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Identification and Characterization of the *Yersinia enterocolitica gsrA* Gene, Which Protectively Responds to Intracellular Stress Induced by Macrophage Phagocytosis and to Extracellular Environmental Stress

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Yersinia enterocolitica **is able to resist the microbicidal mechanisms of macrophages and to grow within phagocytic cells. Some bacteria including *Y. enterocolitica* have been shown to respond to the hostile environment in macrophages by producing a set of stress proteins which are also induced by environmental stresses. To understand the role of stress proteins in intracellular survival of bacteria, we identified and cloned a *Y. enterocolitica* gene, called *gsrA* (global stress requirement). The *gsrA* gene was identified because its insertional inactivation by a transposon resulted in the inability of the organism to grow at an elevated temperature and to survive within macrophages after phagocytosis. The *gsrA* gene was sequenced and shown to encode a basic, 49,500-Da protein. The GsrA protein shows significant amino acid sequence homology to the HtrA stress protein which was originally identified in *Escherichia coli*. Furthermore, the genetically defined *Y. enterocolitica gsrA* mutant was constructed and characterized. The insertional mutation of *gsrA* resulted in inhibition of growth at temperatures above 39°C and greatly increased susceptibility to oxidative and osmotic stresses. The mutant additionally lost the ability to survive and replicate within macrophages. These results, taken together, indicate that the *gsrA* gene is an essential component of the protection mechanism employed by *Y. enterocolitica*, allowing it to respond to the intracellular stress in macrophages as well as extracellular environmental stress.**

It is well known that all organisms respond to a sudden increase in temperature by transient acceleration in synthesis of a group of proteins called "heat shock proteins" (for a review, see reference 32). The heat shock proteins are collectively known as stress proteins that are induced by a variety of different stimuli including ethanol, oxidative products, high salt concentration, nutrient limitation, and damage caused by toxic chemicals and physical agents. The effectiveness of these stimuli varies; that is, they induce a similar, yet different, sets of proteins (10, 33). It is therefore believed that several stress responses overlap and the extent of the overlap varies in different overlapping responses.

Upon bacterial infection, pathogens are exposed to various stressful conditions, e.g., a temperature increase when they encounter a susceptible host or microbicidal mechanisms associated with the host immune system. In particular, the intracellular environment of phagocytic cells is hostile; in it the viability of the organism is threatened by oxidative or nonoxidative microbicidal mechanisms. We have previously demonstrated that *Yersinia enterocolitica* responds to phagocytosis by macrophages by expression of several stress proteins which are also induced by heat shock and oxidative stress in vitro (55). N-terminal amino acid sequencing confirmed homologs of GroEL, GroES, and DnaK among the proteins induced by the intracellularly grown bacteria. *Y. enterocolitica* is capable of surviving and growing in macrophages after phagocytosis, and its ability to grow intracellularly is thought to be an important process in the pathogenesis of this bacterium (23, 47, 51, 52). It also has been reported that some facultative intracellular pathogens, e.g., *Salmonella typhimurium* (4), *Legionella pneumophila* (21), and *Brucella abortus* (24), generally express elevated levels of a set of proteins within macrophages after phagocytosis. It is speculated that the enhanced expression of stress proteins may give intracellular bacteria a selective advantage in their ability to survive in the phagocytes, resulting in their multiplication and dissemination throughout the host.

We have been interested in the function of stress proteins in the expression of pathogenicity of facultative intracellular bacteria. As mentioned above, a similar set of proteins were expressed in *Y. enterocolitica* after different stresses such as heat shock, hydrogen peroxide, superoxide anion, and intracellular stimuli associated with macrophage phagocytosis, indicating definite overlaps between the bacterial stress response in macrophages and three environmental stress responses. To understand the role of stress proteins in bacterial survival and growth in phagocytic cells, a study has been initiated to define new genes whose products are required for growth under both extracellular environmental stress and intracellular stress due to macrophage phagocytosis. We have taken a genetic approach to isolate mutants susceptible to global stresses by transposon mutagenesis. Our approach consists of (i) creating libraries of *Y. enterocolitica* carrying mini-Tn*10* insertions, (ii) screening such libraries for the presence of insertions which result in sensitivity to high temperature, and (iii) screening the growth thermosensitive (GTS) mutants for presence of insertions which affect the viability of bacteria in macrophage phagocytes. In this way, we discovered among 1,800 derivatives with mini-Tn*10* insertions at least five independent genes whose inactivation by transposons results in the inability of *Y. enterocolitica* to grow at 39°C and within macrophages after phagocytosis (54). We call the alleles to these mutations *gsr* (global-stress requirement). In this work, we have identified and characterized the *gsrA* gene, which can complement the mutation of the derivative GTS186 mutant. Furthermore, we have constructed a defined null mutation in *gsrA* and charac-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Y. enterocolitica		
WA314	Serotype O:8, virulence plasmid	14
WA314-RR	Rifampin-resistant and restriction-deficient derivative of WA314	This study
GTS186	GTS mutants caused by mini-Tn*10* insertion	54
KY133	*gsrA*::*kan* mutant	This study
E. coli		
JM109	*recA endA gyrA thi hsdR supE recA* Δ(*lac-proAB*)/F′ (*traD proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	57
SM10	*thi thr leu tonA lacY supE recA*::RP4-2Tc::Mu Km^r	43
SM10λ*pir*	λ*pir* lysogen of SM10	Our collection
KE606	Contains *pir* of R6K	20
Plasmids		
pACYC184	Cloning vector	6
pUC18	Cloning vector	57
pTKY228	*Bam*HI deletion of pBR322	This study
pM17	Carries only oriγ of R6K as a replication origin	This study
pSUP201	pBR325 carrying *oriT* of RP4	43
pTKY229	pMI7 carrying *oriT* of RP4 from pSUP201-1	This study
pTKY240	pACYC184 with a 11.5-kb *Bam*HI fragment containing *gsrA*	This study
pTKY245	pUC18 with a 2.8-kb *Sal*I-*Nru*I fragment containing *gsrA*	This study
pTKY300	pACYC184 with a 6.3-kb *Hin*dIII fragment containing *gsrA*	This study
pTKY301	pACYC184 with a 3.6-kb *Hin*dIII-*Nru*I fragment containing *gsrA*	This study
pTKY302	pACYC184 with a 2.8-kb *Sal*I-*Nru*I fragment containing *gsrA*	This study
pTKY303	pACYC184 with a 2.8-kb *Sal*I-*Nru*I fragment containing the truncated *gsrA*	This study
pTKY304	pACYC184 with a 1.2-kb *Sal*I-*Pst*I fragment containing the truncated *gsrA*	This study
pTKY306	pTKY228 with a 4.7-kb *Nru*I fragment carrying *gsrA*::*kan*	This study
pTKY307	pTKY229 with a 2.7-kb *Nco*I-*Eco*R521 fragment carrying *gsrA*::*kan*	This study
pTKY308	pACYC184 with a 1.7-kb *Taq*I-*Eco*521 fragment carrying *gsrA*	This study

terized it. The results demonstrated that the *gsrA* gene is essentially involved in the protective response to a variety of environmental stresses such as heat, oxidative products, and high salt concentration and to the intracellular stresses associated with macrophage phagocytosis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are shown in Table 1. Bacteria were routinely grown in brain heart infusion broth (Eiken, Tokyo, Japan) and on heart infusion agar (Eiken). RGMC broth (43) was used for conjugation. The antibiotics used for selection were chloramphenicol (25 $\mu g \cdot ml^{-1}$), tetracycline (25 $\mu g \cdot ml^{-1}$), ampicillin (50 $\mu g \cdot ml^{-1}$), kanamycin (50 $\mu g \cdot ml^{-1}$), and rifampin (50 $\mu g \cdot ml^{-1}$).

DNA manipulations. DNA purification, ligation, restriction analysis, and gel electrophoresis were carried out as described by Sambrook et al. (42). Restriction enzymes, T4 DNA ligase, Klenow enzyme, and DNA linkers were products of Takara Shuzo (Otsu, Japan) and Nippon Gene (Toyama, Japan).

Southern blot and colony hybridizations. Genomic DNAs were isolated from the *gsrA* mutant and the isogenic wild-type parent according to the previously outlined procedure (56). After restriction enzyme digestion, approximately 1 μg of DNA was electrophoresed through a 1% agarose gel and transferred to a membrane. The DNA probe was labeled with [α-^{32}P]dCTP (Amersham) by use of the Multiprimer DNA Labelling System (Amersham). Hybridization was performed in buffer containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50 mM Na-phosphate buffer (pH 6.5), 1× Denhardt's solution, 1 mM EDTA, and denatured salmon sperm DNA (250 $\mu g \cdot ml^{-1}$) at 42°C overnight. Washing of the filter was carried out in 2× SSC containing 0.2% sodium dodecyl sulfate (SDS) at room temperature for 20 min with changes of the washing solution every 5 min, followed by 0.1× SSC containing 0.2% SDS at 50°C twice for 30 min each time. Filters for colony hybridization were prepared essentially as described by Sambrook et al. (42). Hybridization was performed in buffer containing 50% formamide, 2× SSC, 10 mM Tris-HCl (pH 7.5), 0.1% SDS, and denatured salmon sperm DNA (500 $\mu g \cdot ml^{-1}$) at 42°C overnight. Blots were washed in 2× SSC containing 50% formamide three times for 10 min each time at 42°C and then in 2× SSC twice for 10 min each time at room temperature.

DNA sequencing. A series of nested exonuclease III deletions was generated in the 2.8-kb *gsrA*-complementing *Sal*I-*Nru*I fragment carried on plasmid pTKY245 (Table 1 and Fig. 1), by using the Deletion kit (Takara Shuzo). When necessary for sequencing complementary strands, specific subclones were constructed. Sequencing was done with Sequenase (version 2.0; United States Biochemicals) and the universal and reverse primers for the pUC18 plasmid.

Construction of a *Y. enterocolitica* strain harboring a defined mutation in *gsrA*. Plasmid pTKY240 (Fig. 1) was digested with *Nru*I, and the fragment containing the entire *gsrA* gene was ligated to the *Pvu*II site of pTKY228. The resulting plasmid, pTKY305, was cleaved at a *Pvu*II site situated 501 bp downstream from the ATG start codon of *gsrA*, ligated to *Bam*HI linkers, and digested with *Bam*HI. The kanamycin gene block (Pharmacia Biotech) was digested with *Bam*HI. Insertion of the kanamycin gene block in the *Bam*HI site of *gsrA* gave pTKY306 (Fig. 3). Plasmid pTKY306 was cleaved at the *Nco*I site situated 52 bp downstream from the ATG start codon and at the *Eco*R52I site situated 106 bp downstream from the TAA stop codon of *gsrA*, and the overhanging ends were filled in with Klenow enzyme. The generated *gsrA*::*kan* fragment was ligated to the filled EcoRI site of pTKY229, a transferable suicide vector constructed by us. It consists of plasmid pMI7 (15) and the fragment (*mob*) encoding *oriT* of RP4 from plasmid pSUP201-1 (43). A suicide feature of pMI7 is based on one of the origins of replication of R6K, oriγ. oriγ is functional only in a host when the π protein encoded by the *pir* gene is provided (20). The resulting mutator plasmid, pTKY307 carrying *gsrA*::*kan*, was introduced into a λ*pir* lysogen of strain SM10 (43) by transformation. SM10λ*pir* can mobilize pTKY307 into *Y. enterocolitica* WA314-RR by conjugation because it carries a derivative of RP4 integrated in the bacterial chromosome which provides conjugative functions in *trans* to the *mob* site on pTKY307. Conjugative crosses were carried out as previously reported (54). The chromosomal *gsrA* gene was replaced by the *gsrA*::*kan* construct by double recombination. The selection of the *gsrA* mutant was based on resistance to kanamycin and rifampin. Allelic exchange was finally checked by Southern hybridization.

Assay for *Y. enterocolitica* survival within macrophages. A continuous macrophage-like cell line, J774-1, derived from a reticulum cell sarcoma (35) was provided by the Japanese Cancer Research Resources Bank and grown in RPMI 1640 (GIBCO BRL, Grand Island, N.Y.) supplemented with 10% fetal calf serum. A total of 5×10^5 J774-1 cells in each well of a 24-well plate were challenged with *Y. enterocolitica* strains at a multiplicity of infection of 5. The plates were centrifuged at 250 × *g* for 10 min at room temperature to enhance and synchronize infection and then incubated for 20 min at 30°C to permit phagocytosis. The free bacteria were removed by three washes with phosphate-buffered saline (PBS). RPMI 1640 supplemented with 2% fetal calf serum and gentamicin (6 $\mu g \cdot ml^{-1}$) was added, and the cells were incubated at 30°C. Wells were sampled at various times by aspirating the medium, lysing the macrophages with 0.5 ml of 1% deoxycholate, and rinsing each well with 0.5 ml of PBS. Triplicate samples were plated individually.

Effect of high temperature on bacterial growth. Bacterial cultures grown overnight in brain heart infusion broth were diluted 1:100 into fresh broth and incubated with shaking at 30°C to a cell concentration of approximately $2 \times 10^8 \cdot ml^{-1}$. The cultures were shifted to 39°C at time zero (Fig. 4). Bacterial growth was monitored by measuring optical density at 600 nm.

Sensitivity to oxidative and osmotic stresses. Bacterial cultures were grown in M9 minimum medium at 30°C to exponential phase, harvested, and resuspended into the same volume of the minimum medium. Hydrogen peroxide was added to a final concentration of 8.8 mM. For measuring sensitivity to osmotic stress, cells were resuspended to M9 minimum medium containing 2.5 M NaCl. Bacterial cells were diluted in PBS at the various times indicated in Fig. 5 and then plated on heart infusion agar. Viable counts were determined after incubation of the plates for 24 h at 30°C.

Nucleotide sequence accession number. The nucleotide sequence data reported in this article will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number D78376.

RESULTS

Cloning of the *Y. enterocolitica gsrA* gene. An allele of *gsrA* was identified in a search for mutations in genes that are required for growth under environmental stress such as high temperature (39°C) and under intracellular stress associated with macrophage phagocytosis. A mutant, GTS186, was isolated from a library that was created in the wild-type *Y. enterocolitica* WA314 by random insertion of mini-Tn*10* carrying the kanamycin resistance gene as previously reported (18, 54). Southern blot analysis indicated that the mini-Tn*10* inactivated gene(s) of mutant GTS186 was likely to reside on a 12-kb *Eco*RI fragment in the chromosome (data not shown). In order to clone the transposon-inactivated gene(s), chromosomal DNA from GTS186 was digested with *Eco*RI and the DNA fragments in the 9- to 15-kb size range were eluted after separation by agarose gel electrophoresis. The DNA fragments were cloned into pUC18, and clones containing the expected fragment were isolated by selecting for kanamycin-resistant transformants. The positive clones contained mini-Tn*10* and, additionally, a fragment of the transposon-interrupted target gene. Using a labeled 5-kb *Bam*HI-*Eco*RI fragment containing the target gene generated from the cloned 12-kb *Eco*RI DNA as a probe in Southern blot analysis against the chromosomal DNA of the wild-type parent resulted in the identification of an 11.5-kb *Bam*HI band and a 6.2-kb *Hin*dIII band which were likely to contain the entire *gsrA* gene. The 11.5-kb *Bam*HI fragment was cloned into pACYC184 and identified by colony hybridization using the same 5-kb DNA probe. The cleavage map of the 11.5-kb *Bam*HI fragment in the resulting clone is shown in Fig. 1A. Plasmid pTKY240 carrying the fragment was confirmed to encode the entire *gsrA* gene by its ability to complement the thermosensitivity of the GTS186 mutant (Fig. 1B).

Sequence of *gsrA*. To define the region encoding the *gsrA* gene in the 11.5-kb *Bam*HI fragment, various fragments derived from pTKY240 DNA were subcloned into pACYC184 and then tested for their ability to complement the growth thermosensitivity of GTS186 (Fig. 1). While the subclone, pTKY302, carrying the 2.8-kb *Sal*I-*Nru*I fragment was capable of complementing the GTS186 mutation, pTKY303 with the 2.1-kb *Pvu*II-*Nru*I fragment and pTKY304 with the 1.2-kb *Sal*I-*Pst*I fragment could not complement the thermosensitivity of GTS186. These results located the *gsrA* gene in the region containing the *Pvu*II and *Pst*I restriction sites on the cleavage map of pTKY240. The 2.8-kb *Sal*I-*Nru*I fragment (Fig. 1A) was subcloned into pUC18 for DNA sequencing. Overlapping deletions were constructed toward the *Nru*I site from the *Sal*I site of the *gsrA* region on pTKY245 and then sequenced.

The resulting sequence of the *gsrA* gene is shown in Fig. 2. Analysis of the 1,800-bp sequence shows that there is an open reading frame of 1,437 bp starting at bp 244 and ending at bp 1680. This open reading frame encodes a protein of 478 amino-acid residues with a predicted molecular mass of 49,500 Da and predicted isoelectric point of 8.56. Comparison of this amino acid sequence indicated that the GsrA protein is a homolog of the HtrA stress response proteins of *Escherichia coli* (26) and *S. typhimurium* (16), as shown in Fig. 2. Within this sequence, three prominent structures characteristic of the previously described HtrA protein were detected. Amino acid residues 1 to 27 represent a potential signal peptide for protein export (34). Towards the middle of the protein sequence between residues 236 and 240, these proteins have the sequence Gly-Asn-Ser-Gly-Gly which is a consensus serine protease catalytic domain (2). Near the carboxy terminus, these three proteins have the Arg-Gly-Asp (RGD) motif which has been characterized as being important for cell binding in some adhesive proteins such as fibronectin and collagens (41). These proteins are recognized by at least one member of a family of structurally related receptors, integrins. The role of this sequence, if any, in the function of the HtrA protease is unknown.

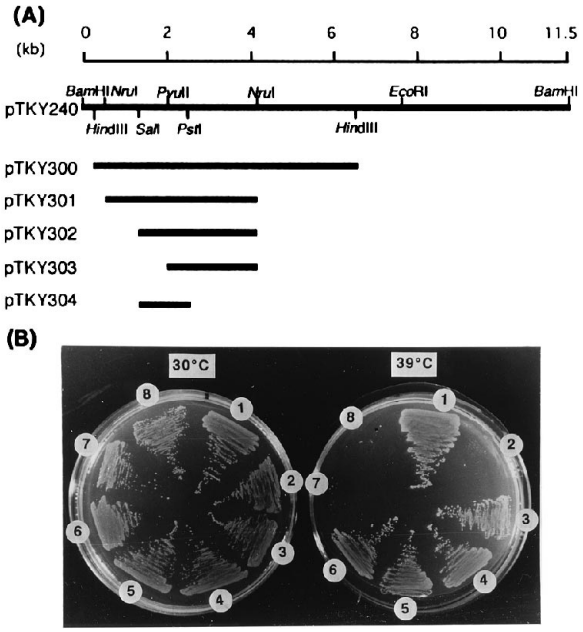

FIG. 1. Cloning and complementation of the *Y. enterocolitica gsrA* gene. (A) Restriction map of the *gsrA* region and structures of various derivatives. Thick lines represent the inserted DNA fragments of the recombinant plasmids. The 6.3-kb *Hin*dIII-*Hin*dIII fragment (for pTKY300), 3.6-kb *Hin*dIII-*Nru*I fragment (for pTKY301), 2.8-kb *Sal*I-*Nru*I fragment (for pTKY302), 2.1-kb *Pvu*II-*Nru*I fragment (for pTKY303), or 1.2-kb *Sal*I-*Pst*I fragment (for pTKY304) was cloned into plasmid pACYC184. (B) Strains grown at 30 and 39°C on a brain heart infusion agar plate are indicated as follows: 1, *Y. enterocolitica* wild-type bacteria; 2, GTS186 mutant; 3 to 8, GTS186 mutant bacteria carrying pTKY240, pTKY300, pTKY301, pTKY302, pTKY303, or pTKY304, respectively. Each plasmid DNA was introduced into *Y. enterocolitica* GTS186 by electroporation using the method of Conchas and Carniel (7).

Construction of a genetically defined *Y. enterocolitica gsrA* mutant. Since we isolated only a single *gsrA* allele, *gsrA*::Tn*10*, in strain GTS186, an additional null allele was constructed as follows (Fig. 3A). First, the kanamycin resistance gene block was inserted in the *Pvu*II site of the *gsrA* gene. Then, the *gsrA*::*kan* DNA on the suicide delivery vector was introduced into *Y. enterocolitica* WA314-RR by conjugal transfer. A *Y. enterocolitica gsrA*::*kan* mutant was generated by allelic exchange (see Materials and Methods). The allelic replacement by double recombination in the resultant mutant, KY133, was verified by Southern blot analysis. As the hybridization pattern

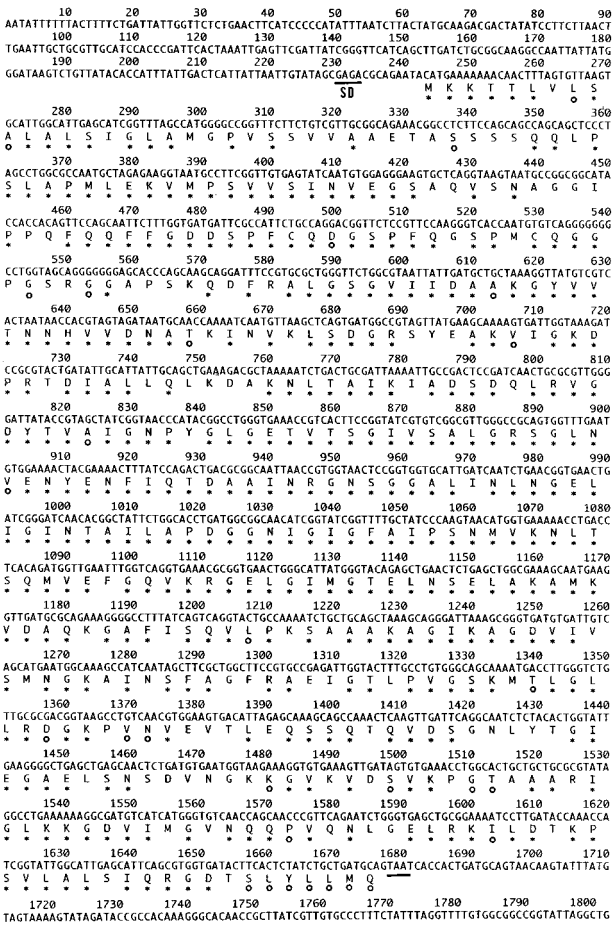

FIG. 2. Nucleotide sequence of the 1,800-bp fragment encoding the *Y. enterocolitica gsrA* gene and the deduced amino acid sequence of the GsrA protein. The reading frame encoding the GsrA protein begins at nucleotide 244. The stop codon, TAA, begins at nucleotide 1678 and is underlined. A putative Shine-Dalgarno (SD) sequence is underlined at bases 230 to 233. Amino acid residues conserved across three proteins, GsrA, *S. typhimurium* HtrA, and *E. coli* HtrA (*), and residues conserved between two proteins (GsrA and an HtrA) (○) are indicated.

in Fig. 3B indicates, use of the 1.2-kb *Bam*HI fragment encoding the kanamycin resistance gene as a probe detected the insertion of the resistance gene on the chromosome of mutant strain KY133 (lane a). Hybridization with a *gsrA*-specific probe has confirmed that the *Y. enterocolitica* WA314 wild-type *gsrA* gene resides on an 11.5-kb *Bam*HI fragment (lane d). Digestion of the mutant strain KY133 chromosome with *Bam*HI and hybridization with a *gsrA*-specific probe indicate that the *gsrA* DNA is cleaved into two fragments with approximate sizes of 2 and 9.5 kb (lane c). This result is due to the insertion of the kanamycin resistance gene block at the *Bam*HI sites newly created in the *gsrA* locus.

Stress response of the *Y. enterocolitica gsrA* mutants. The resultant *gsrA*::*kan* mutant, KY133, was characterized for its response to extracellular environmental stresses and intracellular stresses associated with macrophage phagocytosis. It is well known that *Y. enterocolitica* has a 70-kb plasmid that is highly conserved and essential for yersinia virulence (3, 8). Since presence or absence of the virulence plasmid in the different strains could have significant implications for the growth rate of yersiniae at different temperatures as well as their ability to interact with macrophages (8, 47, 51), we have

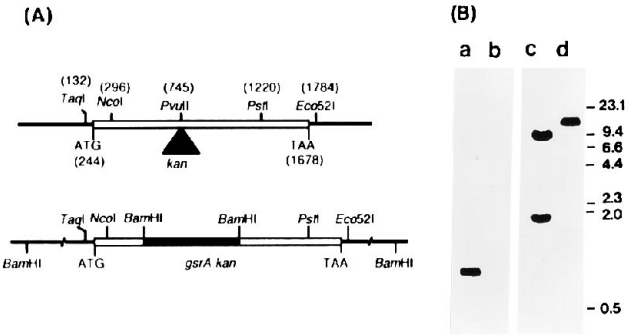

FIG. 3. (A) Restriction cleavage maps of *gsrA* regions on plasmid pTKY307 (upper diagram) and the chromosome of insertion mutant KY133 (lower diagram). Open bars, *gsrA* open reading frame; shaded triangle and bar, kanamycin gene block. (B) Southern hybridization analysis of the *gsrA* mutant KY133 (lanes a and c) and the wild-type strain WA314 (lanes b and d). Chromosomal DNAs were digested with *Bam*HI and separated on a 1% agarose gel by electrophoresis. As a probe for hybridization, the kanamycin resistance gene block (lanes a and b) or the 0.9-kb fragment generated from the *gsrA* region by digestion with *Nco*I and *Pst*I (lanes c and d) was used.

confirmed that the wild-type strain and KY133 mutant strain harbor a 70-kb plasmid by agarose gel electrophoresis of plasmid DNAs prepared from both strains (data not shown). Furthermore, complementation analysis of the GsrA$^-$ phenotype was performed with a plasmid (pTKY308) carrying only the *gsrA*$^+$ gene.

(i) Effects of thermal stress. To study the effect of the *gsrA* mutation on cell growth at high temperature, KY133 mutant cells were grown to exponential phase in brain heart infusion broth at 30°C, shifted to 39°C, and continuously incubated. The growth curves are shown in Fig. 4. When the culture of KY133

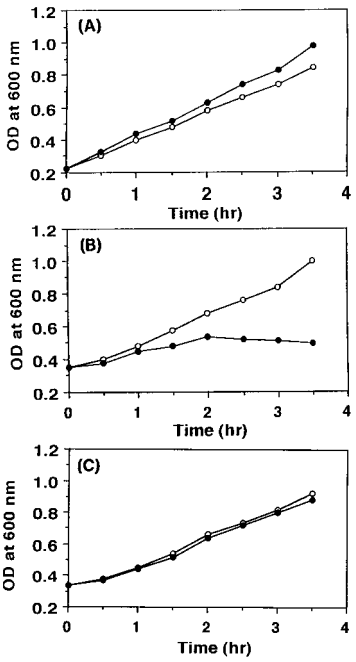

FIG. 4. Effect of temperature on cell growth of WA314 (wild type) (A), *gsrA* mutant KY133 (B), and KY133 carrying pTKY308 (C). Exponentially growing cultures were either incubated continuously at 30°C (○) or shifted to 39°C (●) at time zero. Bacterial growth was monitored by measuring optical density (OD) at 600 nm.

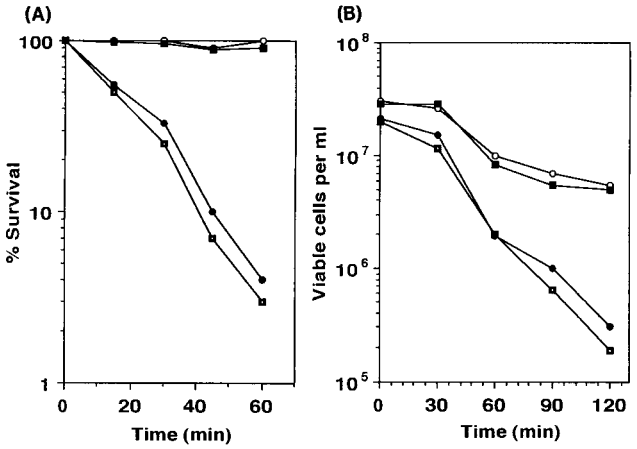

FIG. 5. Sensitivity of WA314 (wild type), *gsrA* mutants GTS186 and KY133, and KY133 carrying pTKY308 to oxidative and osmotic stresses. Cells were exposed to 8.8 mM hydrogen peroxide (A) or osmotic stress in the presence of 2.5 M NaCl (B). Symbols: ○, WA314; ●, GTS186; ◘, KY133; ■, KY133 carrying pTKY308.

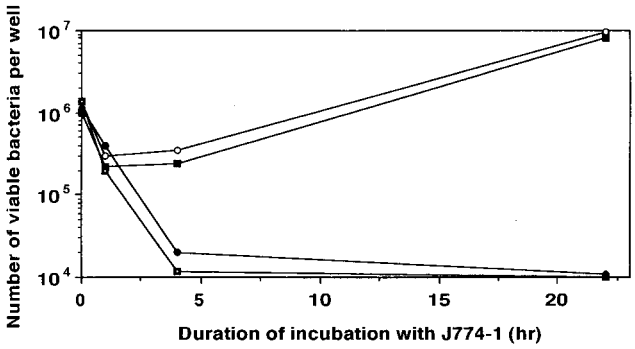

FIG. 6. Fate of WA314 (wild type), *gsrA* mutants GTS186 and KY133, and KY133 carrying pTKY308 within J774-1 macrophage. The numbers of bacteria were determined as described in Materials and Methods. The data are averages of triplicate determinations. Symbols: ○, WA314; ●, GTS186; ◘, KY133; ■, KY133 carrying pTKY308.

was incubated at 39°C following a shift, the cells continued to grow at the 30°C rate for 1 h. Then, cells began to lyse, with a concomitant decrease in the optical density of the culture. Cell lysis was also confirmed in the culture at 3 h after a temperature shift by light microscopy (data not shown). The extent of complementation of growth thermosensitivity by *gsrA* is shown in Fig. 4C. The bacteria carrying pTKY308 grew as well as wild-type cells at 39°C. These results have confirmed that the *gsrA* product is essentially required for bacterial survival and growth at elevated temperature, while being dispensable at low temperature.

(ii) Effects of oxidative stress. It is known that expression of some heat shock genes is induced by oxidative stress such as hydrogen peroxide and superoxide anion (10, 31, 53, 55). It also has been reported that a deletion in the *rpoH* gene encoding the heat shock sigma factor greatly sensitized *E. coli* to oxidative stress, suggesting that stress proteins induced by heat shock play roles in defense against not only thermal stress but oxidative stress (19). We therefore examined the effects of the *gsrA* mutation on sensitivity to oxidative stress by exposure to hydrogen peroxide. Figure 5A shows the sensitivity to hydrogen peroxide of mutant strains GTS186 and KY133 compared with the isogenic wild-type parent. The mutant strains appeared to be more susceptible to hydrogen peroxide than the wild-type parent, such that the survival of mutants decreased approximately 50-fold relative to that of the parental strain after exposure for 60 min to 8.8 mM hydrogen peroxide. The susceptibility to hydrogen peroxide of the KY133 mutant was complemented by coexistence of plasmid pTKY308 carrying *gsrA* (Fig. 5A). These results suggest that the *gsrA* gene is required for protection of *Y. enterocolitica* against oxidative stress.

(iii) Effects of osmotic stress. We then examined the sensitivity of mutant strains GTS186 and KY133 to osmotic stress induced by high salt concentration compared with the wild-type parent. As shown in Fig. 5B, the mutants appeared to be more sensitive to osmotic shock after exposure to 2.5 M NaCl than the wild-type parent. This phenotype of the mutant was complemented by the *gsrA* gene, suggesting that the *gsrA* gene is also required for protection against osmotic stress.

Survival of *Y. enterocolitica gsrA* mutants within macrophages after phagocytosis. *Y. enterocolitica* has been demonstrated to survive and multiply within peritoneal macrophages from diverse animal sources such as mice, humans, and rabbits (51, 52). We previously demonstrated the validity of the macrophage-like cell line J774-1, derived from mouse reticulum, to examine the interaction of the *Y. enterocolitica* WA314 with macrophages (55). As shown in Fig. 6, J774-1 cells were initially bactericidal to the phagocytized bacteria and subsequently permitted growth of *Y. enterocolitica*. The intracellular growth of the *gsrA* mutants GTS186 and KY133 and isogenic wild-type parent within J774-1 cells was monitored up to 22 h after phagocytosis. In the wild-type parent, there was a decrease in the number of viable bacteria during the first hour, which indicated that the initial interaction with the macrophage was the most bactericidal. After this initial drop, the wild-type parent grew and increased in number almost 20-fold in macrophages 22 h after phagocytosis. On the other hand, GTS186 and KY133 mutants showed a decrease in viability within J774-1 cells relative to the wild-type parent after 22 h. The loss of viability of the mutant in macrophage was complemented by the cloned *gsrA* gene (Fig. 6). The mutant tested had numbers of viable intracellular bacteria that were similar to those of the parental strain immediately after phagocytosis, suggesting that the mutant was phagocytized as well as the wild-type parent. These results suggest that the *gsrA* gene is essentially involved in the protecting response to the stress in the macrophage phagosome, as well as to environmental stresses.

DISCUSSION

In an attempt to define stress response genes whose products are required for survival of *Y. enterocolitica* within macrophages after phagocytosis, we have isolated two mutations in the *gsrA* gene. Mutant alleles were created by the random insertion of a mini-Tn*10* transposon in vivo and by the defined mutagenesis of the cloned *gsrA* gene by insertion of the kanamycin resistance gene into the cloned *gsrA* gene in vitro, followed by allelic exchange with wild-type *Y. enterocolitica*. Both alleles exhibited identical mutant phenotypes that led to bacterial death at temperatures above 39°C and increased susceptibility to hydrogen peroxide and high salt concentration in laboratory medium. Furthermore, both mutations resulted in

inability to survive within macrophages. The wild-type *gsrA* gene has been cloned and sequenced. Nucleotide sequence analysis has revealed that the *Y. enterocolitica gsrA* gene encodes a basic, 49,500-Da protein which shows significant amino acid sequence identity with the HtrA stress protein of *E. coli* (26) and *S. typhimurium* (16). A homolog of HtrA also has been identified as an immunoreactive antigen of *B. abortus* (37). The *E. coli* HtrA protein is also known as DegP (49) and is necessary for degradation of abnormally folded proteins transported into the periplasmic space (48). A biochemical study has demonstrated that the *E. coli* HtrA protein has serine protease activity (27). It has also been demonstrated that the previously described *E. coli* protease Do and the HtrA protein are the same protein (50). The *Y. enterocolitica* GsrA protein has two characteristic features, a signal peptide and a serine protease active site, both of which are conserved in the HtrA proteins of *E. coli* (26), *S. typhimurium* (16), and *B. abortus* (37). On the basis of these findings, it was proposed that the GsrA protein serves as a stress protein by degrading abnormal peptides generated from exposure to stress before they accumulate to toxic levels in the periplasmic space.

The hypothesis that stress proteins play an important role in the successful adaptation of intracellular pathogens to the hostile environment of the macrophage phagosome was originally based on indirect evidence, such as induction of the DnaK, GroEL, and GroES homologs in *Y. enterocolitica*, *S. typhimurium*, *L. pneumophila*, and *B. abortus* grown in macrophages (16, 17, 21, 22, 37, 55). DnaK, GroEL, and GroES are essential for bacterial growth and viability at all temperatures in normal bacteria (5, 11). It also has been shown that DnaK and GroEL bind to unfolded polypeptides and function as "molecular chaperones" in cells (9). Substrates for this activity include newly synthesized polypeptides emerging from the ribosome, polypeptides destined for translocation, and globular proteins which have become unfolded following heat shock or other stresses. These stress proteins stabilize the unfolded structure, protecting it from aberrant folding and from nonspecific interactions with other proteins. In response to environmental stress or intracellular stress associated with phagocytosis, induced levels of both chaperones would be required in order to cope with the accumulation of partially unfolded or denatured proteins in the cell. In contrast to these molecular chaperones, the proteolytic stress proteins Lon (28) and HtrA (25) are required only for survival at high temperature in laboratory culture. The *Y. enterocolitica gsrA* gene is also dispensable at low temperatures. For the intracellularly growing bacteria, these proteolytic activities would be required for degrading abnormal peptides accumulated by the action of the microbicidal mechanisms of the macrophage.

In *E. coli*, the heat shock response is under the control of two alternative sigma factors, σ^{32} and σ^{E}. While σ^{32} is absolutely required for transcription of the classical heat shock genes, e.g., *dnaK*, *dnaJ*, *grpE*, *groEL*, and *groES*, σ^{E} is required for transcription of *htrA* (36, 40, 58). Recent studies suggest that the activity of σ^{E} is induced by overproduction of outer membrane proteins, suggesting that the signal to which σ^{E} responds is generated by events occurring in the extracytoplasmic compartment of the cell (29). From the present result, the σ^{E} regulon appears to deal with a feature particularly relevant to life of bacteria within the macrophage.

In the step of ingestion and killing of bacteria by phagocytosis, bacteria are first engulfed by endocytosis into phagosomes, which then fuse with lysosomes to form phagolysosomes. The lysosome contains oxygen-dependent and -independent mechanisms for killing the invading bacteria. The oxygen-dependent mechanism is based on the toxic metabolites of oxygen including hydrogen peroxide and superoxide produced by the respiratory burst and highly contributes to the bactericidal capacity of phagocytes. The oxygen-independent mechanism includes acidification of the phagosome and degradation of its contents by polypeptides with antimicrobial activity. In the present study, we have demonstrated that a mutation in the *gsrA* gene of *Y. enterocolitica* results in increased sensitivity to oxidative stress with hydrogen peroxide, which mimics the oxidative killing mechanism of macrophages. Sensitivity to the oxidative killing mechanism may account for the low recovery of the *gsrA* mutants from the macrophage phagosome. A critical role of stress proteins in survival of intracellular bacteria in host phagosomes was originally demonstrated with *S. typhimurium* in macrophages: an HtrA-deficient mutant was found among mutants with reduced virulence in mice after oral administration (16, 30). *Y. enterocolitica* and *S. typhimurium* have been shown to grow slowly within macrophages and increase 10- to 20-fold during a 24-h incubation after phagocytosis (4, 55). Since these bacteria are exposed to various microbicidal mechanisms in phagosomes or phagolysosomes for a long period, they should respond to the hostile environment by producing an increased amount of stress proteins. On the contrary, we have recently reported that the elevated expression of stress proteins is absent in *Listeria monocytogenes* associated with macrophage phagocytosis (13). *L. monocytogenes* grows rapidly in macrophage cells by escaping from the stressful phagosome into the cytoplasm of the macrophage. Because of this ability for rapid escape, *L. monocytogenes* probably has adapted to survive and grow without the induction of a set of stress proteins.

It has been demonstrated that all pathogenic strains of *Y. enterocolitica* carry a plasmid, pYV, which is essential for virulence and highly conserved among *Yersinia* spp. (3, 8). Of the various pYV-encoded proteins, YadA, YpkA, YopM, YopH, and YopE have been identified as independent virulence determinants of *Yersinia* spp. (8, 47). The in vitro expression of these Yops is coordinately regulated in response to environmental temperature and Ca^{2+}. Maximal expression of Yops is obtained in Ca^{2+}-depleted media at 37°C. YopH is a protein phosphotyrosine phosphatase which acts on proteins in macrophages and epithelial cells and contributes to cytotoxicity and resistance to phagocytosis (1). YopE is a cytotoxin which disrupts the actin microfilament structure of mammalian cells, also contributing to cytotoxicity and resistance to phagocytosis (38). It has been demonstrated that YopE and YopH are secreted by extracellular yersiniae, translocated across the cellular membrane, and subsequently internalized into the target cells, suggesting that yersiniae can grow extracellularly in the host (38, 39, 45). Accordingly, genetic evidence and microscopic evidence have demonstrated that the multiplication of yersiniae occurs predominantly extracellularly in vivo and also in vitro with the expression of large amount of Yops (12, 44). However, the evidence that yersiniae can multiply intracellularly within cultured peritoneal macrophages from diverse animal sources also has been accumulated (46, 51, 52, 55). This discrepancy with the in vitro findings might be explained on the basis of the environmental conditions encountered by bacteria in host tissues. The Yop expression system appears to be involved in extracellular survival of yersiniae in the hostile environment of the mammalian reticuloendothelial system. Yersiniae may exposed to the intracellular environment only during the initial stages of infection in vivo. It is possible that the GsrA protein would contribute to the survival and growth of *Y. enterocolitica* within macrophage phagocytes in the early

stages of infection, resulting in their proliferation and dissemination throughout the host.

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