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The *Yersinia enterocolitica* GsrA Stress Protein, Involved in Intracellular Survival, Is Induced by Macrophage Phagocytosis

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The *Yersinia enterocolitica gsrA* gene is a stress protein gene which was originally identified as essential for protecting cells under both extracellular environmental stress and intracellular stress in macrophages due to phagocytosis. The *gsrA* gene was shown to be a member of the *htrA* class of genes and to possess a sequence homologous to that of the promoter recognized by a stress-induced σ factor, σ^E. In order to study the induction of the potentially σ^E-controlled *gsrA* gene in *Y. enterocolitica* after phagocytosis by macrophages, we identified GsrA by overproducing the protein using a T7 promoter-*gsrA* fusion. We found that it is translated as an unstable 49,500-Da protein which is processed by removal of an amino acid fragment consisting of 27 residues, resulting in a stable 46,800-Da protein. By radiolabeling proteins specific to bacteria in the J774-1 macrophage-like cell line, we found that the production of GsrA protein is indeed enhanced in bacterial cells growing within macrophage phagosomes. Transcriptional activation of the *gsrA* gene was determined by using the *gsrA* promoter-*lacZ* fusion system. This work provides the first piece of evidence that the σ^E regulon responds to the stressful environment found in macrophages.

Bacteria in general exhibit a rapid molecular response with coordinate gene expression when they are exposed to survival-threatening conditions, including sudden elevated temperature, oxidative damage, nutrient limitation and starvation, variations in osmolarity and pH, and chemical stress (for a review, see reference 25). Regarding bacterial infections, one of the most survival-threatening environments encountered by invading bacteria is the intracellular environment of macrophages, where bacteria are threatened by oxidative or nonoxidative microbicidal mechanisms. However, some bacteria, such as *Yersinia enterocolitica* (37, 41, 42), *Salmonella typhimurium* (2, 8), *Legionella pneumophila* (13, 14), and *Listeria monocytogenes* (35), have adapted well to survive within phagocytic cells. They have developed various strategies to withstand the bactericidal mechanisms associated with phagocytosis: (i) resistance to phagocytosis (7, 37), (ii) escape from the phagosome into the cytoplasm (35), (iii) inhibition of phagosome-lysosome fusion (2, 13), and (iv) resistance to low pH (14) and to antimicrobial proteins in the phagolysosome (8). In addition to these mechanisms for escape, it has been hypothesized that bacterial heat shock proteins, which are collectively known as stress proteins, play an important role in allowing these organisms to successfully adapt to the hostile environment of the host phagosome. This hypothesis was originally based on indirect evidence, such as the elevated expression of homologs of the stress proteins DnaK, GroEL, and GroES in *S. typhimurium*, (1), *Y. enterocolitica* (44), *L. pneumophila* (17), and *Brucella abortus* (19), which grow intracellularly in macrophages after phagocytosis. Later, this hypothesis was directly supported by a report in which the insertional mutation of the *S. typhimurium* homolog of the stress protein gene, *htrA*, resulted in the inability to survive inside macrophages (15). An essential role for members of the HtrA class of stress proteins in survival of intracellular bacteria has also been shown by the identification and characterization of insertional mutants of GsrA (stands for global stress requirement) of *Y. enterocolitica* (45) and the HtrA homolog of *Brucella melitensis* (27).

As mentioned above, several facultative intracellular bacteria generally induce the expression of a similar set of stress proteins during intracellular growth after phagocytosis by macrophages. Induced levels of the stress proteins are required to cope with the intracellular stress accumulated by action of the microbicidal mechanisms in the macrophage. In *Escherichia coli*, the induction of stress proteins by heat shock is under the control of two alternative factors, σ^{32} and σ^E, which are sigma subunits of RNA polymerase (6, 9, 47). The sigma factor σ^{32} is absolutely required for the transcription of a set of heat shock genes with products that consist primarily of chaperones, such as DnaK, GroEL, and GroES, and proteases. The evidence that the synthesis of DnaK, GroEL, and GroES homologs is induced in *S. typhimurium* (1), *Y. enterocolitica* (44), *L. pneumophila* (17), and *B. abortus* (19) growing in macrophages after phagocytosis suggests that the potential σ^{32} heat shock regulons of these pathogenic bacteria indeed respond to the hostile environment in macrophage phagosomes. In contrast, σ^E is required for the transcription of *htrA* in *E. coli* (4, 22). Though much less is known about the σ^E regulon, one of four promoters upstream of the *rpoH* gene encoding σ^{32} is known to be transcribed only by RNA polymerase E containing σ^E ($E\sigma^E$) under conditions of severe stress, such as 50°C or 10% ethanol (5). Since σ^{32} is unstable (39), continued expression of *rpoH* by $E\sigma^E$ is probably essential for cell survival under these severe conditions.

The *htrA* gene was initially identified as essential for protecting cells at high temperatures, i.e., above 42°C (21). The *gsrA* gene was originally identified as an essential component of the protection mechanism employed by *Y. enterocolitica*

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TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristic(s)	Reference or source
Bacterial strains		
Y. enterocolitica WA314	Serotype O:8, virulence plasmid	11
E. coli JM109	*recA endA gyrA thi hsdR supE recA* Δ(*lac-proAB*) *F′* (*traD proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*)	46
Plasmids		
pTKY248	pBluescript SK derivative with its *gsrA* gene under the control of the T7 promoter	This study
pTKY257	2.8-kb *gsrA*$^+$ *Sal*I-*Nru*I fragment from *Y. enterocolitica* in pACYC184	45
pTKY258	Km^r cassette in the *Bam*HI site of pTKY257	This study
pTKY259	pTKY258 derivative with a deletion of the 2.9-kb *Nco*I fragment	This study
pTKY260	Promoterless *lacZ* gene from Z1918 in the *Nco*I site of pTKY259	This study
pTKY261	Same as pTKY260 but its *lacZ* gene is in the opposite orientation	This study
pACYC184	Cm^r Tc^r plasmid	3
pBluescript SK	Ap^r plasmid with its T7 promoter reading into the polylinker	Stratagene
pGP1-2	Km^r plasmid encoding T7 RNA polymerase under the control of *c*I857	40
Z1918	Ap^r plasmid carrying the promoterless *lacZ* reporter gene	34

under intracellular stress in macrophages as well as extracellular environmental stress. It was shown to be a member of the *htrA* class of heat shock genes (45). A potential σ^E type heat shock promoter sequence as well as *htrA* homologs of *S. typhimurium* (15) and *B. abortus* (30) was detected upstream of the cloned *gsrA* gene. Because Eσ^E is the only form of RNA polymerase that transcribes *htrA* in *E. coli* (4), it is possible that a σ^E-like factor responds to intracellular signals associated with macrophage phagocytosis and results in the enhanced expression of GsrA and HtrA homologs after phagocytosis. To investigate this possibility, we initially identified the GsrA protein of *Y. enterocolitica* and then examined the enhanced expression of the GsrA protein in *Y. enterocolitica* growing within macrophage phagosomes. Furthermore, the transcriptional activation of the *gsrA* gene was examined in the environment of the macrophage after phagocytosis. This work provides the first evidence that the potential σ^E regulon responds to stresses in the environment of the macrophage.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were routinely grown in brain heart infusion broth (Nissui, Tokyo, Japan) and on heart infusion agar (Nissui). M9 medium supplemented with glucose (0.2%), thiamine (2 μg/ml), $MgSO_4$ (1 mM), $MgCl_2$ (3 mM), $CaCl_2$ (0.1 mM), $FeCl_3$ (0.3 mM), and all L-amino acids except methionine and cysteine was used for the labeling experiments with [^{35}S]methionine. When necessary, the media were supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), and/or tetracycline (25 μg/ml).

DNA subcloning. All DNA subcloning procedures were done essentially as described by Sambrook et al. (33). Restriction enzymes, T4 ligase, and Klenow fragment enzyme were products of Takara Shuzo (Otsu, Japan).

Construction of a T7 promoter-*gsrA* fusion and a *gsrA* promoter-*lacZ* fusion. To construct a plasmid derivative carrying the T7 promoter-*gsrA* fusion, the 1,653-bp *Taq*I-*Eco*52I DNA fragment encoding *gsrA* from pTKY257 (see Fig. 1B and C) was cloned into the pBluescript SK vector digested with *Acc*I and *Not*I to yield plasmid pTKY248. The structure of the resulting plasmid is shown in Fig. 1A. The construction of a plasmid derivative carrying the *gsrA* promoter-*lacZ* fusion was performed as follows. The 1,265-bp cassette encoding the Km^r gene was inserted into the *Bam*HI site of pTKY257 (see Fig. 1C) to yield plasmid pTKY258. Digestion of pTKY258 with *Nco*I followed by self-ligation generated pTKY259. The 3,214-bp fragment carrying the promoterless *lacZ* gene was isolated from Z1918 (34) digested with *Bam*HI. After end repair with Klenow fragment enzyme, this fragment was ligated into the *Nco*I site of pTKY259 to yield pTKY260 and pTKY261, which are identical except for the orientations of their *lacZ* genes with respect to that of the *gsrA* promoter. The structure of pTKY260 is shown in Fig. 1A.

Exclusive radiolabeling of GsrA protein. Selective radiolabeling of plasmid-encoded proteins was achieved by using the T7 RNA polymerase-promoter system established by Tabor and Richardson (40). Plasmid pTKY248 or the pBluescript SK vector as a control was transferred into *E. coli* JM109 bacteria already containing plasmid pGP1-2, which encodes T7 RNA polymerase under the control of *c*I857. These transformants were grown in broth containing ampicillin and kanamycin at 30°C to an optical density at 600 nm of 0.3. Cells (2 ml) were centrifuged, washed with M9 medium, and resuspended in 1 ml of the same medium. Cells were grown at 30°C for 60 min and then shifted to 42°C for 15 min to induce the expression of the T7 RNA polymerase gene under the control of *c*I857. Rifampin was added at a final concentration of 200 μg/ml, and the culture was then incubated at 42°C for an additional 10 min. The culture was shifted to 30°C for 20 min and subsequently labeled with [^{35}S]methionine (50 μCi) for 5 min at 30°C.

Macrophages and phagocytosis. A continuous macrophagelike cell line, J774-1, derived from a reticulum cell sarcoma (29), was grown in RPMI 1640 (GIBCO BRL, Grand Island, N.Y.) supplemented with 10% fetal calf serum. A total of 2×10^6 J774-1 cells grown in each well of a six-well plate were challenged at a bacterium/J774-1 cell ratio of 50:1. The plates were centrifuged at $250 \times g$ for 10 min at room temperature to enhance and synchronize infection and then incubated for 30 min at 37°C to permit phagocytosis. The free bacteria were removed by three washes with phosphate-buffered saline.

Radiolabeling of bacterial proteins in macrophages after phagocytosis. The monolayer J774-1 cells were challenged with *Y. enterocolitica* WA314. After phagocytosis as described above, the culture was incubated in methionine-free RPMI 1640 (GIBCO BRL) containing gentamicin (6 μg/ml) and cyclohexamide (50 μg/ml) at 37°C. After incubation for 3 h, bacterial proteins were subsequently labeled with [^{35}S]methionine (50 μCi) for 1 h at 37°C and extracted as described previously (10, 44).

Transcription of the *gsrA* gene in *Y. enterocolitica* after phagocytosis. The monolayer J774-1 cells were challenged with *Y. enterocolitica* WA314 carrying pTKY260 or pTKY261. Phagocytosis was allowed by the procedure described above, and the cultures were incubated in RPMI 1640 supplemented with gentamicin (6 μg/ml) and 2% fetal calf serum at 37°C. The 0.5-h time point corresponds to adherent and intracellular bacteria after extensive washing with phosphate-buffered saline. At various times after exposure to bacteria, macrophages were lysed with 0.5% deoxycholate and plated for determination of CFU. The remainder of the sample was diluted into Z buffer to assay β-galactosidase activity as described by Miller (24). Triplicate samples were assayed individually.

Resolution of radiolabeled proteins by one- and two-dimensional gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of radiolabeled proteins was done essentially by the method of Laemmli (18) on 12.5% polyacrylamide gels. The procedure used for isoelectric nonequilibrium focusing in the first dimension was that of O'Farrell et al. (26), and SDS-PAGE in the second dimension was performed as described previously (44). After electrophoresis, the gels were dried, exposed to film (Fuji RX100) at room temperature for 2 days, and analyzed by using the software package MELANIE II of Bio-Rad (Hercules, Calif.).

Protein isolation and N-terminal sequence analysis. Selected protein spots from two-dimensional gels were isolated for N-terminal sequencing. After electrophoresis, proteins were transferred from gels to polyvinylidene difluoride (Immobilon; Millipore) membranes by the procedure described by Hirano (12). The membranes were stained with Coomassie brilliant blue R-250 (Sigma) for 1 min, destained in 50% methanol–10% acetic acid for a few minutes, and then rinsed with water and air dried. Stained protein spots were cut out of the polyvinylidene difluoride membrane and analyzed with a protein sequencer (model 473A; Applied Biosystems).

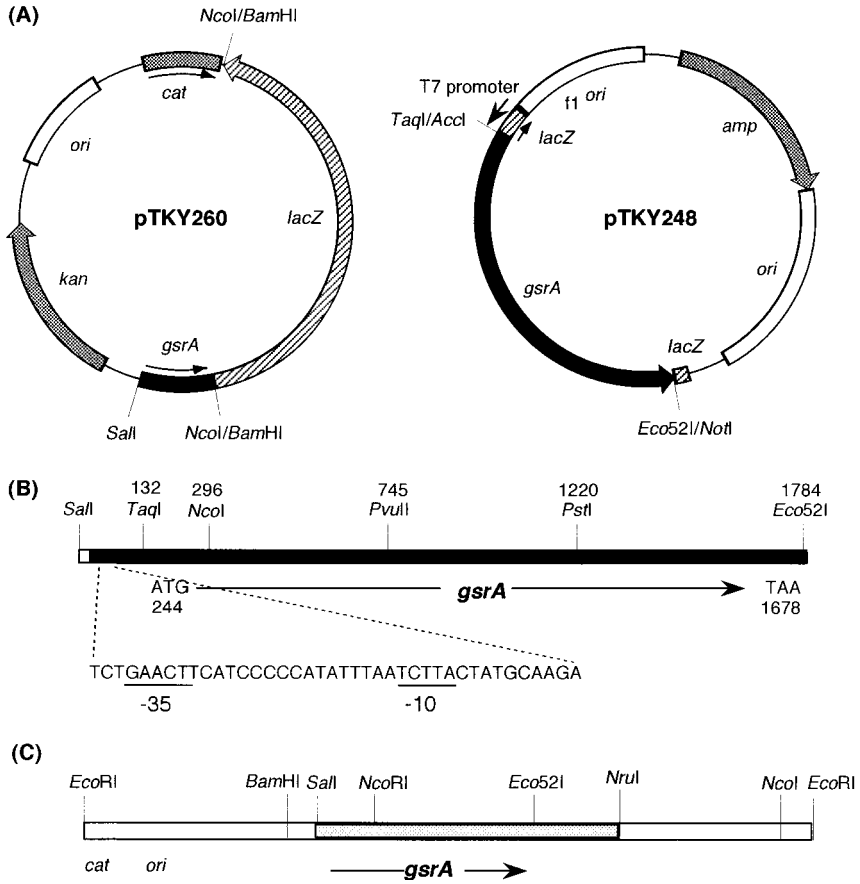

FIG. 1. (A) Physical map of pTKY248 carrying the T7 promoter-*gsrA* fusion and pTKY260 carrying the *gsrA* promoter-*lacZ* fusion. (B) Restriction map of the *gsrA* gene and the adjacent region. A thick line represents the nucleotide sequence of the 1,800-bp fragment encoding *gsrA* (45). The −35 and −10 sequences are underlined. (C) Restriction map of pTKY257 carrying the 2,800-bp fragment encoding the *gsrA* gene in pACYC184. Abbreviations: *cat*, chloramphenicol acetyltransferase gene; *kan*, kanamycin resistance gene; *amp*, ampicillin resistance gene; *ori*, origin of replication of the vector plasmid.

RESULTS

Identification of the *Y. enterocolitica* GsrA protein. In order to identify the GsrA protein, we constructed a strain which overproduces GsrA by using the T7 polymerase-promoter system (Fig. 1). This plasmid possesses a T7 promoter that is transcribed into the polylinker for cloning. Proteins under the control of T7 RNA polymerase can be selectively produced by inhibiting *E. coli* RNA polymerase with rifampin. This experiment was carried out in the *E. coli* JM109 derivative carrying pGP1-2, which encodes the T7 RNA polymerase and pTKY248, whose structure is shown in Fig. 1A. The specific induction of a protein of approximately 50 kDa was observed by SDS-PAGE (Fig. 2, lane c). This protein was absent in a control extract from cells transformed with the pBluescript SK vector instead of pTKY248 (Fig. 2, lane d). This overproduced protein corresponds to one of the proteins induced by heat shock in *Y. enterocolitica* cells (Fig. 2, lane b). The same extract from cells carrying pTKY248 was analyzed by two-dimensional nonequilibrium PAGE (Fig. 2, gel e). We consequently found that two prominent proteins of approximately 50 and 47 kDa are synthesized in the GsrA-overproducing cells. The 47-kDa protein was overproduced so abundantly that it could be visualized simply by staining the gel with Coomassie brilliant blue (data not shown). Since the previous nucleotide sequence analysis showed that the *gsrA* gene does not have sufficient coding capacity for both a 50- and a 47-kDa protein (45), it is possible

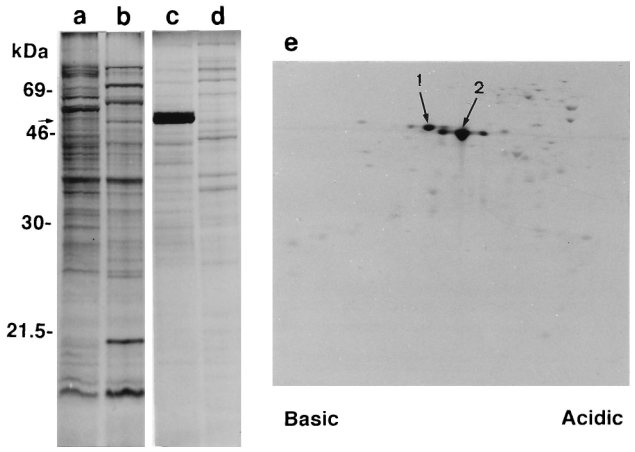

FIG. 2. Exclusive expression of the GsrA protein. *Y. enterocolitica* cells were grown in M9 medium at 28°C and labeled for 10 min with [^{35}S]methionine (50 μCi/ml) at 28°C (lane a) or transferred to 42°C, incubated for 5 min, and then labeled for 10 min at 42°C (lane b). *E. coli* cells carrying either pTKY248 (lane c) or the vector pBluescript SK (lane d) were labeled with [^{35}S]methionine (50 μCi/ml) by using the T7 polymerase-promoter system to selectively label plasmid proteins (see Materials and Methods). Extracts from cells were separated by SDS–12.5% PAGE. The sample used in lane c was subjected to two-dimensional gel electrophoresis (gel e). Autoradiographs of the dried gels are shown. An arrow at the left side of the gel points to the position of the processed 47-kDa GsrA protein. The proteins indicated by arrows 1 and 2 have molecular sizes of 50 and 47 kDa, respectively.

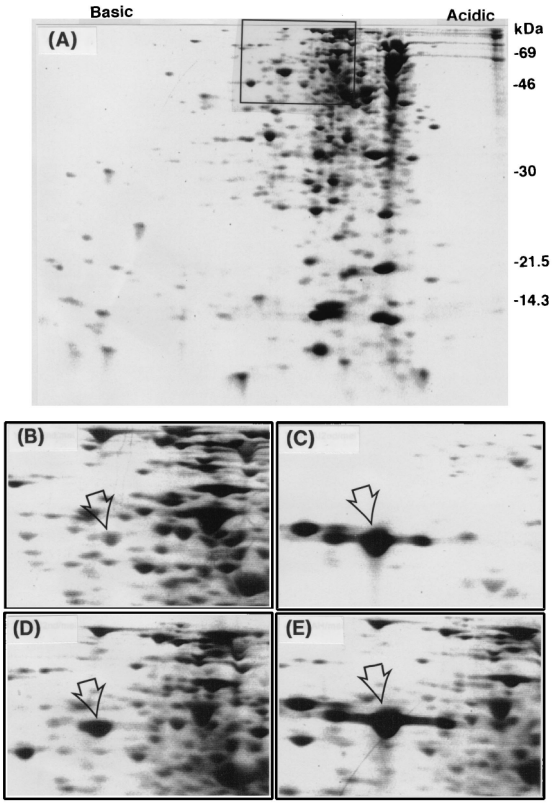

FIG. 3. Identification of the GsrA protein on a two-dimensional gel. *Y. enterocolitica* cells were labeled at 42°C for 10 min, following a shift from 28 to 42°C for 5 min (A and D), or radiolabeled for 10 min at 28°C (B). *E. coli* cells carrying pTKY248 and pGP1-2 were radiolabeled by using the T7 polymerase-promoter system (C). Extracts from cells were subjected to two-dimensional gel electrophoresis. Autoradiographs of the dried gels are shown. Mixed extracts of cells in panels C and D are shown in panel E. The region shown in a rectangle in panel A corresponds to the area shown in panels B to E. The arrows point to the position of the processed 47-kDa GsrA protein.

that these peptides are precursor products from the same gene. To confirm this possibility, we analyzed the amino acid sequence of each protein. Consequently, the N-terminal amino acid sequences of the proteins were MKKTTLVLSALALSIG and AETASSSSQQLPSLAPMLG for the 50- and 47-kDa proteins, respectively. Since the amino acid sequence deduced from the nucleotide sequence of the *gsrA* gene previously reported (45) was MKKTTLVLSALALSIGLAMGPVSSVVA AETASSSSQQLPSLAPMLG, these results suggest that the 50-kDa protein is the precursor product expressed from the intact *gsrA* gene and that the 47-kDa polypeptide is the protein processed by removal of an N-terminal fragment consisting of 27 amino acid residues. This agrees with the previously reported suggestion that amino acid residues 1 to 27 of GsrA may be a signal peptide for protein export (45). To verify that both the 50- and 47-kDa proteins are truly produced from the chromosomal copy of *Y. enterocolitica* under physiological conditions, a heat shock extract from *Y. enterocolitica* was mixed with an extract from the GsrA-overproducing cells and then the mixture, as well as each extract alone, was electrophoresed on two-dimensional gels. Results presented in Fig. 3 show that in *Y. enterocolitica* cells, the 47-kDa protein, which comigrates with the 47-kDa protein overproduced by the T7 promoter-polymerase system in *E. coli* cells, is present. We have therefore concluded that the 47-kDa protein is the posttranslationally modified *gsrA* gene product of *Y. enterocolitica*. Furthermore, the GsrA protein was confirmed to be induced by heat shock at 42°C (Fig. 3B and D).

Enhanced expression of the GsrA protein in intracellularly grown *Y. enterocolitica* after phagocytosis by macrophages. To examine whether the *Y. enterocolitica* GsrA protein is induced by macrophage phagocytosis, the proteins synthesized in bacteria growing within macrophage cells were labeled with [^{35}S]methionine in the presence of cyclohexamide. As a control, when the heat-inactivated cells of *Y. enterocolitica* were phagocytized and radiolabeled, neither bacterial proteins nor host proteins incorporated [^{35}S]methionine, suggesting that the cyclohexamide completely inhibits the synthesis of the macrophage protein under these conditions (44). The extract from bacteria growing within macrophage phagosomes, as well as that from bacteria grown in culture medium, was analyzed by two-dimensional nonequilibrium gel electrophoresis. The results are shown in Fig. 4. As previously reported (44), the protein profile of the phagocytized bacteria is apparently different from that of bacteria grown in the medium. Many proteins whose syntheses are enhanced or reduced as a result of phagocytosis (Fig. 4A and B) were observed. Those induced by phagocytosis were proteins that are related to *Y. enterocolitica* stress proteins, which include the very prominent DnaK, GroEL, and GroES proteins. Visual scanning of the regions shown in Fig. 4C and D revealed that the synthesis of the GsrA protein is significantly higher in intracellularly growing bacteria after phagocytosis by macrophages than in extracellularly

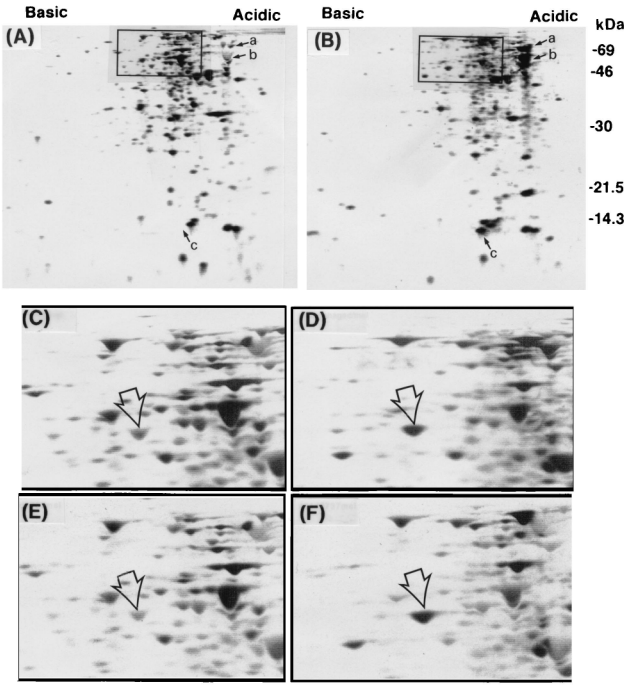

FIG. 4. Enhanced expression of the *Y. enterocolitica* GsrA protein within macrophage phagosomes. (A and C) Bacterial cells were labeled in methionine-free RPMI medium for 1 h at 37°C. (B and D) Bacterial cells were labeled in the presence of cyclohexamide within J774-1 cells between 3 and 4 h after phagocytosis at 37°C. (E and F) Bacterial cells were radiolabeled for 10 min at 28°C (E) and at 42°C between 5 and 15 min after the temperature shift (F). A portion of the lysate was subjected to two-dimensional gel electrophoresis. Autoradiographs of the dried gels are shown. The regions shown in rectangles in panels A and B correspond to the area shown in panels C to F. The arrows point to the position of the 47-kDa GsrA protein. Arrows labeled a, b, and c indicate DnaK, GroEL, and GroES, respectively.

TABLE 2. Features of the relevant stress proteins synthesized in *Y. enterocolitica* growing in vitro or in macrophage cells

Protein	pI[b]	Amt of protein (vol)[a]	
		In vitro	In macrophages
GsrA[c]	7.06	1.191	2.868
DnaK	4.68	2.205	12.20
GroEL	4.73	2.886	15.00
GroES	5.60	0.119	7.449

[a] Amount of protein was due to a quantitative analysis of the X-ray films shown in Fig. 4 by using the software package MELANIE II of Bio-Rad. Volume is the integration of optical density over the spot's area.

[b] The pIs of GsrA, GroEL, and GroES were deduced from the amino acid sequences previously reported (43, 45). The pI of DnaK was estimated from the pattern determined by two-dimensional gel electrophoresis with MELANIE II.

[c] A processed form of GsrA.

growing bacteria in the culture medium. The amounts of GsrA, DnaK, GroEL, and GroES synthesized in *Y. enterocolitica* growing in vitro and in macrophage cells were quantified and are shown in Table 2. From these results, we conclude that the synthesis of the GsrA protein is substantially induced in *Y. enterocolitica* after phagocytosis by macrophages.

Transcriptional activation of the *Y. enterocolitica gsrA* gene after phagocytosis by macrophages. To determine whether the enhanced production of GsrA protein in *Y. enterocolitica* growing in macrophage phagosomes is based on the transcriptional activation of the *gsrA* gene, we constructed the *lacZ* transcriptional gene fusion containing the promoter region of *gsrA*. We previously noted the σ^E-type heat shock promoter sequence upstream of the *gsrA* gene (45), and the sequence is shown in Fig. 1B. The fragment containing the putative promoter was fused to the promoterless *lacZ* gene to generate a plasmid, pTKY260, whose structure is shown in Fig. 1A. We then quantitatively analyzed *gsrA* transcription in *Y. enterocolitica* by monitoring phagocytosis by macrophages, using β-galactosidase activity to monitor *gsrA* promoter activity. Results are shown in Table 3. Controls with uninfected macrophages and macrophages infected with wild-type *Y. enterocolitica* WA314 showed no measurable β-galactosidase activity (data not shown). Immediately after phagocytosis (the 0.5-h time point in Table 3), β-galactosidase activity in cells carrying pTKY260 was comparable to that in extracellular bacteria (the 0 time point in Table 3). However, 2.5 h after phagocytosis, a significant increase in *gsrA* transcription was observed. The enhanced expression from the *gsrA* promoter was observed at least until 4.5 h after phagocytosis. As a control, the effect of phagocytosis on transcription from the promoter of the *cat* gene was examined with plasmid pTKY261. Plasmids pTKY260 and pTKY261 are identical except for the orientation of their *lacZ* genes with respect to that of the *gsrA* promoter. No intracellular activation of transcription of the *cat* promoter-*lacZ* fusion was observed. Therefore, we concluded that *gsrA* transcription in *Y. enterocolitica* was significantly and specifically activated within macrophages.

TABLE 3. Transcriptional activation of the *gsrA* gene after macrophage phagocytosis as judged by the activity of *lacZ* fused to the *gsrA* promoter

Fusion carried by *Y. enterocolitica*	β-Galactosidase activity at time point after phagocytosis (h)[a]:			
	0	0.5	2.5	4.5
gsrA-lacZ	25 ± 4	27 ± 3	67 ± 5	98 ± 11
cat-lacZ[b]	92 ± 8	88 ± 5	89 ± 6	84 ± 8

[a] Bacterial cells were taken by lysing J774-1 cells 0, 0.5 (see Materials and Methods), 2.5, and 4.5 h after phagocytosis. Activity is expressed as picomoles of *o*-nitrophenyl β-galactoside hydrolyzed per minute per CFU of bacteria. The 0 time point corresponds to bacteria grown in medium that were not phagocytosed by macrophages. Values are means ± standard deviations.

[b] *cat*, chloramphenicol acetyltransferase gene in pACYC184.

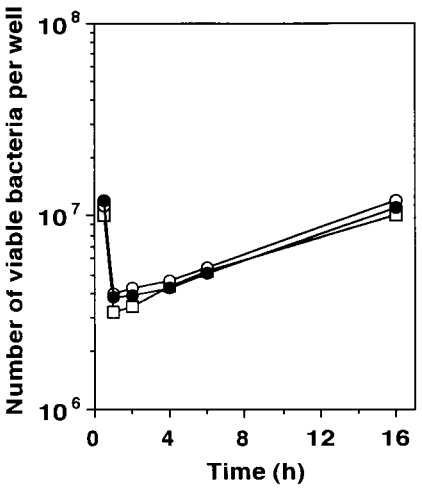

FIG. 5. Growth curves of *Y. enterocolitica* strains in J774-1 macrophages. J774-1 cells were challenged with *Y. enterocolitica* WA314 (□) and the derivatives carrying pTKY260 (○) or pTKY261 (●). The numbers of bacteria that survived were determined as previously described (45). The data are averages of triplicate determinations.

Figure 5 shows the growth curves of various strains of *Y. enterocolitica* in J774-1 macrophage cells. All of the comparisons between intracellularly and extracellularly grown bacteria were made during the log phase in both culture systems. However, it is possible that the protein expression observed in bacteria growing within macrophage cells was simply related to a difference in growth phase. Accordingly, we examined the protein profiles of *Y. enterocolitica* grown in RPMI medium and harvested during early log phase, middle log phase, and late log phase. Furthermore, we examined β-galactosidase activity to monitor *gsrA* transcription in *Y. enterocolitica* harvested during the same phases. Examinations showed that none of the phenotypic changes associated with intracellular growth were observed during these phases of growth in medium (data not shown).

DISCUSSION

In this study, we identified the *Y. enterocolitica* GsrA protein on two-dimensional gels by using the extract from cells in which the protein was exclusively produced under the T7 promoter-polymerase system. The results suggested that the GsrA protein was translated as an unstable precursor of 50 kDa and then was processed to a stable protein of approximately 47 kDa by removal of an N-terminal fragment consisting of 27 amino acid residues (Fig. 2). This indeed supports the previously reported hypothesis that GsrA is an extracytoplasmic protein and that amino acid residues 1 to 27 of the protein form a possible signal peptide for protein export (45). On the basis of the amino acid sequence deduced from the nucleotide sequence, the 50- and 47-kDa proteins estimated by SDS-PAGE can be assigned to proteins of 49,500 and 46,800 Da, respectively. The precursor protein could not be detected in *Y. enterocolitica* cells carrying a single copy of the *gsrA*$^+$ gene (Fig. 3A and D). It is probable that processing in normal cells is very efficient when compared with that in overproducing cells.

We found that the synthesis of GsrA protein was significantly enhanced in *Y. enterocolitica* intracellularly grown within macrophages after phagocytosis (Fig. 4 and Table 2). Furthermore, it was demonstrated that this enhanced production was due to the transcriptional activation of the *gsrA* gene (Table 3). Since there are multiple stimuli, e.g., sublethal oxidative stress, lysosomal contents, acid shock, and starvation, the enhanced expression of the GsrA protein perhaps represents the summation of the actions of various stress responses of *Y. enterocolitica* within macrophage phagosomes. Members of the *htrA* class of genes, including *gsrA*, have been shown to possess the consensus promoter sequence recognized by the σ factor of RNA polymerase, σ^E (15, 30, 45). σ^E was initially discovered in a study examining the transcription of *rpoH* at a lethal temperature, 50°C (5). At 50°C, almost all transcription of *rpoH* occurs from the P3 promoter, which is wholly transcribed by $E\sigma^E$. The only other *E. coli* gene known to be transcribed by $E\sigma^E$ is *htrA* (4, 22). A recent study demonstrated that the transcription of *htrA* in *E. coli* increased fourfold at 10 min after a shift to 42°C and eightfold at 10 min after a shift to 50°C (32). In contrast, there was only a slight (~1.5-fold) increase in transcription at the promoter of *rpoE* encoding σ^E at 10 min after a shift to 42°C and even less of an increase after a shift to 50°C (28, 32). The small increase in transcription of σ^E is unlikely to completely account for the immediate increase in transcription of *htrA* by temperature upshift, which implies additional levels of regulation in controlling $E\sigma^E$ activity. Though the method of regulation of the σ^E of the facultative intracellular bacteria within macrophage phagosomes is not clear at present, it can be concluded that the potential σ^E regulon, which includes *gsrA* and *htrA* homologs, is apparently induced in response to the environmental stresses found in macrophages.

In general, the heat shock proteins, either chaperones or proteases, prevent aggregation following thermal damage, protect proteins from further degradation, and may target proteins for proteolysis (6). In response to intracellular stress associated with phagocytosis, induced levels of stress proteins are required in order to cope with the accumulation of partially unfolded or denatured proteins in intracellular bacteria. Recently, the participation of DnaK in intracellular growth of *Brucella suis* within macrophages was reported (16). A member of the HtrA class of stress proteins has been shown to encode a periplasmic serine protease which is necessary for the degradation of abnormally folded fusion proteins transported into the periplasmic space (20, 38). It is therefore proposed that the GsrA or HtrA protein serves as a stress protein to protect extracytoplasmic compartments from stress. Interestingly, recent studies have demonstrated that the expression of σ^E is induced by overproduction of outer membrane proteins, suggesting that the signal to which $E\sigma^E$ responds is generated by events occurring in the extracytoplasmic compartment of the bacterial cell (23).

Yersinia spp. are able to resist the microbicidal mechanisms of and multiply within phagocytic cells, as shown by many studies (41, 42, 44). In addition to the characteristics of a facultative intracellular pathogen, it is well known that the multiplication of yersiniae occurs predominantly extracellularly in vitro with the expression of large quantities of *Yersinia* outer proteins (Yops), which are encoded by a virulence plasmid (7, 37). The maximal expression of Yops in vitro is obtained in Ca^{2+}-depleted media at 37°C. It has been demonstrated that Yops are secreted by extracellular yersiniae, translocated across the cellular membrane, and subsequently internalized into target cells, such as macrophages, suggesting that yersiniae can grow extracellularly in the host (31, 36). This discrepancy with the in vitro findings might be explained on the basis of the environmental conditions encountered by bacteria in host tissues. It may be that yersiniae are exposed to the intracellular environment only during the initial stages of infection in vivo. It is possible that the GsrA protein contributes to the growth of *Y. enterocolitica* within macrophage phagocytes in the early stages of infection, resulting in their proliferation and dissemination throughout the host. The Yop expression system appears to be involved in extracellular survival of yersiniae in the hostile environment of the mammalian reticuloendothelial system. One may expect that during the overproduction of Yops, the expression of GsrA is induced in response to the events occurring in the extracytoplasmic compartment of *Y. enterocolitica* cells.

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