0.9
Menadione		
15 mM	97.2 ± 2.8	45.6 ± 7.1
50 mM	57.0 ± 22.3	5.0 ± 5.1
50°C	89.9 ± 4.5	41.6 ± 17.1

[a] Geometric mean percent ± 2 standard deviations for three experiments.

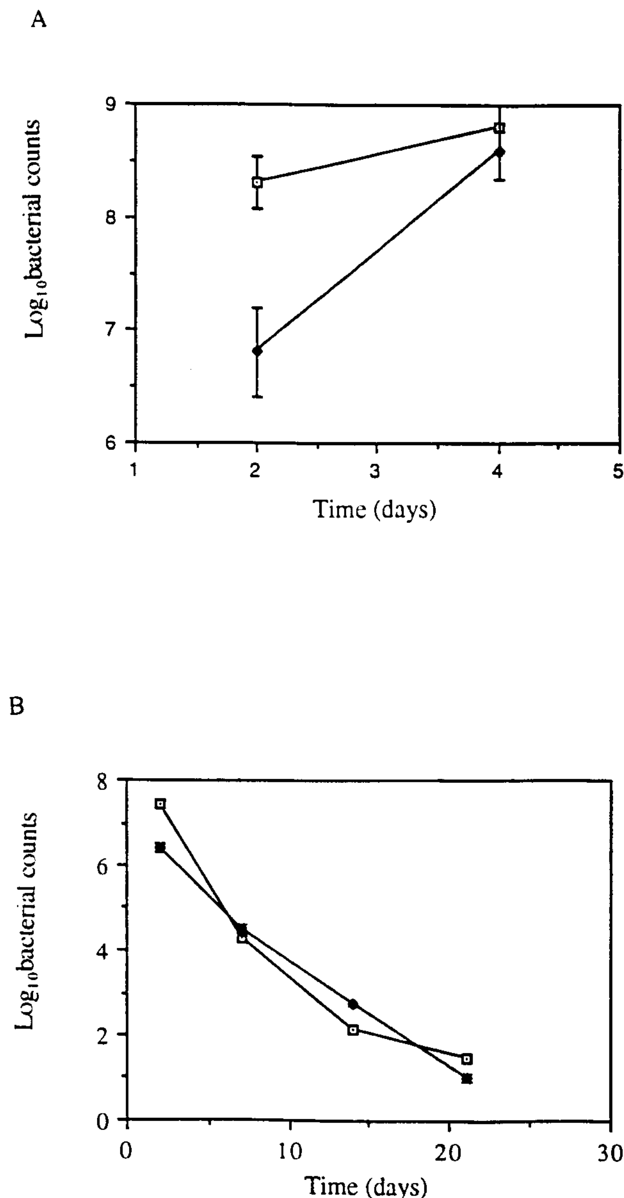

FIG. 3. Colonization of liver (□) and spleen (◆) tissues of BALB/c mice following oral administration of 8081v (mice which had not already been sacrificed were dead by day 5 postinfection) (A) and YHT67 (B). Each point represents the geometric mean ± 2 standard errors for 10 mice.

sumably functional Yops. Bacterial counts in liver and spleen tissues confirmed that YHT67 was cleared more efficiently than 8081v, which always caused death by 5 days postinfection (Fig. 3). However, more than 10^3 YHT67 bacteria persisted in liver and spleen tissues significantly longer than 8081v cured of the virulence plasmid (5, 16), suggesting that the Yops also contribute to the persistence of YHT67 in mice. In vitro studies confirmed that YHT67 and 8081v had comparable growth rates at 37°C, but YHT67 showed sensitivity to killing by high temperature (50°C), H_2O_2, and O_2^-. The sensitivity to oxidative stress could account for the low recovery of the mutant from infected tissues. If YHT67 and 8081v have equal abilities to colonize and invade host tissues, YHT67 may be more susceptible to killing by phagocytosis, which may explain its reduced virulence. Given that YHT67 is sensitive to oxidative stress and is rapidly cleared by the host, the expression of HtrA by *Y. enterocolitica* may provide a mechanism, independent of the Yops, by which the organism counteracts the oxidative burst during phagocytosis. This protection is not simply correlated with in vitro sensitivity to oxidative stress. Badger and Miller (2) have identified a *Y. enterocolitica* 8081v *rpoS* mutant that is also sensitive to H_2O_2-induced stress at 37°C but which is as virulent as the parent strain in BALB/c mice. A possible explanation for this discrepancy is that the *htrA* mutant was more than twice as sensitive as the *rpoS* mutant to H_2O_2-induced stress.

Johnson et al. (26) have also shown that *S. typhimurium htrA* mutants were able to confer protection against lethal challenge with virulent organisms. *S. typhimurium htrA* mutants have subsequently been shown to be excellent live attenuated vaccine strains (9, 43). In this study, 7 of the 10 mice were protected by YHT67 against a lethal challenge of 10^8 virulent *Y. enterocolitica* organisms. This protection was greater than that afforded by an *aroA* mutant to mice also challenged by 10^8 8081v organisms (5). Several doses of the *aroA* mutant were required for measurable protection, which contrasts with an *S. typhimurium aroA* mutant that, like the *htrA* mutant, conferred protection after a single dose (33). The greater protection conferred by the *Y. enterocolitica htrA* mutants than the *aroA* mutants correlates with bacterial count data showing that the *aroA* mutant was cleared after day 5 postinfection (5), compared with the 21 days required to clear the *htrA* mutant in this study.

The reverse-genetics PCR-based strategy described in this report is proving to be particularly useful for the construction of defined *Yersinia* mutants, as well as mutants in other bacteria, especially for species for which transposon mutagenesis is not established (52). Furthermore, the approach, which amplifies and mutates gene fragments, is not dependent on the availability of cloned genes. Therefore, potentially important gene products, which may be lethal when expressed in *E. coli*, are not excluded from mutagenesis analysis. In this study, for example, we were unable to clone the *Y. enterocolitica htrA* gene in *E. coli*, but were able to construct a defined *htrA* mutant.

It has been suggested that bacterial stress response proteins play an important role in allowing pathogenic organisms to adapt physiologically to the hostile environment of the host pathogen. As the *Y. enterocolitica htrA* mutant shows increased sensitivity to oxidative killing and shows attenuated virulence in BALB/c mice, further studies with the *htrA* mutant should improve our understanding of the mechanisms by which *Yersinia* spp. resist host defenses. Studies are in progress to compare the effects of the oxidative burst and to measure the survival of YHT67 and 8081v and cured derivatives in cultured neutrophil and macrophage cells. These studies and the construction and evaluation of further *Yersinia* mutants may help to dissect the mechanisms by which *Y. enterocolitica* resists phagocytosis and may help to resolve the controversies regarding the intracellular location of the organism during different stages of the infective process.

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