INFECTION AND IMMUNITY, Oct. 2002, p. 5404–5411 Vol. 70, No. 10
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.10.5404–5411.2002
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Characterization of a Novel Intracellularly Activated Gene from *Salmonella enterica* Serovar Typhi

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Received 22 April 2002/Returned for modification 2 May 2002/Accepted 27 June 2002

A *Salmonella enterica* serovar Typhi gene that is selectively up-regulated upon bacterial invasion of eukaryotic cells was characterized. The open reading frame encodes a 298-amino-acid hydrophobic polypeptide (30.8 kDa), which is predicted to be an integral membrane protein with nine membrane-spanning domains. The protein is closely related (87 to 94% reliability) to different transport and permease systems. Gene expression under laboratory conditions was relatively weak; however, sevenfold induction was observed in a high-osmolarity medium (300 mM NaCl). The growth pattern in a laboratory medium of a serovar Typhi strain Ty2 derivative containing a 735-bp in-frame deletion in this gene, named *gaiA* (for gene activated intracellularly), was not affected. In contrast, the mutant was partially impaired in intracellular survival in murine peritoneal macrophages, as well as in human monocyte-derived macrophages. However, in the case of human macrophages, this survival defect was modest and evident only at late infection times (24 h). Despite the distinct intracellular survival kinetics displayed in macrophages of different species, the *gaiA* null mutant was significantly affected in its potential to trigger apoptosis in both murine and human macrophages. Provision of the *gaiA* gene in *trans* resulted in complementation of these phenotypes. Interestingly, the absence of a functional *gaiA* gene caused a marked attenuation in the mouse mucin model, as shown by the increase (3 orders of magnitude) in the 50% lethal dose of the mutant strain over that of the parental strain Ty2 ($P \leq 0.05$). Altogether, these data indicate that the product encoded by the *gaiA* gene is required for triggering apoptosis and bacterial survival within murine macrophages, which is consistent with the in vivo results obtained in the mouse mucin model. However, *gaiA* was not required for initial intracellular survival in human cells, indicating that its role in the natural host might be more complex than is suggested by the studies performed in the murine system.

Infections caused by *Salmonella enterica* serovars constitute a major public health problem worldwide (1, 20). These pathogens can affect both humans and animals, causing food-borne diseases ranging from mild gastroenteritis to life-threatening systemic infections, such as those caused by *S. enterica* serotype Typhi in humans (19, 20, 41). The clinical management of patients infected with *S. enterica* serovars is rendered difficult due to the emergence of multidrug-resistant strains (47).

S. enterica serovar Typhi has a particularly complex infection cycle, in which the microorganism transits through different niches (41, 55). It has been demonstrated that during infection, the expression of bacterial products is tightly regulated according to environmental signals (10). This allows the pathogen to optimize the expression of the virulence factors required in each phase, avoiding the additional energetic cost associated with the production of unnecessary products. Better understanding of the molecular basis of *Salmonella* infections has led to identification of bacterial products which are essential for pathogenicity, such as virulence factors, regulatory proteins, and secretion systems (15, 19). The corresponding genes constitute potential targets for the development of attenuated strains, which can be used either as live vaccines against salmonellosis or as carriers for heterologous antigens (7, 32).

Serovar Typhi promoters that are activated mainly upon bacterial entry into eukaryotic cells have been identified previously (58). It is likely that the genes controlled by these promoters are involved in the infection process to some extent. Precedents exist in *S. enterica* serovar Typhimurium demonstrating that genes induced inside cultured macrophages or epithelial cells are essential for virulence. This is the case for the *spv* operon and the *mig-14* gene (13, 61). In this work we describe the characterization of a novel serovar Typhi gene driven by one of those promoters. The results obtained suggest that this gene is involved in the interactions between bacteria and phagocytic cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used in this study are described in Table 1. Bacteria were grown in Luria-Bertani (LB) broth (48) or LB agar plates. Plasmids were maintained in the *Escherichia coli* strain XL1-Blue, and the INVαF′ strain was used as a recipient for cloning fragments amplified by PCR and cloned into the pCR2.1 vector. Media were supplemented with chloramphenicol (50 $\mu g\ ml^{-1}$), ampicillin (200 $\mu g\ ml^{-1}$), nalidixic acid (20 $\mu g\ ml^{-1}$), or streptomycin (50 $\mu g\ ml^{-1}$) when required.

DNA manipulations. Plasmid DNA isolation, restriction endonuclease digestion, ligation, transformation, agarose gel electrophoresis, and other standard DNA techniques were carried out as described by Sambrook et al. (48). Oligo-

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TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Relevant genotype or characteristics	Reference or source
Strains		
E. coli		
XL1-Blue	Tc^r; *endA1 recA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 gyrA96 relA1 lac* [F′ *proAB lac1*^{q}ZΔM15 Tn*10*]	Stratagene
INVαF′	F′ *endA1 recA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 gyrA96 relA1* φ80 *lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR* λ$^-$	Invitrogen
SM10 (λpir)	Km^r; *thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu-Km::Tn*7* λ*pir*	34
Serovar Typhi		
Ty2	Wild type	ATCC 19430
Ty2 Na1^r	Na1^r; spontaneous derivative of Ty2	This work
Δ*gaiA*	Na1^r; Ty2 Na1^r derivative containing an in-frame deletion of the *gaiA* gene	This work
Serovar Typhimurium		
MT189	*recA1 galE*496/F′ 100-12 λ*pir* (*gal*$^+$ *bio*$^+$)	24
LT2	Wild type	ATCC 15277
Plasmids		
PCR2.1	Ap^r Km^r; high-copy-number vector for cloning PCR products	Invitrogen
pKNG101	Sm^r; broad-host-range π-dependent suicide vector	30
pHOB35	Sm^r; pKNG101 derivative containing a PCR fragment encompassing the *gaiA* gene with a 735-bp internal deletion	This work
pVDL8	Cm^r; derivative of pHSG575 with additional *Not*I sites flanking the polylinker	60
pHOB38	Cm^r; pVDL8 derivative containing a PCR fragment encompassing the gaiA ORF, generated with primers 5102XBA and SEQAPOR2	This work
pUJ9TT	Ap^r; multicopy promoter probe vector to generate fusions with the *lacZ* gene	29
pHOB700	Ap^r; pUJ9TT derivative containing a 431-bp *Eco*RI/*Bam*HI PCR fragment generated with primers 5102ECO and 3LACZBAM	This work
pLS102	Ap^r; pCB182 derivative containing an in vivo activated promoter from the *Salmonella* strain Ty2	58

nucleotides (Table 2) were synthesized by GIBCO. Colony PCR, extraction of PCR products, and cloning experiments were performed according to standard protocols (48). Inverse PCR was carried out as described by Ochman et al. (40). DNA sequencing was performed by using a *Taq* dye-deoxy terminator cycle sequencing kit and a model 373A automatic DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, Calif.), according to the manufacturer's instructions. Restriction and modification enzymes were purchased from New England Biolabs (Frankfurt, Germany). Electroporation was carried out with a gene pulser (Bio-Rad Laboratories, Richmond, Calif.) as described by O'Callaghan and Charbit (39). Searches in databases for nucleotide and amino acid sequence homologies were performed by using the BLASTP (2), BLASTP plus BEAUTY (2, 66), FASTA3 (42), and PSORT (36) algorithms.

Construction of a nonpolar mutation. Overlap extension PCR (27) was used to generate an in-frame deletion in the *gaiA* gene. Two PCR fragments were obtained by using the primer pairs 5102XBA and 3Δ172 (447 bp) and 5Δ1823 and SEQAPOR2 (510 bp). The resulting products contain the first 87 bp and the last 72 bp of the *gaiA* open reading frame (ORF), respectively. An 18-bp overlap in their sequences allowed the amplification of a 934-bp fragment during a second PCR using the primer pair 5102XBA and SEQAPOR2. The resulting product, which encompasses a *gaiA* gene containing an internal 735-bp deletion, was digested with *Xba*I and cloned into *Xba*I-digested pKNG101 (30), generating pHOB35. This plasmid was transformed into strain SM10 (λ*pir*) and then transferred by conjugation (25) into the recipient *S. enterica* serovar Typhi strain Ty2 (Nal^r). Cointegration and excision of the suicide vector were performed as previously described (30). The in-frame deletion contained in the serovar Typhi *gaiA* mutant resulting from the allelic exchange was confirmed by PCR analysis using primers homologous to regions encompassed in the deleted fragments or to adjacent external sequences (data not shown). Primers 5102XBA and SEQAPOR2 were used to amplify the full-length *gaiA* gene and 360 bp of the region located upstream of the start codon, which was subsequently cloned into the low-copy-number vector pVDL8 (60), generating pHOB38, which was used for complementation studies.

TABLE 2. Oligonucleotides used for PCR

Name	Sequence (5′→3′)
5102XBA	GGTCTAGAGATCACATTTTGTAATGTTACAC
5102ECO	GGGAATTCGATCACATTTTGTAATGTTACAC
5102B	GGAAGCTTTTTACGCTGCTATTGCTG
5Δ1823	GCGATGTCGCTTTCCGGCGGTCTGATG
3Δ172	GCCGGAAAGCGACATCGCCCAGAA
SEQAPOR2	CGCGGAATTTACGACATCATCA
3LACZBAM	CCGGATCCAAGACAGGCGCGGCC
REVSD	GGCTCGAGTCAGCAAAACGGCGAACAG

Construction of a *gaiA′*-*′lacZ* fusion. For construction of a *gaiA′*-*′lacZ* fusion, a 431-bp *Eco*RI/*Bam*HI-fragment amplified by PCR using primers 5102ECO and 3LACZBAM, which contains 375 bp of the upstream sequence of *gaiA* and 56 bp of the *gaiA* gene, was cloned into pUJ9TT (29), generating pHOB700. β-Galactosidase activity was quantified by the method of Miller (33).

Tissue culture methods, invasion tests, and in vivo studies. *S. enterica* serovar Typhi strains were tested for the ability to survive in Henle 407 cells (ATCC CCL-6) and the macrophage-like cell line J774A.1 (ATCC TIB 67). Primary macrophages were either obtained from the peritoneal cavities of BALB/c mice or derived from human peripheral blood mononuclear cells obtained from healthy volunteers as previously described (43). Henle 407 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 25 mM HEPES, 10% (vol/vol) fetal calf serum (FCS), and 5 mM glutamine (GIBCO). Macrophages were maintained in DMEM (Sigma Chemie GmbH, Deisenhofen, Germany) supplemented with 4.5 g of glucose/liter, 10% FCS, 5 mM glutamine, and 1.5 g of $NaHCO_3$/liter in an atmosphere containing 5% CO_2 at 37°C. Cells seeded at a concentration of approximately 5×10^4 per well in 24-well tissue culture plates (Inter Med NUNC, Roskilde, Denmark) were infected with bacteria grown overnight in static LB broth cultures supplemented with 17.53 g of NaCl/liter during 90 min (Henle cells), 60 min (human macrophages), or 30 min (murine macrophages and J774A.1 cells), as previously described (22, 29). The number of apoptotic cells was determined by using an in situ cell death detection kit with fluorescein (Boehringer Mannheim GmbH) according to the manufacturer's instructions. The mouse mucin model described by Powell et al. (44) was used for determination of the 50% lethal dose (LD_{50}) (45).

Statistical analysis. The statistical significance of the results obtained was evaluated by the chi-square test.

Nucleotide sequence accession numbers. The nucleotide sequences reported here have been deposited in the EMBL database under accession number AJ006101.

RESULTS AND DISCUSSION

Identification of an *S. enterica* serovar Typhi gene that is activated upon infection of eukaryotic cells. A previous study allowed the identification of serovar Typhi promoters that were activated upon invasion of eukaryotic cells (58). By use of the gene encoding chloramphenicol acetyltransferase (*cat*) as a reporter, it was estimated that the activity of the promoter contained in vector pLS102 was increased 93-fold when bacteria entered eukaryotic cells (58). This vector carries a 548-bp fragment of serovar Typhi DNA containing a promoter motif reminiscent of σ^{54}-dependent promoters, a Shine-Dalgarno sequence, and the start of an ORF at position 372 (58).

Total DNA from the serovar Typhi strain Ty2 was digested with different restriction endonucleases recognizing sequences present in the insert from pLS102 and was subsequently analyzed by Southern blotting using the digoxigenin-labeled insert from pLS102 as a probe. A 1,600-bp fragment that reacted specifically with the probe was identified by using *Apo*I. To identify the sequences located downstream of the truncated ORF present in pLS102, this 1,600-bp fragment was amplified by inverse PCR using primers REVSD and 5102B, with *Apo*I-digested DNA as a template. Sequence analysis of the resulting fragment allowed us to design primer SEQAPOR2, corresponding to the 3′ end, which was used together with primer 5102XBA to PCR amplify the whole ORF by using chromosomal DNA from the serovar Typhi strain Ty2 as a template.

The gene present in the cloned fragment, which was named *gaiA* (for gene activated intracellularly), encodes a 298-amino-acid hydrophobic polypeptide with a predicted molecular mass of 30.8 kDa and a pI of 10.02. A potential leader peptide with a cleavage site at positions 18 and 19 (ACL-GL) was detected by using the SignalP algorithm (37). Use of the TopPred II algorithm (9) suggested with 95% reliability that the GaiA protein is an integral membrane protein with nine membrane-spanning domains (Fig. 1). Interestingly, nine predicted N-myristoylation sites were also detected; one of them was located at the predicted leader peptide cleavage site (GLALGG; positions 19 to 24). In gram-negative bacteria the myristoylation of lipid A is essential for its proinflammatory properties, which are essential for a virulent phenotype (23, 56, 57). However, the covalent attachment of a myristoyl group to an NH_2-terminal glycine residue seems to occur exclusively in eukaryotic cells (3, 53). Nevertheless, recent studies have proved that proteins exported from prokaryotic cells can also be N myristoylated by the machinery of target cells (38, 54).

Searches for homologies in databases showed that the product encoded by the *gaiA* gene exhibits 88% identity (293-amino-acid overlap) with the putative transport protein coded by the *f451* ORF from *E. coli* (YICM_ECOLI; P31438) and 87% identity with the corresponding allelic variant of the *E. coli* strain EDL933 (AAG588858). The *E. coli yicM* gene is located in the intergenic fragment between the *uhpT* and *nlpA* genes (5). *uhpT* is involved in the uptake of phosphorylated sugars (14, 63, 64), whereas *nlpA* is a lipoprotein attached to the cytoplasmic membrane (68). The location of the *gaiA* gene was examined in the genomes of *S. enterica* serovars Typhi and Typhimurium by using the "Enteric" server (http://galapagos.cse.psu.edu/enterix/enteric/enteric.html). This analysis revealed that, unlike its *E. coli* homologue, *gaiA/yicM* is flanked by several ORFs of unknown function that are absent in the *E. coli* genome (10 and 9 ORFs in the upstream and downstream regions, respectively). Moreover, 12 ORFs upstream of *gaiA/yicM* is the *mgtC* gene, which has been shown to be the first gene of a 17-kb region encompassing *Salmonella* pathogenicity island 3 and required for intramacrophage survival (4). This evidence suggests that *gaiA/yicM* has been conserved between the two genera but that in the case of *Salmonella*, extensive chromosomal rearrangement of the genome has occurred in its flanking regions, including insertion of a pathogenicity island.

The product encoded by the *gaiA* gene was also analyzed by using the Propsearch algorithm (28), which detects functional or structural homologues belonging to putative protein families by using 144 properties (e.g., amino acid composition, molecular weight, content of bulky or small residues, average hydrophobicity, and charge). The Euclidian distance between the product encoded by *gaiA* and other database sequences suggested that this protein is related with a 87 to 94% reliability to different transport systems (e.g., sugars, cytosine, ABC transporters, arsenic pumps, branched amino acids, Na^+/H^+ antiporter). This suggests that the product encoded by this gene may be involved in the transport of substrates required for bacterial metabolism.

Transcriptional regulation of the *gaiA* gene. Previous results demonstrated that the promoter driving the expression of the *gaiA* gene is activated intracellularly (58). To gain further knowledge about the potential environmental signals mediating gene activation, a translational fusion was generated between *gaiA* and the gene coding for β-galactosidase (*lacZ*), which was used as a reporter. A DNA fragment spanning nucleotides −375 to +56 (with respect to the *gaiA* ATG start codon) was fused to the *lacZ* gene present in pUJ9TT (see Materials and Methods), generating plasmid pHOB700. This fragment includes the promoter and upstream regions, containing potential binding sites for regulatory factors, and maintains intact the translational initiation region in order to avoid potential artifacts deriving from affected translational initiation (50). The pHOB700 plasmid was highly unstable in wild-type *Salmonella* strains, even when passed through an intermediate *rec*-negative mod^+ serovar Typhimurium strain, suggesting that this may be partly due to recombination events. Thus, transcriptional activation studies were performed by using *Salmonella* strain MT189 (*recA1*), in which the construct was stable, as a recipient.

In the initial studies the influence of the growth phase on the expression of the reporter gene was analyzed (Fig. 2A), since this factor seems to play a key role in *Salmonella* invasion (11, 12, 31, 59). A minor increase in the expression of the reporter was observed at the early-exponential phase, followed by a second increment at the late-exponential and early-stationary phases (Fig. 2A). In contrast, when the effect of changes in osmolarity was analyzed, a clear increment of about sevenfold in the expression of *lacZ* was observed at 0.3 M NaCl (Fig. 2B). Interestingly, bacterial growth at lower and higher osmolarities resulted in reduced expression. This is in agreement with what was previously observed for the adhesive and invasive phenotypes (optimal at 0.3 M NaCl) and the expression of different virulence factors and type III secretion systems (15, 16, 59). Since the availability of Mg^{2+} has been also reported to affect *Salmonella* virulence, via the response regulator PhoP/PhoQ

A

```
         5102ECO →
         5102XBA →
gatcacattttgtaatgttacactgcgtggattttccgcactaaaacgacgcaggtaaccacaatgaatgaaaata
tcgcagaaaaattccgcgctgatggggttgccaggcctaactggtcagccgtttttggcgtggcgttttgtgtcgc
ctgcctgattaccgttgagtttttgccgttagtctgttgacgccgatggcgcaggatctggggatctccgagggcg
tcgccggtcagtcggtcaccgttacggtttgtcgcgatgttttccagcctgttcattacccagattattcaggcga
                               ← REVSD
ccgacaggcgttatatcgttattctgttcgccgttttgctgacggcttcttgtctg ATG GTC TCC TTT GCC
                 5102B →          ← 3LACZBAM              M   V   S   F   A
AAC AGC TTT ACG CTG CTA TTG CTG GGC CGC GCC TGT CTT GGG TTG GCG CTG GGC GGA
 N   S   F   T   L   L   L   L   G   R   A   C   L   G   L   A   L   G   G
    ← 3Δ172
TTC TGG GCG ATG TCG[GCG TCG CTG ACC ATG CGA CTG GTT CCC GCG CGT ACC GTG CCG
 F   W   A   M   S   A   S   L   T   M   R   L   V   P   A   R   T   V   P
AAA GCG CTG TCG GTG ATT TTT GGC GCG GTC TCC ATC GCG TTA GTG ATC GCC GCG CCG
 K   A   L   S   V   I   F   G   A   V   S   I   A   L   V   I   A   A   P
CTG GGC AGT TTT TTG GGC GGT ATT ATT GGC TGG CGT AAT GTC TTT AAC GCC GCT GCG
 L   G   S   F   L   G   G   I   I   G   W   R   N   V   F   N   A   A   A
GTG ATG GGC GTA CTG TGC GTC ATC TGG GTG GTG AAA TCA CTG CCG TCG CTG CCG GGC
 V   M   G   V   L   C   V   I   W   V   V   K   S   L   P   S   L   P   G
GAA CCT TCT CAC CAG AAA CAG AAT ATG TTT AGC CTG TTG CAA CGC CCT GGC GTG ATG
 E   P   S   H   Q   K   Q   N   M   F   S   L   L   Q   R   P   G   V   M
GCC GGG ATG ATC GCC ATC TTT ATG TCT TTT GCC GGG CAG TTC GCT TTC TTT ACC TAT
 A   G   M   I   A   I   F   M   S   F   A   G   Q   F   A   F   F   T   Y
ATT CGC CCG GTC TAT ATG AAT CTG GCG GGC TTT GAC GTT GAT GGT CTG ACG CTG GTG
 I   R   P   V   Y   M   N   L   A   G   F   D   V   D   G   L   T   L   V
CTG CTA AGT TTT GGT ATT GCC AGC TTC GTT GGC ACT TCT TTC TCC TCT TAC GTC CTG
 L   L   S   F   G   I   A   S   F   V   G   T   S   F   S   S   Y   V   L
AAA CGT TCG GTA AAA CTG GCG CTG GCC GGT GCG CCG CTG CTA CTG GCG CTG AGC GCG
 K   R   S   V   K   L   A   L   A   G   A   P   L   L   L   A   L   S   A
CTG ACG CTC ATT GTG TGG GGA AGC GAC AAA ACC GTG GCG GGG GGA ATA GCG ATT ATC
 L   T   L   I   V   W   G   S   D   K   T   V   A   G   G   I   A   I   I
TGG GGA CTG GCG TTT GCG TTG GTG CCG GTG GGA TGG TCA ACG TGG ATC ACT CGT TCT
 W   G   L   A   F   A   L   V   P   V   G   W   S   T   W   I   T   R   S
CTT GCC GAT CAG GCG GAA AAA GCC GGT TCC ATC CAG GTC GCG GTG ATT CAA CTG GCA
 L   A   D   Q   A   E   K   A   G   S   I   Q   V   A   V   I   Q   L   A
AAT ACC TGT GGC GCG GCG GTG GGC GGT TAT GCG CTC GAC AAT TTC GGG CTG CTT TCG
 N   T   C   G   A   A   V   G   G   Y   A   L   D   N   F   G   L   L   S
                 5Δ1823 →
CCG CTG GCG]CTT TCC GGC GGT CTG ATG CTG TTG ACG GCG TTA GTC GTG GCG GCG AAA
 P   L   A   L   S   G   G   L   M   L   L   T   A   L   V   V   A   A   K
GTC CGT ATT ACG CCA ATG AGT TGA tattgctgccatttgtaggccggatacgacgttagtcgttatcc
 V   R   I   T   P   M   S   *
ggcaataataaacgcttaatcaaaaccgttatgctctgcggtggcggcaaaccattctccgctttttttcaccgtg
cgcgtttgggtggctaaatccagttgtacaaaaccatagcgatttttatagccgttcagccatgaccagttatcga
tcaaggtccacatgtggtagccaagacagtgacagccctcgctaatgcctttatgcagccatttaagatgctcaga
gataaagtcaatccggtaatcatcgtgaatctgtcccgcttgcacaaaacgctgctcgttctcaacgcccatcccg
                                                       ← SEQAPOR2
ttttcggagataaaacagcgcggattgccgtaattatcccgcaggttggtgatgatgtcgtaaattc
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B

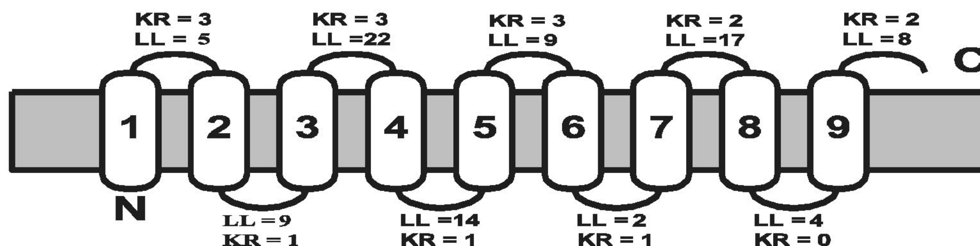

FIG. 1. Identification of the product encoded by the *gaiA* gene from serovar Typhi. (A) Nucleotide sequence and predicted translation product of the *gaiA* locus. Amino acids are given in one-letter code, start and stop codons are boldfaced, sequences deleted in the Δ*gaiA* mutant are marked off by square brackets (with amino acids in italics), primer sequences are double underlined, and arrows indicate direction. (B) Predicted topology of the product encoded by the *gaiA* gene. The predicted leader sequence peptide has been removed for the prediction. The topology corresponding to the cytoplasmic location of the COOH terminus is shown. Abbreviations: LL, loop length; KR, lysine-plus-arginine profile.

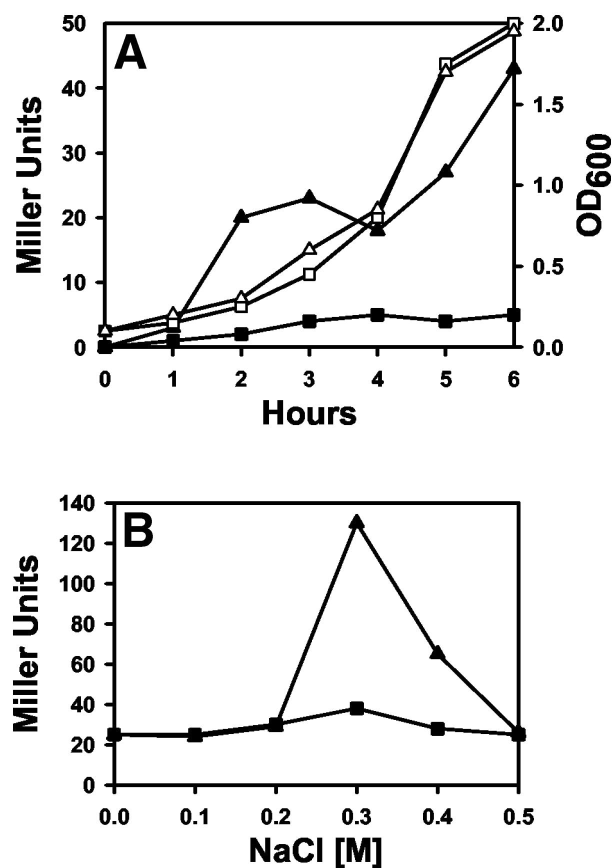

FIG. 2. Activation of the *gaiA* promoter in response to different growth conditions. Serovar Typhimurium MT189 bacteria carrying either plasmid pUJ9TT (■) or plasmid pHOB700 (▲) were grown either in LB medium (A) or in MM63 minimal medium supplemented with different concentrations of NaCl (B), and β-galactosidase production was monitored. Growth rates are indicated by open symbols (optical density at 600 nm [OD_{600}]). Results are expressed as Miller units; standard deviations were lower than 5%.

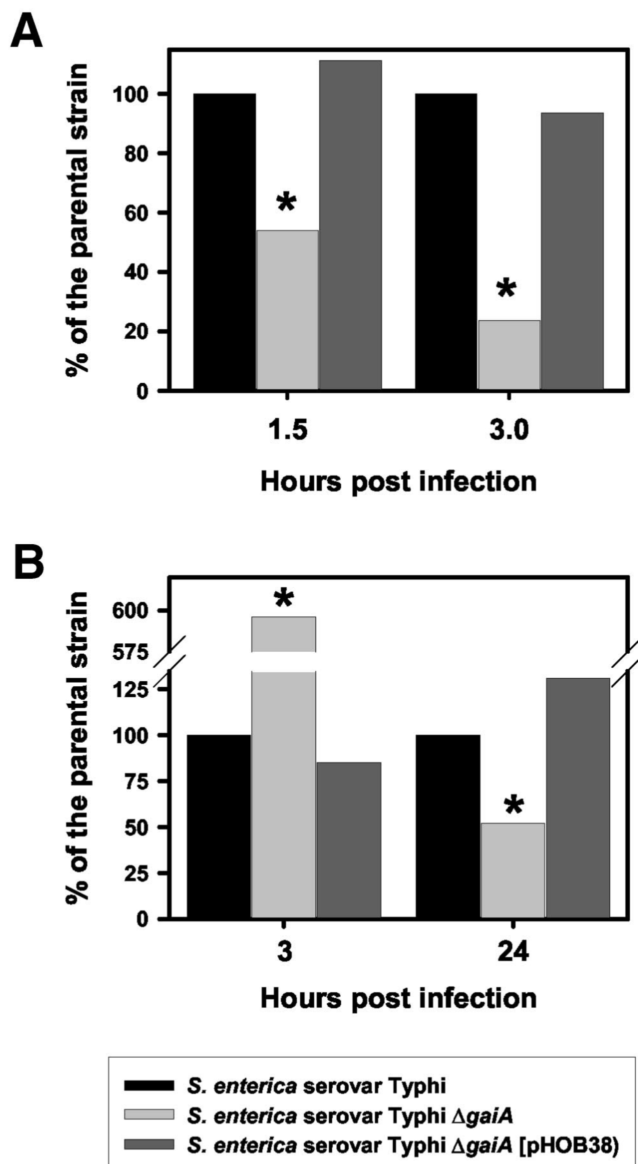

FIG. 3. Interaction of the serovar Typhi Δ*gaiA* mutant with macrophages. The capacities of the serovar Typhi strain Ty2, its Δ*gaiA* derivative, and the Δ*gaiA* mutant complemented with pHOB38 to survive within mouse peritoneal macrophages (A) or monocyte-derived human macrophages (B) were evaluated. The CFU recovered per well was compared with the number of viable bacteria harvested from cells infected with the wild-type strain Ty2. Results are expressed as CFU relative to the values obtained for the parental strain. Asterisks indicate that the differences from the control values were considered significant ($P \leq 0.05$).

(17), we investigated whether bacterial growth in the presence of different concentrations of Mg^{2+} affects the expression of the *gaiA-lacZ* fusion. However, no significant differences were observed (data not shown).

GaiA is involved in the interactions between serovar Typhi and macrophages. To assess whether the product encoded by *gaiA* is involved in the pathogenesis process, a serovar Typhi Ty2 mutant containing a 735-bp in-frame deletion in this gene was generated as described in Materials and Methods. The serovar Typhi Δ*gaiA* strain did not differ from the parental strain in terms of growth pattern in LB medium, morphology (by light microscopy), or expression of the Vi antigen (data not shown).

The capacity of the null mutant to infect and survive within epithelial cells was then evaluated. No statistically significant differences were observed in the number of viable intracellular bacteria recovered following 4, 6, 8, and 24 h of infection of Henle 407 epithelial cells (data not shown). Then we analyzed whether the Δ*gaiA* mutant was able to survive within professional phagocytes. Upon infection of mouse peritoneal macrophages, the serovar Typhi Δ*gaiA* strain displayed a 46 to 77% reduction ($P \leq 0.05$) in the number of viable intracellular bacteria from that for the wild type at 1.5 and 3 h postinfection, respectively (Fig. 3A). To confirm the role played by the product encoded by the Δ*gaiA* gene in the phenotype observed, a plasmid containing a PCR fragment encompassing the full-length *gaiA* gene and 360 bp of the regions located upstream of the ATG start codon (pHOB38) was introduced into the serovar Typhi Δ*gaiA* mutant. The provision of *gaiA* in *trans* resulted in full complementation of the mutant phenotype, re-

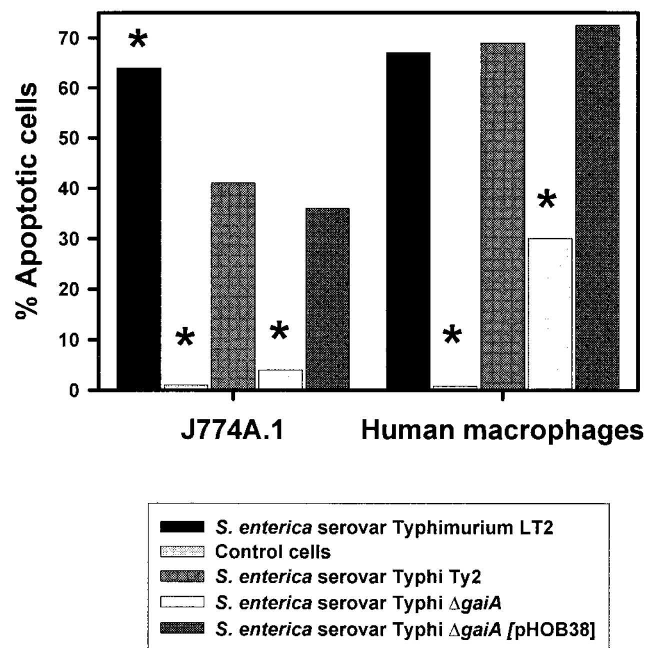

FIG. 4. *Salmonella*-mediated apoptosis of infected cells. The capacities of the serovar Typhi strain Ty2, its Δ*gaiA* derivative, and the Δ*gaiA* mutant complemented with pHOB38 to trigger apoptosis in J774A.1 cells and monocyte-derived human macrophages were evaluated and compared with that of the positive-control serovar Typhimurium strain LT2. Results are expressed as percent apoptotic cells. Asterisks indicate that the differences from results for the parental strain *S. enterica* serovar Typhi Ty2 were considered significant ($P \leq 0.05$).

storing intracellular survival levels to those of the wild-type parental strain (Fig. 3A). It is unlikely that the differences observed were due to impaired infectivity, since the number of viable bacteria recovered per well after 30 min of infection was in the range of 1×10^5 to 2×10^5 for all strains tested.

It has been demonstrated that *S. enterica* serovar Typhimurium is able to trigger apoptosis of infected macrophages (6, 8, 26, 35, 49, 69). This process seems to play a key role also in elicitation of immune responses to *Salmonella* (67, 69). Since the intracellular survival of the mutant strain was reduced only in macrophages, not within epithelial cells, we decided to assess whether the capacity of the serovar Typhi Δ*gaiA* mutant to stimulate programmed cell death was also affected. The murine macrophage-like cell line J774A.1 was infected with serovar Typhi Ty2 or its Δ*gaiA* derivative, and the number of apoptotic cells was determined after 8 h by a terminal deoxynucleotidyltransferase-mediated dUTP-fluorescein nick end labeling (TUNEL) assay (Fig. 4). Approximately 41% of cells infected with serotype Typhi Ty2 underwent apoptosis after 8 h of infection. In contrast, only 4% of the cells infected with the Δ*gaiA* mutant exhibited signs of apoptosis. The capacity to promote apoptosis was restored in the Δ*gaiA* mutant complemented with pHOB38.

Infected macrophages play a key role in *Salmonella* infections (21, 41). However, intracellular survival is host dependent. While serovar Typhimurium can survive within both mouse and human macrophages, serovar Typhi survives at reasonable rates only within human macrophages (51, 62). Serovar Typhi also exhibits a particular survival kinetics in human macrophages, which is characterized by an initial phase of bacterial death followed by a second phase of persistent survival. Furthermore, serovar Typhimurium usually causes more apoptosis than serovar Typhi (51). Therefore, it has been proposed that serovar Typhi can survive chronically within human macrophages by causing little damage, thereby favoring systemic dissemination.

Humans are the natural hosts for serovar Typhi. Therefore, to further characterize the role played by the product encoded by the *gaiA* gene, monocyte-derived human macrophages were infected. Surprisingly, significantly higher numbers of viable bacteria were recovered from cells infected with the Ty2 Δ*gaiA* mutant than from those infected with the parental strain ($P \leq 0.05$) after 3 h of infection (Fig. 3B). This consistent increment in survival was abolished by providing the *gaiA* gene in *trans* (Fig. 3B). However, the initial survival pattern was reverted 24 h after infection, with the Δ*gaiA* mutant exhibiting a slightly lower viability than the wild-type strain. Provision of the *gaiA* gene in *trans* resulted in full complementation of the mutant phenotype (Fig. 3B). To better characterize the dynamics of the intracellular survival process for each strain tested, survival indexes (the number of viable bacteria after 24 h divided by the number of viable bacteria after 3 h) were calculated. The survival index of the wild-type strain was 4.2, showing that there was a significant increment in the number of viable bacteria after 24 h. In contrast, the survival index of the Δ*gaiA* mutant was 0.48, demonstrating that there was a marked reduction in the number of viable bacteria during the course of infection. When the *gaiA* gene was provided in *trans*, the phenotype of the deletion mutant reverted, and the survival index was even higher than that of the wild-type strain (6.6).

Thus, it seems that during short-term infection of human macrophages the *gaiA* gene is not required for intracellular survival, and indeed, its loss causes an apparent abolishment of the initial phase of bacterial death. To evaluate whether this phenotype could be related to differences in the capacity to trigger macrophage apoptosis, we monitored the percentages of apoptotic cells in human macrophages infected with the wild-type and mutant strains. The results obtained (Fig. 4) demonstrated that the ability of the Δ*gaiA* mutant to trigger apoptosis was significantly reduced from that of the wild-type strain ($P \leq 0.05$). When the *gaiA* gene was provided in *trans*, full complementation of the mutant phenotype was observed (Fig. 4). Thus, it seems that the difference in survival kinetics displayed by the Δ*gaiA* mutant in murine and human macrophages is not related to an impaired capacity to trigger apoptosis. Interestingly, it has been demonstrated that *spvA*, another *Salmonella* gene which is induced in intracellular bacteria, follows a differential kinetics of induction in both macrophages and epithelial cells, displaying maximal induction (≥100-fold) 6 to 8 h postinfection (65). Considering our results, it is tempting to postulate a potential role for *gaiA* in bacterial survival within human macrophages during late infection.

S. enterica serovar Typhi is avirulent for mice. This seems to reflect its inability to grow in murine macrophages. However, the use of iron-enriched mucin results in iron overloading, which increases bacterial growth within phagocytic cells (52).

In an attempt to further characterize the role of *gaiA*, the in vivo virulence of the serovar Typhi Δ*gaiA* mutant was evaluated by using the mouse mucin model. Under these conditions, the LD_{50} calculated for the Δ*gaiA* mutant (2.1×10^7 CFU) was approximately 3 orders of magnitude higher than that for the parental strain Ty2 (4.78×10^4 CFU). This result suggests that the presence of a functional *gaiA* gene is required in vivo for expression of a fully virulent phenotype in the murine mucin model, which agrees with the observed defects of the Δ*gaiA* mutant in triggering apoptosis and in surviving within murine macrophages. However, we should be cautious when evaluating these data, since mice are not the natural hosts for serovar Typhi.

Salmonella genes which are selectively activated upon infection of eukaryotic cells are likely to be either housekeeping genes or genes encoding novel virulence factors. Preliminary studies suggest that GaiA may be involved in the transport or utilization of carbon sources (data not shown). The cytoplasm of infected cells might be a nonpermissive environment for intracellular pathogens, unless they are able to express special metabolic genes. Although serovar Typhi is a pathogen that resides in membrane-bound compartments of the host cell, its proliferation within these compartments must rely on the acquisition of nutrients that are probably present in the cytoplasm. The potential involvement of GaiA in the transport or utilization of carbohydrates, as well as in bacterial interactions with phagocytes, suggests that it might play an essential role in bacterial nutrition or as a factor coupled to the expression of genes involved in the pathogenesis process. A connection between a nutrition-related function and the induction of virulence genes has been shown for *Listeria monocytogenes* (18, 46), a pathogen that proliferates in the host cell cytoplasm.

However, with regard to the real role played by the *gaiA* product, our studies do not reveal what the exact biological function of the GaiA protein during the infection process could be. In fact, direct proof of the involvement of GaiA in pathogenesis in its natural host is still lacking. Serovar Typhi is extremely sensitive to murine macrophages, and the mutant analyzed in this study showed only a slight survival defect at late infection times in monocyte-derived human macrophages. Additional work focused on analysis of the interaction of the serovar Typhi *gaiA* mutant with other human immune-system cells, such as dendritic cells, or, alternatively, analysis of the capacity of the corresponding serovar Typhimurium mutant to trigger disease in mice might provide further insights into the overall function of this gene during natural infections.

ACKNOWLEDGMENTS

We are particularly grateful to K. N. Timmis for his generous support and encouragement and to Bianka Karge, Astrid Müller, Karina Watzke, and Urte Winckler for outstanding technical help.

Part of this work was supported by a grant from the European Community (EU project "Multicomponent *Salmonella* live vaccines: optimising molecular, cellular and immunological parameters to enhance vaccine safety and immunogenicity," QLK2-CT-1999-00310).

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Editor: D. L. Burns