# 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

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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 PCRbased 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  $-80^{\circ}$ C in Luria broth (LB) containing 25% (vol/vol) glycerol and were grown on *Yersinia* selective agar (CIN agar; Oxoid, Basingstoke, England). At  $28^{\circ}$ C, *Y. enterocolitica* was cultured in LB, and at  $37^{\circ}$ C, 5 mM CaCl<sub>2</sub> 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

Strain or plasmid	Relevant characteristics <sup>a</sup>	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 <sup>r</sup> Y. enterocolitica 8081 htrA double crossover	This study	
E. coli			
XL2-Blue MRF'	Cloning strain	Stratagene	
CC118-λpir	Donor strain in tri-cross mating experiments	31	
S17::pNJ5000	Tri-cross mating strain with Tet <sup>r</sup> helper plasmid	21	
Plasmids			
pUC19	Ap <sup>r</sup>	Pharmacia	
pUC4K	Kn <sup>r</sup> ; source of Kn <sup>r</sup> BamHI cassette	Pharmacia	
pACYC184	Cm <sup>r</sup> Tet <sup>r</sup> ; source of Cm <sup>r</sup> for suicide vector	New England Biolabs	
pGP704	Derivative of pJM703.1; oriR6K mobRP4 Apr	31	
pSC1	Cm <sup>r</sup> suicide vector; 838-bp BsaBI-BsaAI pACYC184 fragment in PvuII-DraI pGP704	This study	
pNJ5000	Tet <sup>r</sup> , for triple mating	21	
pYHT38	pUC19 plus 1.3 kb of htrA	This study	
pYHT43	pYHT38 with 53-bp deletion in <i>htrA</i>	This study	
pYHT46	pYHT43 plus Kn <sup>r</sup>	This study	
pYHT58	Donor plasmid for conjugation; Cm <sup>r</sup> Kn <sup>r</sup> pYHT46 <i>Pvu</i> II- <i>Xba</i> I fragment containing the mutated <i>htrA</i> gene cloned into <i>Eco</i> RV- <i>Xba</i> I pSC1	This study	

TABLE 1.	Bacterial	strains and	plasmids	used in	this study
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<sup>a</sup> Abbreviations: Ap<sup>r</sup>, ampicillin resistant; Cm<sup>r</sup>, chloramphenicol resistant; Kn<sup>r</sup>, kanamycin resistant; Tet<sup>r</sup>, tetracycline resistant.

with 20 mM sodium oxalate and 20 mM MgCl<sub>2</sub> (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  $\mu$ g/ml), chloramphenicol (40  $\mu$ g/ml), kanamycin (50  $\mu$ g/ml), and tetracycline (20  $\mu$ g/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 *Hind*III and *Pst*1, 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 *Bg*/II site were engineered into the *htrA* gene

A 53-bp deletion and unique *Bg*/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 *Bg*/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  $\mu$ l 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-*xpir* cells to form 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-

Oligonucleotide	PCR method	Strand	Sequence $(5' \rightarrow 3')^a$
H10	PCRDOP	+	GAACTGCAGTATATGAARAARACNAC
H8	PCRDOP	-	TGAAGCTTNGCNCCNATDATNACRTCNCC
H13	IPCRM	-	TG <u>AGATCT</u> CGATAGCCTKACGGTATAATC
H14	IPCRM	+	TG <u>AGATCT</u> AGTGGTTTGAATGTGGAAA
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

TABLE 2. Oligonucleotides used for PCR

<sup>*a*</sup> Underlined nucleotides represent *Hin*dIII (H8), *Pst*I (H10), and *Bg*III (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 108 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 108 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 EcoRV, HindIII, EcoRI, and BamHI 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, tricross mating experiments resulted in 60 kanamycin-resistant Y. enterocolitica exconjugants per ml of donor strain E. coli CC118- $\lambda pir$ . 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<sup>8</sup> 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<sup>8</sup> 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

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MKKTTL-	AMSAL	ALSIGLA	ISPIS	SVVAABTASS- ATAAKTSSSAM	SSOOTESTAT		SVVSINVEGSAL SVVSINVEGSAL	UV TTV7
MKKTTL-	ALSRL	ALSLGLA	ISPIS	ATAAETSSAT-	TACOMPSLAI	MLEKVMPS	SVVSINVEGST	īv
MSRARIS	NYRKGVAAV	ALSAALAGAF	VVTGPLGALN	EARAEAVHVTP	PPQAGFAI	DLVEKVRP	AVVSVRVKKDV	QE
MVKK	TFFTTLAAV	SFSAALETAL	FFSGCGSSLW	<b>TTKAHANSVFS</b>	SLMQQOGFAL	DIVSQVKPA	AVVSVQVKSNKI	ĸĸ
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SNAGG	IPPOFOO	FFGDDSPF	COD-GSPFOG	i SPMCOGGPGSK	GEAPSKO-DI	RALGSGV		NNH
-NTPR	MPRNFQQ	FFGDDSPF	CQD-GSPFQN	SPFCQGGGNGG	NGGO-OO-KI	MALGSGV	IIDAAKGYVVT	NNH
-NTPR	MPRNFQQ	FFGDDSPF	CQE-GSPFQS	SPFCQGGQGGN	IGGGQ-QQ-KI	MALGSGV	IIDADKGYVVT	NNH
TSNRGPC	FFGPPGFDQ	LPDGHPLKRF	FRDFGMEPRG	DSRSDNRRGKA	NKPRPGHERI	PVAQGSGF	VI-SEDGYVVT	NNH
REWFFSC	FFSTPGFDQ	LPDQHPLKKF	FQDF	YNRDKPSNK	SLORSHRLRI	PIAFGSGF	FI-SSDGYIVT	NNH
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VVDNATK	INVKLSDGR	S-YEAKVIGR	UPRTDIALLQ	LKDAK-NLTAI	KIADSDQLR	VGDYTVAI	GNPYGLGETVT	SGI
VVDNAmv	TKAUTences	K-FDAKMUCH	OPRSDIALIQ	TONDE -NLTAI	TEMADODALR		GNET GLGETVI CNDRCT CIRIN	SGT
VVSDGDA	YTVVLDDG-	TELDAKLIGA	DPRTDLAVIK	INAPKRKFVYV	AFGDDNKVR	VGDWVVAV	CNPFGLGGTVT	SGI
VISDGTS	YAVVLDDG-	TELNAKLIGT	DPRTDLAVLK	VNE-KRKFSYV	DFGDDSKLR	VGDWVVAI	GNPFGLGGTVT	AGI
VVDDADT	ITVNLPGSD	IEYKAKLIGK	DPKTDLAVIK	IEA-N-NLSAI	TFTNSDDLM	GDVVFALO	INPFGVGFSVT	SGI
VIDGADK	IKVTIPGSNI	KEYSATLVGT	DSESDLAVIR	ITK-D-NLPTY	KFSDSNDIS	GDLVFAI	GNPFGVGESVIX	QGI
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VSALCRS	GLNVENYEN	FIQTDAAINR	GNSGGALINL	NGELIGINTAI	LAPDGGNIG:	IGFAIPSN	MVKNLTSOMVE	FGO
VSALGRS	GLNAENYEN	FIQTDAAINR	GNSGGALVNL	NGELIGINTAL	LAPDGGNIG	IGFAIPSN	MVKNLTSQMVE	YGQ
VSALGRS	GLNVENYEN	FIQTDAAINF	RGNSGGALVNL	NGELIGINTAJ	LAPDGGNIG	IGFAIPSN	MVKNLTSQMVE	YGO
VSARCR-	5765 mmm	TTOTOS 31000						
VOIDCOR	DIGAGPYDD	FIQIDAAVNE	GNSGGPAFDL	SGEVIGINTAI	FSPSGGSVG	IAFAIPSS	TAKOVVDOLIK	KGS
VSARGR-	DIGTGVYDD	FIQIDAAVNF FIQIDAAVNF FIOTDASINF	(GNSGGPAFDL (GNSGGPTFDL (GNSCGALVDS)	SGEVIGINTAI NGKVVGVNTAI RGVIVGINSAI	FSPSGGSVG	IAFAIPSS IAFAIPAA	TAKQVVDQLIK TANEVVQQLIE	KGS KGL
VSARGR- ISALNKD VSALNKS	DIGTGVYDD DIGTGVYDD NIGLNQYEN GIGINSYEN	F IQIDAAVNF F IQIDAAVNF F IQTDASINF P IQTDASINF	(GNSGGPAFDL (GNSGGPTFDL (GNSGGALVDS) (GNSGGALIDS)	SGEVIGINTAI NGKVVGVNTAI RGYLVGINSAI RGGLVGINTAI	FSPSGGSVG FSPSGGNVG LSRGGGNNG ISKTGGNHG	iafaipss' Iafaipaa Igfaipsni Igfaipsni	TAKQVVDQLIK TANEVVQQLIE MVKDIAKKLIE MVKDIVTOLIK	KGS KGL KGK TGK
VSARGR- ISALNKD VSALNKS	DIGTGVYDD DIGTGVYDD NIGLNQYEN GIGINSYEN	F IQIDAAVNF F IQIDAAVNF F IQTDAS INF P IQTDAS INF	(GNSGGPAFDL (GNSGGPTFDL (GNSGGALVDS) (GNSGGALIDS)	SGEVIGINTAI NGKVVGVNTAI RGYLVGINSAI RGGLVGINTAI	FSPSGGSVG FSPSGGNVG LSRGGGNNG ISKTGGNHG	iafaipss' Iafaipaa Igfaipsni Igfaipsni	TAKQVVDQLIK TANEVVQQLIE MVKDIAKKLIEI MVKDIVTQLIK	KGS KGL KGK TGK
VSARGR- ISALNKD VSALNKS	DIGAGPYDD DIGTGVYDD NIGLNQYEN GIGINSYEN	FIQIDAAVNF FIQIDAAVNF FIQTDASINF PIQTDASINF 330	GNSGGPAFDL GNSGGPTFDL GNSGGALVDS GNSGGALIDS 340	SGEVIGINTAI NGKVVGVNTAI RGYLVGINSAI RGGLVGINTAI 350	FSPSGGSVG FSPSGGNVG LSRGGGNNG ISKTGGNHG 360	IAFAIPSS IAFAIPAA IGFAIPSNI IGFAIPSNI 370	TAKQVVDQLIK TANEVVQQLIE MVKDIAKKLIE MVKDIVTQLIK 380	kgs Kgl Kgk Tgk
VSARGR- SALNKD VSALNKS	DIGAGPYDD DIGTGVYDD NIGLNQYENI GIGINSYENI 320	FIQIDAAVNF FIQIDAAVNF FIQTDASINF PIQTDASINF 330	(GNSGGPAFDL GNSGGPTFDL GNSGGALVDS) GNSGGALIDS) 340 	SGEVIGINTAI NGKVVGVNTAI RGYLVGINSAI RGGLVGINTAI 350	FSPSGGSVG FSPSGGNVG LSRGGGNNG: ISKTGGNHG 360	IAFAIPSS IAFAIPAA IGFAIPSN IGFAIPSN 370	TAKQVVDQLIK TANEVVQQLIE MVKDIAKKLIE MVKDIVTQLIK 380	kgs Kgl Kgk Tgk
VSARGR- ISALNKD VSALNKS	DIGAGPYDD DIGTGVYDD NIGLNQYENI GIGINSYENI 320           	FIQIDAAVN FIQIDAAVNF FIQTDASINF PIQTDASINF 330       	GNSGGPAFDL GNSGGPTFDL GNSGGALVDS GNSGGALIDS 340     EEGPLSSOGT	SGEVIGINTAI NGKVVGVNTAJ RGYLVGINSAI RGGLVGINTAI 350     - -	FSPSGGSVG FSPSGGNVG LSRGGGNNG ISKTGGNHG 360     •     LKRGDV VS	IAFAIPSS IAFAIPAA IGFAIPSNI IGFAIPSNI 370           	TAKQVVDQLIK TANEVVQQLIE MVKDIAKKLIEI MVKDIVTQLIK 380           	KGS KGL KGK TGK
VSARGR- ISALNKD VSALNKS   /KRGELG	DIGAGPYDD DIGTGVYDD NIGLNQYENI GIGINSYENI 320   	FIQIDAAVN FIQIDAAVN FIQTDASIN PIQTDASIN 330       LAKPMKVDAQ LAKAMKVDAQ	(GNSGGPAFDL GNSGGPTFDL (GNSGGALVDS) (GNSGGALIDS) 340   +   ERGPLSSQGT/ -RCAFVSOVM	SGEVIGINTAI NGKVVGVNTAJ RGYLVGINSAI RGGLVGINTAI 350     - - AQNLAAAKSRG PNS-SAAKA-G	FSPSGGSVG FSPSGGNVG LSRGGGNNG ISKTGGNHG 360     .   IKRGDVIVSN IKRGDVIVSN	IAFAIPSS IAFAIPAA IGFAIPSNI 370   	TAKQVVDQLIK TANEVVQQLIE MVKDIAKKLIE MVKDIVTQLIK 380       FAGPRAEIGTLI FAGPRAEIGTLI	KGS KGL KGK TGK PVG
VSARGR- ISALNKD VSALNKS / /KRGELG /KRGELG	DIGAGPYDD DIGTGVYDD NIGLNQYENI GIGINSYENI 320       IMGTELNSEI IMGTELNSEI IMGTELNSE	FIQIDAAVNF FIQIDASINF JIQIDASINF 330     LAKPMKVDAQ LAKAMKVDAQ LAKAMKVDAQ	(GNSGGPAFDL (GNSGGALVDS) (GNSGGALIDS) 340     ERGPLSSQGT. RGAFVSQVM 2-RGAFVSQVL	SGEVIGINTAI NGKVVGVNTAI RGYLVGINSAI 350     - - AQNLAAAKSRG PNS-SAAKA-G PNS-SAAKA-G	FSPSGGSVG FSPSGGNVG ISRGGNNG ISKTGGNHG 360   	IAFAIPSS IAFAIPAA IGFAIPSNI IGFAIPSNI 370   	TAKQVVDQLIK TANEVVQQLIE MVKDIAKKLIE MVKDIVTQLIK 380       FAGFRAEIGTII FAALRAQVGTM FAALRAQVGTM	KGS KGL KGK TGK PVG PVG
VSARGR- ISALNKD /SALNKS /KRGELG /KRGELG /KRGELG /ERGWIG	DIGAGYYDD DIGTGVYDD NIGLNQYEN GIGINSYEN 320         IMGTELNSE IMGTELNSE IMGTELNSE MGTELNSE	FIQIDAAVNF FIQIDASINF PIQTDASINF 330     LAKPMRVDAQ LAKAMKVDAQ LAKAMKVDAQ IAASLGL-AF	(GNSGGPAFDL (GNSGGALVDS) (GNSGGALIDS) 340     ERCPLSSQT. -RCAFVSQVM 2-RCAFVSQVL EKCAIVASPQ	SGEVIGINTAI NGKVVGVNTAI RGYLVGINSAI 350     AQNLAAAKSRG PNS-SAAKA-G DD-GPAAKAG	FSPSGGSVG FSPSGGNVG LSRGGNNG: ISKTGGNHG: 360        IKRGDVIVSI IKRGDVITSI IKAGDVITSI SIKAGDVITSI	IAFAIPSS IAFAIPAA IGFAIPSNI IGFAIPSNI 370   	TAKQVVDQLIK TANEVVQQLIE WVKDIAKKLIE 380          FAGFRAEIGTL FAALRAQVGTM FRALRAQVGTM FRALRAQVGTM	KGK KGK TGK PVG PVG PVG
VSARGR- ISALNKD /SALNKS /KRGELG /KRGELG /KRGELG /ERGWIG /QRGWIG	DIGAGYYDD DIGTGVYDD NIGLNQYEN GIGINSYEN 320         IMGTELNSE IMGTELNSE UMGTELNSE VQIQPVTKD VQIQPVTKD	FIQIDAAVNF FIQIDASINF PIQTDASINF 330     LAKPMKVDAQ LAKAMKVDAQ LAKAMKVDAQ IAASIGL-AR ISDSIGL-KR	Consegpaped Consegatives Conseg	SGEVIGINTAI NGKVVGVNTAI RGYLVGINSAI 350     AQNLAAAKSRG PNS-SAAKA-G PNS-SAAKA-G DD-GPAAKAG KGPAAKAG	FSPSGGSVG FSPSGGNVG ISRTGGNHG: 360     •    IKRGDVIVSI IKRGDVITSI IKAGDVITSI IKAGDVITSI	IAFAIPSS IAFAIPAA IGFAIPSNI IGFAIPSNI 370 i i INGKAINSJ LNGKPISSJ LNGKPISSJ VNGBTVQD VNGBKIND	TAKQVVDQLIK TANEVVQQLIE WVKDIAKKLIE 380         FAGFRAEIGTLI FAALRAQVGTMI FAALRAQVGTMI FAALRAQVGTMI FAALRAQVGTMI	KGS KGL KGK TGK PVG PVG PVG APG
VSARGA- ISALNKD VSALNKS VSALNKS VKRGELG VKRGELG VKRGELG VERGWIG VDRGFLG IDRGFLG	DIGAGYYDD DIGTGVYDD NIGLNQYENI GIGINSYENI 320         IMGTELNSE IMGTELNSE SIMGTELNSE VQIQPVTKD VQIQPVTKD VQIQPVTKE ASILALQGD	FIQIDAAVNF FIQIDASINF PIQTDASINF 330     LAKPMRVDAQ LAKAMKVDAQ LAKAMKVDAQ IAASIGL-AF ISDSIGL-KF TKKAXK	KGNSGGPAFDL KGNSGGALVDS GNSGGALVDS 340             RGPLSSQT   -RGAFVSQVM   -RGAFVSQVM     KGALVASPQ     KGALITDPL   QEGALITDPL	SGEVIGINTAI NGKVVGVNTAI RGYLVGINSAI RGGLVGINTAI 350     AQNLAAAKSRG PNS-SAAKA-G PNS-SAAKA-G DD-GPAAKAG KGPAAKAG	FSPSGGSVG FSPSGCNVG LSRGGNNG: ISKTGGNHG: 360          IKRGDVITS: IKRGDVITS: IKAGDVITS: IKAGDVITS: IKAGDVITS: IKAGDVITS:	IAFAIPSS IAFAIPAA IGFAIPSNI 370       MNGKAINSJ LINGKPISSJ LINGKPISSJ VNGETVQD VNGEKIND VNGEKIND	TAKQVVDQLIK TANEVVQQLIE MVKDIAKKLIE 380         FAGFRAEIGTLI FAALRAQVGTM FAALRAQVGTM PRDLARKVANI VRDLAKKIANM PIDLKNYIGTLI	KGS KGL KGK TGK PVG PVG PVG APG SPG EIG
VSARGR- USALNKD VSALNKS VKGELG VKGELG VKGELG VRGELG VRGFLG ZRGYLG ZRGYLG	DIGAGYYDD DIGTGVYDD NIGLNQYEN GIGINSYEN 320         IMGTELNSE IMGTELNSE SIMGTELNSE VQIQPVTKD VQIQPVTKE ASILALQGD VGLQDLSGD	FIQIDAANN FIQIDASINF PIQTDASINF 330     LAKPMKVDAQ LAKAMKVDAQ LAKAMKVDAQ LAKAMKVDAQ LAKAMKVDAQ LAKAMKVDAQ LASLGL-AR ISDSIGL-KR TKKAYKN LQNSYDN	CONSEGDATEDL CONSEGALIDS CONSEGALIDS CONSEGALIDS 340     ERCPLSSQET. -RCAFVSQVM CRCAFVSQVM EKCAIVASPQ EXCALIDPL QEGALITDPL QEGALITDVQ IKEGAVVISVE	SGEVIGINTAI NGKVVGVNTAI RGYLVGINSAI 350     AQNLAAAKSRG PNS-SAAKA-G PNS-SAAKA-G DD-GPAAKAG KGPAAKAG KGSSADEAG KDSPAKKAG	FSPSGGSVG FSPSGGNVG ISRTGGNHG: 360     •    IKRGDVIVSI IKRGDVITSI IKAGDVITSI IKAGDVITSI IKAGDVITSI IKAGDVITSI IKAGDVITSI IKAGDVITSI	IAFAIPSS IAFAIPAA IGFAIPSNI 370       MNGKAINSJ LINGKPISSJ LINGKPISSJ VNGETVQD VNGEKIND VNGEKIND	TAKQVVDQLIK TANEVVQQLIE MVKDIAKKLIE 380         FAGFRAEIGTLI FAALRAQVGTMI FAALRAQVGTMI PRDLARKVANI VRDLAKKIANM PIDLKNYIGTLI TNELRNLIGSM	KGS KGL KGK TGK PVG PVG PVG PVG SPG EIG LPN
SALNKO SALNKO SALNKS SALNKS KRGELG KRGELG KRGELG KRGELG ZORGWLG DRGFLG ERGY-G 90	DIGAGYYDD DIGTGVYDD NIGLNQYENI GIGINSYENI 320         IMGTELNSE! IMGTELNSE UMGTELNSE VQIQPVTKD VQIQPVTKE ASILALQGD VGLQDLSGD 400	FIQIDAANN FIQIDASINF PIQTDASINF 330     LAKPMKVDAQ LAKAMKVDAQ	CONSEGRATEDL CONSEGRALIDS CONSEGRALIDS 340     ERCPLSSQT. -RCAFVSQVM CRCAFVSQVM EKCAIVASPQ EKCAIVASPQ EKCAITDPL QEGALITDPL QEGALITDPL 220	SGEVIGINTAI NGKVVGVNTAI RGYLVGINSAI 350     AQNLAAAKSRG PNS-SAAKA-G DD-GPAAKAG KGPAAKAG KGSSADEAG KDSPAKKAG 430	FSPSGGSVG FSPSGGNVG ISRGGNNG: ISRTGGNHG: 360     •    IKRGDVITS: IKRGDVITS: IKAGDVITS: I	IAFAIPSS IAFAIPAA IGFAIPSNI 370      INGKAINSJ LINGKPISSJ LINGKPISSJ UNGETVQD VNGEKIND VNGEKIND VNGEKIND VNGEKVN 100KVIKSJ	TAKQVVDQLIK TANEVVQQLIE MVKDIAKKLIE 380         FAGFRAEIGTLI FAALRAQVGTMI FAALRAQVGTMI PRDLARKVANI VRDLAKRIANM PIDLKNYIGTLI TNELRNLIGSM 460	KGS KGL KGK TGK PVG PVG PVG PVG SPG EIG LPN
SARGR- SALNKD SALNKS SALNKS KRGELG KRGELG KRGELG FRGWIG QRGWLG DRGFLG ERGY-G 90	DIGAGYYDD DIGTGVYDD NIGLNQYENI GIGINSYENI 320         IMGTELNSE IMGTELNSE SIMGTELNSE SIMGTELNSE VQIQPVTKD VQIQPVTKE ASILALQGD VGIQDLSGD 400 	FIQIDAAVNF FIQIDASINF PIQTDASINF 330     LAKPMKVDAQ LAKAMKVDAQ	KGNSGGPAFDL KGNSGGALVDS GNSGGALIDS 340     ERGPLSSQT -RGAFVSQVM -RGAFVSQVM EKGAIVASPQ LAKGALITDPL QEGALITDVQ KEGAVVISVE 420 	SGEVIGINTAI NGKVVGVNTAI RGYLVGINSAI 350     AQNLAAAKSRG PNS-SAAKA-G PNS-SAAKA-G DD-GPAAKAG KGPAAKAG KGSSADEAG KDSPAKKAG 430 	FSPSGGSVG FSPSGGNVG ISRGGNNG: ISRTGGNHG: 360       : IRAGDVITS: IRAGVITS: IRAG	IAFAIPSS IAFAIPAA IGFAIPSNI IGFAIPSNI 370 i i MKKAINSJ LNGKPISSJ LNGKPISSJ UNGETVQD VNGETVQD VNGETVQD VNGEKIND VNGEKIND VNGEKIND	TAKQVVDQLIK TANEVVQQLIE MVKDIAKKLIE MVKDIVTQLIK 380        FAGFRAEIGTLI FAALRAQVGTM FAALRAQVGTM PRDLAKKVANI VRDLAKKVANI VRDLAKKIANM PIDLKNYIGTLI TNELKNLIGSM 460 	RGS KGL KGK TGK PVG PVG PVG PVG SPG SPG LPN
SARGR- SALNKD SALNKS SALNKS KRGELG KRGELG KRGELG FRGWIG QRGWLG DRGFLG ERGY-G 90	DIGAGYYDD DIGTGVYDD NIGLNQYENI GIGINSYENI 320         IMGTELNSE IMGTELNSE SIMGTELNSE VQIQPVTKD VQIQPVTKE ASILALQGD VGIQPLSGD 400   	FIQIDAAVNF FIQIDASINF PIQTDASINF 330     LAKPMKVDAQ LAKAMKVDAQ LAKAMKVDAQ LAKAMKVDAQ LAKAMKVDAQ LAKAMKVDAQ LAKAJKN LONSYDN 410   	KGNSGGPAFDL KGNSGGALVDS GNSGGALIDS 340     ERGPLSSQGT. -RGAFVSQVM -RGAFVSQVM EKGAIVASPQ LAKGALITDPL QEGALITDVQ KEGAVVISVE 420 	SGEVIGINTAI NGKVVGVNTAI RGYLVGINSAI 350   AQNLAAAKSRG PNS-SAAKA-G PNS-SAAKA-G DD-GPAAKAG KGPAAKAG KGSSADEAG KDSPAKKAG 430 	FSPSGGSVG FSPSGGNVG ISRGGNNG: ISKTGGNHG: 360   	IAFAIPSS IAFAIPSNI IGFAIPSNI IGFAIPSNI 370 i i MKGKAINSJ LNGKPISSJ VNGETVQD VNGEKIND VNGEKIND VNGEKIND VNGEKIND VNGEKIND VNGEKIND VNGEKVKN 450 i	TAKQVVDQLIK TANEVVQQLIE MVKDIAKKLIE MVKDIVTQLIK 380        FAGFRAEIGTLI FAALRAQVGTM FAALRAQVGTM PRDLAKKVANI VRDLAKKVANI VRDLAKKIANM PIDLKNYIGTLI TNELRNLIGSM 460 	KGS KGL KGK TGK PVG PVG PVG PVG SPG EIG LPN
SARGR- SALNKD SALNKS SALNKS SALNKS SKRGELG KRGE KRGE KRGE KRGE KRGE KRGE KRGE KRG	DIGAGYYDD DIGTGVYDD NIGLNQYENI GIGINSYENI 320         IMGTELNSEI IMGTELNSE SIMGTELNSE VQIQPVTKD VQIQPVTKD VQIQPVTKE ASILALQGD VGIQDLSGD 400   LRDGKPVNV	FIQIDAAVNF FIQIDASINF PIQTDASINF 330     LAKPMKVDAQ LAKAMKVDAQ	CONSEGRATEDL CONSEGRATEDL CONSEGRATEDS CO	SGEVIGINTAI NGKVUGUNTAI RGYLVGINSAI 350   AQNLAAAKSRG PNS-SAAKA-G PNS-SAAKA-G PNS-SAAKA-G DD-GPAAKAG KGPAAKAG KGSSADEAG KDSPAKKAG 430   GNLYTG	FSPSGGSVG FSPSGGNVG ISRGGNNG: ISKTGGNHG: 360     .    ISKTGGNHG: 310 ISKTGGVITS: ISKAGDVIT	IAFAIPSS IAFAIPSNI IGFAIPSNI IGFAIPSNI 370 i i MNGKAINSJ LNGKPISSJ VNGETVQD VNGEKIND VNGEKIND VNGEKIND VNGEKIND VNGEKIND VNGEKUND VNGEKUND	TAKQVVDQLIK TANEVVQQLIE MVKDIAKKLIE MVKDIVTQLIK 380          FAGFRAEIGTLI FAALRAQVGTM FAALRAQVGTM PRDLAKKVANL VRDLAKRIANM PIDLKNYIGTLI TNELRNLIGSM 460 	KGS KGL KGK TGK PVG PVG PVG SPG SPG SPG SPG LPN 
VSARGR- VSARGR- ISALNKD VSALNKS VRGELG VRGELG VRGELG VRGFLG VRGFLG VRGFLG SKISLGL SKISLGL	DIGAGYYDD DIGTGVYDD NIGLNQYENI GIGINSYENI 320         IMGTELNSEI IMGTELNSE SIMGTELNSE VQIQPVTKD VQIQPVTKD VQIQPVTKD VQIQPVTKE ASILALQGD VGIQDLSGD 400   LRDGKAITVI LRDGKAITVI	FIQIDAAVNF FIQIDASINF PIQTDASINF 330     LAKPMKVDAQ LAKAMKVDAQ	CONSEGRATEDL CO	SGEVIGINTAI NGKVVGVNTAI RGYLVGINSAI 350   AQNLAAAKSRG PNS-SAAKA-G PNS-SAAKA-G DD-GPAAKAG KGPAAKAG KGPAAKAG 430   GNLYTG GNLYTG	FSPSGGSVG FSPSGGNVG ISRGGNNG: ISKTGGNHG: 360   ISKTGGNHG: 360   IKAGDVITS: IKAGD	IAFAIPSS IAFAIPSNI IGFAIPSNI IGFAIPSNI IGFAIPSNI 370 i i MNGKAINSJ LNGKPISSJ VNGETVQD VNGEKIND VNGEKIND VNGEKIND VNGEKIND VNGEKIND VNGEKIND VNGEKIND VNGEKIND VNGEKIND	TAKQVVDQLIK TANEVVQQLIE MVKDIAKKLIE 380        FAGFRAEIGTLI FAALRAQVGTM PRDLAKKVANI VRDLAKKVANI VRDLAKKIANM PIDLKNYIGTLI TNELKNLIGSM 460 	KGS KGL KGK TGK PVG PVG PVG PVG SPG EIG LPN       KPG KAN
VSARGELG VSARGELG VKRGELG VKRGELG VKRGELG VKRGELG VKRGELG IDRGFLG IDRGFLG IDRGFLG IERGY-G 390     SKMTLGL SKLSLGL SKLSLGL	DIGAGYYDD DIGTGVYDD NIGLNQYENI GIGINSYENI 320         IMGTELNSEI IMGTELNSEI IMGTELNSE VQIQPVTKD VQIQPVTKD VQIQPVTKD VQIQPVTKE ASILALQGD VQIQPVTKE ASILALQGD UGLQDLSGD   LRDGKPVNVI LRDGKPVNVI LRDGKPVNVI LRDGKQVNV	FIQIDAAVNF FIQIDASINF PIQTDASINF 2330      AKPMKVDAQ  AKAMKVDAQ  LAKAMKVDAQ  LAKAMKVDAQ	CONSEGRATEDL CO	SGEVIGINTAI NGKVVGVNTAI RGYLVGINSAI 350     AQNLAAAKSRG PNS-SAAKA-G PNS-SAAKA-G DD-GPAAKAG KGPAAKAG KGSSADEAG KDSPAKKAG 430   GNLYTG STIFSG SSIFNG	FSPSGGSVG FSPSGGNVG ISRGGNNG: ISKTGGNHG: 360         IKRGDVIVS: IKAGDVITS: IKAGDVITS: IKAGDVITS: IKAGDVITS: IKAGDVITS: IKAGDLVTKV ILVWDLITEV 440   IEGAELSNS: IEGAELSNS: IEGAEMSNKK IEGAEMSNKK	IAFAIPSS IAFAIPSA IGFAIPSNI IGFAIPSNI IGFAIPSNI 370 i i MNGKAINSJ LNGKPISS VNGETVQD VNGEKIND VNGEKIND VNGEKIND VNGEKIND JNGKVKN 450 j 00	TAKQVVDQLIK TANEVVQQLIE MVKDIAKKLIE MVKDIVTQLIK 380          FAGFRAEIGTLI FAALRAQVGTM PRDLAKKVANI VRDLAKRIANM PIDLKNYIGTLI TNELKNLIGSM 460 	KGS KGL KGK TGK PVG PVG APG SPG EIG LPN       KPG KAN KTG
VSARGR- VSARGR- ISALNKD VSALNKS VKRGELG VRGELG VRGELG VRGELG DRGFIG DRGFIG ERGY-G 390   	DIGAGYYDD DIGTGVYDD NIGLNQYENI GIGINSYENI 320         IMGTELNSEI IMGTELNSEI IMGTELNSE WQIQPVTKD ASILALQGD' VGLQDLSGDI 400   LRDGKPVNVI LRDGKPVNVI LRDGKQVNV WRKNKAEE I	FIQIDAAVNF FIQIDAAVNF FIQIDASINF JQIDASINF JQIDASINF JQIDASINF JQIDASINF JQIDASINF JQIDASINF JQIDASINF JQIDASINF JQIDASINF JQIDASINF JQIDAAVNF JQIDASINF JQI	GNSGGPAFDL GNSGGALVDS GNSGGALIDS 340     PRGPLSSQGT. PRGAFVSQVM PRGAFV	SGEVIGINTAI NGKVVGVNTAI RGYLVGINSAI 350     AQNLAAAKSRG PNS-SAAKA-G DD-GFAAKAG KGPAAKAG KGPAAKAG KGSSADEAG KDSPAKKAG 430   STIFSG STIFSG SSIFNC	FSPSGGSVG FSPSGGNVG ISRGGNNG: ISKTGGNHG: 360        IIKAGDVITS: II	IAFAIPSS IAFAIPSN IGFAIPSN IGFAIPSN IGFAIPSN IGFAIPSN IGFAIPSN INGKAINSI LNGKPISS VNGETVQD VNGEKIND VNGEKIND VNGEKIND VNGEKIND VNGEKIND SQ GL-TVVPS	TAKQVVDQLIK TANEVVQQLIE MVKDIAKKLIE 380          FAGFRAEIGTLI FAALRAQVGTM FAALRAQVGTM PRDLARKVANI VRDLAKRIANM PIDLKNYIGTLI TNELKNLIGSM 460 	KGS KGL FVG PVG PVG PVG SPG SPG EIG LPN       KPG KAN KTGD
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VSARGR- VSARGELG VKRGELG VKRGELG VKRGELG VKRGELG VKRGELG VKRGELG VKRGELG VKRGELG SKLGL SKISLGL	DIGAGYYDD DIGTGVYDD NIGLNQYENI GIGINSYENI 320         IMGTELNSEI IMGTELNSEI IMGTELNSE UMGTELNSE VQIQPVTKD VQIQPVTKD VQIQPVTKD ASILALQGD VGLQDLSGDI 400   LROCKPVNVI LREGKAITVI LRDGKQVNV WRKNRAEE I WKSGKEENI LRDG-KKER	FIQIDAAVNF FIQIDASINF JQTD	GNSGGPAFDL GNSGGALIDS GNSGGALIDS 340     PRGPLSSQGT PRGPLSSQGT PRGAFVSQVM PRGAFVSQVM PRGAFVSQVM PRGAFVSQVM CARFVSQVM	SGEVIGINTAI NGKVVGVNTAI RGYLVGINSAI 350     AQNLAAAKSRG PNS-SAAKA-G DD-GPAAKAG KGS-SAAKA-G DD-GPAAKAG KGS-SADEAG KDSPAKKAG 430   STIFSG SSIFK KSGSQSNDNDG KDGSKYSNEHG GVQSDLIDG QNGAQGQL-NG	FSPSGGSVG FSPSGGNVG ISRGGNNG: ISKTGGNHG: 360      IKAGDVITS: IKAGDVITS: IKAGDVITS: IKAGDVITS: IKAGDVITS: IKAGDVITS: IEGAELSNS:	IAFAIPSS IAFAIPSN IGFAIPSN IGFAIPSN IGFAIPSN IGFAIPSN IGFAIPSN IGFAIPSN INGKAINSI INGK	TAKQVVDQLIK TANEVVQQLIE MVKDIAKKLIE 380        FAGFRAEIGTII FAALRAQVGTM PRDLARKVANI VRDLARKVANI VRDLARKVANI VRDLARKVANI MOLARKVANI ONGKGVKVNSVI DKGVVVSSVI DGGVVVNV EDGKGVVVTDV DDGKGVVVTDV DDGKGVVVTDV KDVNGVLVHSVI DDVQGVLVSOVI	KGS KGL KGK TGK PVG PVG PVG SPG SPG SPG LPN       KPG KAN KTG DPD DKEK NEN
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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. typhi*murium 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

Stross	% Survival <sup>a</sup>	rvival <sup>a</sup>
511055	8081v	YHT67
H <sub>2</sub> O <sub>2</sub>		
10 mM	$91.2 \pm 2.4$	$29.8 \pm 8.9$
40 mM	$61.7 \pm 12.8$	$2.8\pm0.9$
Menadione		
15 mM	$97.2 \pm 2.8$	$45.6 \pm 7.1$
50 mM	$57.0 \pm 22.3$	$5.0 \pm 5.1$
50°C	$89.9 \pm 4.5$	$41.6 \pm 17.1$

<sup>*a*</sup> Geometric mean percent  $\pm 2$  standard deviations for three experiments.

Α



В



FIG. 3. Colonization of liver  $(\boxdot)$  and spleen  $(\blacklozenge)$  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  $\pm 2$  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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