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The YfgL Lipoprotein Is Essential for Type III Secretion System Expression and Virulence of *Salmonella enterica* Serovar Enteritidis▽†

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***Salmonella enterica*, like many gram-negative pathogens, uses type three secretion systems (TTSS) to infect its hosts. The three TTSS of *Salmonella*, namely, TTSS-1, TTSS-2, and flagella, play a major role in the virulence of this bacterium, allowing it to cross the intestinal barrier and to disseminate systemically. Previous data from our laboratory have demonstrated the involvement of the chromosomal region harboring the *yfgL*, *engA*, and *yfgJ* open reading frames in *S. enterica* serovar Enteritidis virulence. Using microarray analysis and real-time reverse transcription-PCR after growth of bacterial cultures favorable for either TTSS-1 or TTSS-2 expression, we show in this study that the deletion in *S. enterica* serovar Enteritidis of *yfgL*, encoding an outer membrane lipoprotein, led to the transcriptional down-regulation of most *Salmonella* pathogenicity island 1 (SPI-1), SPI-2, and flagellar genes encoding the TTSS structural proteins and effector proteins secreted by these TTSS. In line with these results, the virulence of the Δ*yfgL* mutant was greatly attenuated in mice. Moreover, even if YfgL is involved in the assembly of outer membrane proteins, the regulation of TTSS expression observed was not due to an inability of the Δ*yfgL* mutant to assemble TTSS in its membrane. Indeed, when we forced the transcription of SPI-1 genes by constitutively expressing HilA, the secretion of the TTSS-1 effector protein SipA was restored in the culture supernatant of the mutant. These results highlight the crucial role of the outer membrane lipoprotein YfgL in the expression of all *Salmonella* TTSS and, thus, in the virulence of *Salmonella*. Therefore, this outer membrane protein seems to be a privileged target for fighting *Salmonella*.**

Salmonella enterica infections are an important worldwide health problem. *Salmonella* serovars are responsible for diseases ranging from mild gastroenteritis to life-threatening systemic infections. During the course of infection, these serovars use many virulence factors, among which the type III secretion systems (TTSS) play a major role. TTSS-1, encoded by *Salmonella* pathogenicity island 1 (SPI-1), mainly allows intestinal epithelial cell invasion (57), thereby allowing the bacteria to cross the intestinal barrier. TTSS-2, encoded by SPI-2, is required for intracellular survival and multiplication (54) and, consequently, is important for systemic dissemination of the bacteria. The virulence phenotypes associated with SPI-1 and SPI-2 are dependent on the ability of the TTSS to deliver effector proteins into the host cell cytosol. Thus, bacteria hijack the eukaryotic cellular machinery for their own profit. The flagella, which share a common architectural design with TTSS, are involved in the motility of the bacteria and favor the interaction with the intestinal epithelium (31, 47). However, their role in *Salmonella* virulence remains controversial (26, 27, 47).

We previously characterized a *Salmonella enterica* subsp. *enterica* serovar Enteritidis mutant which was altered in motility and in invasion of Caco-2 enterocytes and HD11 macrophages. In line with these phenotypes, the mutant secreted smaller amounts of SPI-1 and flagellar proteins under conditions favorable for SPI-1 expression. The mutant was also shown to colonize fewer ceca and spleens than the wild type in a model using 1-day-old chicks. These phenotypes were the result of the insertion of an *aphT* kanamycin resistance cassette in the *yfgL* open reading frame (ORF) (3). This insertion could have a polar effect on two downstream ORFs, i.e., *engA* and *yfgJ*. The *yfgJ* ORF has no homology in databanks. *engA* (or *yfgK* or *der*) encodes a GTP-binding protein essential for *Thermotoga maritima*, *Escherichia coli*, and *Salmonella* viability (23, 29). Finally, YfgL was recently shown to be an outer membrane lipoprotein associated in a complex with YaeT and two other lipoproteins, namely, YfiO and NlpB. YfgL enhances the YaeT activity of assembling outer membrane β-barrel proteins in *E. coli* (46, 56). Moreover, YfgL is a member of the σ^E

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TABLE 1. Bacterial strains and plasmids used for this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
1009	*S. enterica* serovar Enteritidis wild-type strain (Nal^r Sm^r); avirulent in mouse model	17
LA5	*S. enterica* serovar Enteritidis wild-type strain (Nal^r); virulent in mouse model	1
LK5*hsdR*	*hsdR* mutant of *S. enterica* serovar Enteritidis LK5 (Sp^r)	18
EE631	*S. enterica* serovar Typhimurium EE251 with a *sipC*::*lacZY* fusion (11-6)	5
1009*sipC*::*lacZY*	1009 with a *sipC*::*lacZY* fusion (11-6)	This work
1009*yfgL*::*aphT*	*aphT* insertion into *yfgL* of strain 1009	3
LA5 *yfgL*::*aphT*	*aphT* insertion into *yfgL* of strain LA5	This work
LA5Δ*yfgL*	LA5 isogenic mutant with the *yfgL* gene deleted	This work
1009Δ*yfgL*	1009 isogenic mutant with the *yfgL* gene deleted	This work
1009*invA*::*aph*Δ*T*	*aph*Δ*T* insertion into *invA* of strain 1009	This work
1009Δ*yfgL invA*::*aph*Δ*T*	*aph*Δ*T* insertion into *invA* of strain 1009Δ*yfgL*	This work
Plasmids		
pKD4	Vector carrying an FRT-Km-FRT cassette (Cb^r Km^r)	14
pKD46	Carries λ-Red γ, β, and *exo* genes under the control of P_{araB}; temperature-sensitive replication (Cb^r)	14
pCP20	Temperature-sensitive replication and expression of FLP recombinase gene (Cb^r)	8
pACYC177	Cloning vector (Cb^r Km^r)	7
pUC4K	Source of *aphT* cassette	Pharmacia
pUC18*aph*Δ*T*	Source of *aph*Δ*T* cassette	53
p*Syfg-eng*	Plasmid pSU2718 carrying *yfgM*, *yfgL*, *engA*, and *yfgJ* ORFs	3
pAC*yfgL*	1.7-kb fragment carrying *yfgL* in plasmid pACYC177	This work
pAC*hilA*	2-kb fragment carrying *hilA* in plasmid pACYC177	This work

regulon, and a lack of its expression leads to a defect in outer membrane protein assembly, which in turn acts as an inducing signal for σ^E (38, 42).

The aim of this study was to better characterize the mechanisms involving the *yfg-eng* chromosomal region in *S. enterica* serovar Enteritidis virulence. We demonstrate that YfgL is crucial for *S. enterica* serovar Enteritidis virulence in mice. This phenotype could be related to the transcriptional down-regulation of genes encoding proteins involved in the biosynthesis of all *Salmonella* type III secretion systems, including the central regulators HilA, SsrB, and FlhC, in a Δ*yfgL* mutant. Moreover, although YfgL is involved in the assembly of outer membrane proteins, we show that the regulation of TTSS expression observed is not due to an inability of the Δ*yfgL* mutant to assemble TTSS in its membrane.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. *S. enterica* serovar Enteritidis 1009 is a streptomycin (Sm; 500 μg ml^{-1})-resistant, nalidixic acid (Nal; 100 μg ml^{-1})-resistant strain derived from a strain isolated from human feces (17). The *S. enterica* serovar Enteritidis LA5 wild-type strain (nalidixic acid resistant [Nal at 20 μg ml^{-1}]) is a field isolate from infected chickens (1). Bacteria were routinely grown in Luria-Bertani (LB) broth with shaking at 150 rpm at 37°C overnight. When required, bacteria were grown in LB medium containing 300 mM NaCl, as described by Arricau et al. (4), or in low-phosphate, low-magnesium medium at pH 5.8 (LPM, pH 5.8), as described by Coombes et al. (10). These culture conditions induce SPI-1 gene transcription (4) and expression of SPI-2 genes (10), respectively. Antibiotics were added to the culture medium when necessary, at the following concentrations: spectinomycin (Sp), 100 μg ml^{-1}; kanamycin (Km), 25 μg ml^{-1}; carbenicillin (Cb), 100 μg ml^{-1}; Nal, 20 or 100 μg ml^{-1}; and Sm, 500 μg ml^{-1}. Mutants and the related wild-type strains have similar growth curves under all culture conditions used in this study (data not shown).

β-Galactosidase assays. A *sipC* transcriptional fusion was introduced into *S. enterica* serovar Enteritidis strain 1009, its *yfgL*::*aphT* isogenic mutant, or this mutant harboring the p*Syfg-eng* plasmid, using P22HT105 transduction (50) of the *sipC-lacZY* (11-6) fusion of strain EE631 (5). β-Galactosidase activity was assayed as described by Miller (35) at the end of the exponential growth phase of bacterial culture in LB broth containing 0.3 M NaCl. Average values (± standard deviations) for activity units were calculated from at least three independent assays in each case.

Setting up the DNA microarray. The microarray contained 4,554 of 4,740 ORFs identified by sequencing of the *S. enterica* serovar Typhimurium LT2 genome (96%) (33). Primers designed to amplify the genome of *S. enterica* serovar Typhimurium LT2 in a two-round amplification strategy were purchased from Sigma Genosys. Genes were amplified using specific primers with HotStar *Taq* DNA polymerase (QIAGEN) in 25-μl mixtures (2.5 ng template DNA, 60 pM of each primer, 1.5 mM $MgCl_2$, a 200 μM concentration of each deoxynucleoside triphosphate, and 5 U HotStar *Taq* polymerase), with an annealing temperature of 60°C. The product size was determined by gel electrophoresis prior to dilution of the products 25-fold for use in the second round of amplification. An aliquot (5 μl) of each diluted product was used in the second reaction, using universal primers (TCCTAGGAGCTCTCTTCT for the forward primer and TGCCTAGGGCTCTTCG for the reverse primer) which annealed to the 19-nucleotide overhang generated in the first round. Amplification occurred in a similar manner in a 100-μl reaction volume at 55°C (cmgm.stanford.edu/pbrown/protocols/index.html). PCR products were resuspended in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 1.5 M betaine (Sigma) at a concentration of 400 ng/μl (16). Negative control genes from *Campylobacter jejuni* (*groEL*, *tpx* thiol peroxidase gene, and *fliE*) were included in the microarray. The resuspended PCR products were spotted onto poly-L-lysine-coated slides (coating was done in-house) in duplicate, using a commercially available robotic arrayer (MicroGrid II; BioRobotic) which generates microarrays with a DNA spot size of 150 μm in diameter. The slides were left to rehydrate overnight before being fixed by snap drying for 1 min at 90°C on a heating block and UV fixation at 650 mJ. The slides were blocked in dichloroethane (Aldrich) and succinic anhydride (Sigma), using 1-methylimidazole (Sigma) as a catalyst (16).

RNA extraction and DNase treatment. Bacteria were cultured under known SPI-1- or SPI-2-inducing conditions (4, 10). RNA extractions were performed immediately after recovery of bacteria. Total bacterial RNA was obtained as

TABLE 2. Primers used in this study

Primer name	Sequence (5′ to 3′)[a]
yfgL-P1	CCATTACCGCAGGTTGAAAACCAGTTTACCCCGACCA CTGTCTGGAGCACGTGTAGGCTGGAGCTGCTTC
yfgL-P2	CAAAACGCCCGTCATCGACATTAATCCAGTGCAGATA ACCTTCGCTAT CGCATATGAATATCCTCCTTAG
yfgL-E	GCGAATTCCAGGAAAACGGCCCCTACACCAGGAGC
yfgL-S	ATCGAGCTCCAATTGCGTAAATTACTTCTGCCAGGG
yfgM8	AAGCCGATATCCAGTTGCAGCAGG
yfgK12	TGCGGGTCAGAAGCTTAAATAGCGTGG
tufA-F	TGTTCCGCAAACTGCTGGACG
tufA-R	ATGGTGCCCGGCTTAGCCAGTA
sipA-F	CCAACGCAATGGCGAGTCAC
sipA-R	GCCGTCTCCGTTTGATGCGT
fliD-F	TCACCACCAAAATTGCCACC
fliD-R	CCTTGTAACGGGCAACGGT
invF-F	TTTGCGAGCAGGCCGTTGTC
invF-R	GCGCCATCGATAAATGCCAGT
hilA-F	GGTTTAATCGTCCGGTCGTAGTG
hilA-R	CCTGATCCTGCATCTGAAAAGG
hilA1-C	GCACTATCGATACCAGGATATACGGCAGCGTCCAT
hilA2-S	GCACTCCCGGGCTAAGCAACCAGATTACGATGATA
sseA-F	TTCACCAAATCCGGGCTA
sseA-R	TCTCGGCCTCCTGGTTAA
ssrB-F	CTTAGTCTACCTGGCATCAATGGC
ssrB-R	CGCTAACAGAACTTGCTGACTACTGC

[a] Restriction sites are underlined.

previously described (20). Possible DNA contamination of isolated total RNA was removed by treatment with DNase I (Invitrogen) according to the manufacturer's instructions. The absence of DNA contamination was checked by PCR amplification, using total RNA as a template and primers specific for the *tufA* gene (Table 2). Measuring the optical density at 230, 260, and 280 nm prior to use indicated the purity and concentration of RNA ($A_{260/280}$ values ranged from 1.8 to 2.0). The quality of the RNA was assessed by gel electrophoresis and ethidium bromide staining.

DNA microarray hybridization and analysis. For indirect labeling, 15 μg of DNase-treated total RNA was converted to fluorescently labeled cDNA as described by J. DeRisi (http://derisilab.ucsf.edu), with modifications. In brief, total RNA was transcribed to cDNA, using Superscript II reverse transcriptase (Invitrogen) in the presence of amino-allyl-dUTP, and then labeled with Cy3 or Cy5 dye (Amersham). Equal amounts of the labeled probes were mixed and resuspended in hybridization buffer (4× SSC, 3× Denhardt's solution, 25 mM HEPES, 0.3% sodium dodecyl sulfate [SDS], and 500 ng ml^{-1} of yeast tRNA). Hybridization was carried out at 63°C for 16 h. The slides were then washed, dried, and scanned with an Axon 4000 B scanner. Replicate hybridization experiments for three (SPI-1 conditions) or four (SPI-2 conditions) independent sets of cultures and RNA extractions were carried out on wild-type and mutant strains.

For the analysis of scanned microarray images, GenePix Pro 4.0 software (Axon Instruments) was used to compute Cy3 and Cy5 fluorescence intensities and local background and to identify inadequate spots. The Cy3 and Cy5 values for each spot were normalized so that the mean of the ratios of medians of all features was equal to one. Genes with an intensity of <2 standard deviations above the local background were considered not detected and excluded from subsequent analysis. Differentially expressed genes were obtained using the statistical software SAM (significance analysis of microarrays) (52). This software is specifically used for microarray data analysis, identifying the most statistically significant differentially expressed genes between two groups of samples. It also estimates the median false discovery rate, which is the percentage of genes falsely reported to show statistically significant differential expression. The microarray data analysis procedures used in this study were fully MIAME (minimum information about a microarray experiment) compliant.

Real-time RT-PCR. Real-time reverse transcription-PCR (RT-PCR) was performed to measure the transcriptional levels of the *sipA*, *fliD*, *invF*, *hilA*, *sseA*, and *ssrB* genes and the housekeeping gene *tufA* (34), using a Light Cycler system and SYBR green I (Roche). One microgram of DNase-treated total RNA from three to five independent cultures and RNA extractions was reverse transcribed to cDNA with avian myeloblastosis virus reverse transcriptase (Promega) using random hexamer primers (Promega) according to the manufacturer's protocol. For all genes of interest, specific primer sequences were designed using the Vector NTI software program (Invitrogen) (Table 2). Amplification was performed using Titanium *Taq* DNA polymerase (Clontech). Optimal PCR conditions were determined for each primer pair. Quantification of cDNA samples was achieved by reference to a standard curve generated from a series of dilutions (10 to 10^6) of the positive control pCR2.1 TOPO (Invitrogen) plasmid containing the amplicon of interest. Following amplification, melting curves were generated to verify PCR product identity.

Construction of *S. enterica* serovar Enteritidis mutants. The λ-Red mutagenesis system (14) was used to construct *yfgL* deletion mutants of *S. enterica* serovar Enteritidis, with a few modifications. In brief, a PCR product containing the Km^r cassette of pKD4 was generated using primers yfgL-P1 and yfgL-P2. The PCR product was then introduced into *S. enterica* serovar Enteritidis strain LK5*hsdR* (18) harboring plasmid pKD46 by electroporation with a Micropulser, as recommended by the manufacturer (Bio-Rad). Km-resistant, Cb-sensitive potential deletion mutants were selected and checked by PCR, using primers outside the deletion site (yfgL-E and yfgL-S) (Table 2). Next, a P22HT105*int* lysate was prepared from one of these mutants to carry the *yfgL*::Km mutation into *S. enterica* serovar Enteritidis strain LA5 or 1009. After being checked by PCR with the same primers as before, plasmid pCP20 was introduced by electroporation into the LA5*yfgL*::Km or 1009*yfgL*::Km mutant. Carbenicillin-resistant transformants were selected at 30°C, after which a few were cultured at 42°C without any antibiotics and tested for the loss of carbenicillin and kanamycin resistance. LA5Δ*yfgL* and 1009Δ*yfgL* mutants are Km^s Cb^s and have the right "scar" sequence (14) and deletion limits.

The 1009*invA*::*aph*ΔT mutant carries a nonpolar mutation in *invA* and was obtained as previously described (53). The 1009Δ*yfgL invA*::*aphΔT* and LA5*yfgL*::*aphT* mutants were obtained by P22HT105*int* transduction of the *invA*::*aph*Δ*T* and *yfgL*::*aphT* mutations into 1009Δ*yfgL* and LA5, respectively.

Construction of plasmids pAC*yfgL* and pAC*hilA*. The chromosomal DNA of strain LA5 was amplified by PCR, using primers yfgM8 and yfgK12 (Table 2), to amplify the *yfgL* ORF. After restriction with the EcoRV and HindIII restriction enzymes, the 1.7-kb PCR product was cloned into the SmaI and HindIII sites of pACYC177 vector, generating pAC*yfgL*. Sequencing of the pAC*yfgL* insert was carried out, and the sequence was shown to be correct.

In a similar way, *hilA* of strain LA5 was amplified by PCR, using primers hilA1-C and hilA2-S (Table 2). The 2-kb PCR product obtained was cloned into the ClaI and SmaI sites of the pACYC177 vector, generating pAC*hilA*. The sequence of the *hilA* gene in the recombinant plasmid was checked.

Invasion assays. *S. enterica* serovar Enteritidis strains were grown overnight in 10 ml LB broth with appropriate antibiotics at 37°C without shaking. The human adenocarcinoma cell line HT-29 (ECACC no. 85061109) (19) was used between passages 27 and 67. Cell monolayers were grown to confluence in 24-well plates as previously described (43). Monolayers were further infected for 2 h at a multiplicity of infection of 10. Entry of *S. enterica* serovar Enteritidis into HT-29 cells was quantified by a gentamicin protection assay, as previously described (3). The percentage of intracellular bacteria was calculated by dividing the number of gentamicin-resistant bacteria by the number of bacteria in the original inoculum and then multiplying the result by 100. Results were then compared by one-way analysis of variance and analyzed by the Student-Newman-Keuls test, using Instat software (GraphPad).

Protein analysis. To analyze secreted and intracellular proteins, bacteria were cultured in LB broth containing 0.3 M NaCl until the optical density at 600 nm reached 1.8, and secreted bacterial proteins were then recovered as previously described by Arricau et al. (4). β-Lactoglobulin (0.5 μg ml^{-1}; Sigma) was added to each culture supernatant as a positive internal control of protein precipitation. Intracellular protein extracts were obtained from bacterial pellets directly resuspended in Laemmli sample buffer. Proteins were separated by electrophoresis in 10% (wt/vol) SDS-polyacrylamide gels (30) and either stained with colloidal Coomassie brilliant blue G-250 (37) or transferred to 0.2-μm nitrocellulose membranes (Protran; Schleicher and Schuell). Proteins were analyzed with a polyclonal rabbit anti-SipA antiserum (1:500) or a polyclonal rabbit anti-H:g,m serum (1:500) as previously described (3). They were revealed with peroxidase–goat anti-rabbit antibody and the Uptilight enhanced chemiluminescence substrate as described by the manufacturer (Interchim).

In order to analyze total membrane proteins, bacteria were grown in LB broth containing 300 mM NaCl or in LPM, pH 5.8, until the optical density at 600 nm reached 1 or 0.4, respectively. Bacterial pellets were washed with 25 mM Tris, pH 7.4, and resuspended in 25 mM Tris, pH 7.4. Cells were kept on ice, and lysates were obtained by sonication. Intact cells and debris were removed by two centrifugation steps (7,000 × *g*, 10 min, 4°C). The supernatants were then subjected to centrifugation at 65,000 × *g* for 10 min at 4°C. The pellets, consisting of the total membrane protein fraction, were resuspended directly in Laemmli buffer. After being boiled at 100°C for 5 min, proteins were subjected to electrophoresis

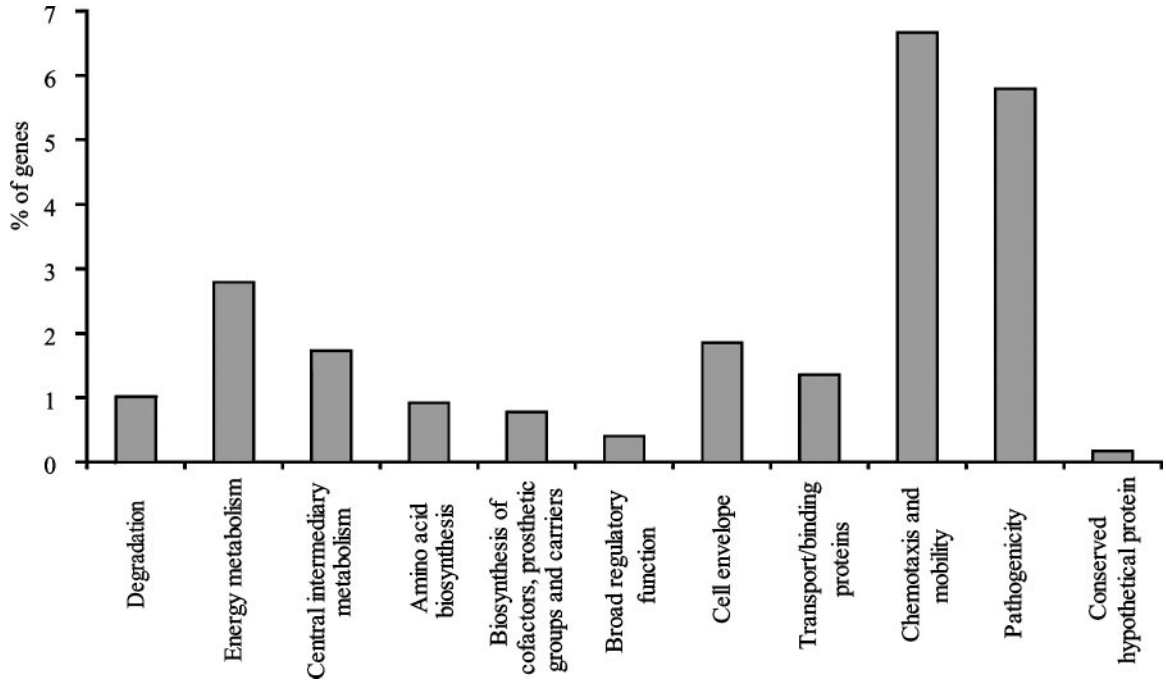

FIG. 1. Functional categories of genes down-regulated in a *yfgL*::*aphT* mutant of *S. enterica* serovar Enteritidis under SPI-1-inducing conditions. Strains were cultured under conditions favorable for SPI-1 gene transcription. After statistical analysis of the microarrays, genes differently expressed in the LA5*yfgL*::*aphT* mutant compared to the wild-type strain were grouped into functional categories based on the *S. enterica* serovar Typhi genome annotation (http://www.sanger.ac.uk/Projects/S_typhi/St_gene_list_hierarchical.shtml) ($P < 0.05$). The histogram shows the percentage of genes affected in each category.

in a 12% (wt/vol) SDS-polyacrylamide gel. Three independent experiments were carried out for each culture condition.

Mouse infection. Six-week-old female BALB/c mice were obtained from the Experimental Unit of the Institut National de la Recherche Agronomique (Nouzilly, France) and maintained in our animal facilities on a diet of mouse chow and water ad libitum. Groups of 10 mice were each inoculated orally with approximately 5×10^8 CFU of *S. enterica* serovar Enteritidis as previously described (40). Spleen colonization was estimated at 6 days postinoculation by plating serial dilutions in phosphate-buffered saline on tryptic soy agar. Results were compared by analysis of variance and analyzed by the Bonferroni-Dunn test. The course of survival was recorded for at least 20 days following inoculation. Results were compared by analysis of variance and analyzed by the Student *t* test. Three independent experiments were carried out. Animal care and handling were conducted in accordance with institutional guidelines.

Antibiotic sensitivity. The sensitivities of the different strains to novobiocin, rifampin, erythromycin, vancomycin, and bacitracin were assessed by a disk diffusion assay using 6-mm filter paper disks (Bio-Rad). A 0.1-ml aliquot of an overnight culture of each strain was seeded into 10 ml of LB broth and cultured for 4 h at 37°C with shaking. This bacterial culture was then poured over an LB agar plate, and disks containing the aforementioned antibiotics were placed on it. Plates were incubated for 20 h at 37°C. The diameter of the growth inhibition zone around each disk was recorded in millimeters.

RESULTS

***sipC* transcription is down-regulated in an *S. enterica* serovar Enteritidis *yfgL*::*aphT* mutant.** A *yfgL*::*aphT* mutant of *S. enterica* serovar Enteritidis strain 1009 (1009*yfgL*::*aphT*) has previously been shown to secrete smaller amounts of invasion effectors and flagellar proteins than its wild-type parent strain (3). Since the genes encoding these proteins have common transcriptional regulatory circuits (24), we analyzed the transcription of the *sipC* ORF, which encodes an effector protein for invasion, using a *sipC-lacZY* fusion. Bacteria were cultured in LB broth containing 0.3 M NaCl until the end of the exponential growth phase. Under these conditions, which are favorable for SPI-1 gene transcription (4), the *S. enterica* serovar Enteritidis wild-type strain 1009 containing the *sipC-lacZY* fusion expressed 3,450 ± 362 Miller units of β-galactosidase, whereas the β-galactosidase level was 12 times lower in the *yfgL*::*aphT* mutant (292 ± 57 Miller units). Introduction of the *yfg-eng* locus (harboring the *yfgM*, *yfgL*, *engA*, and *yfgJ* ORFs) in *trans* partially restored the expression of *sipC* (913 ± 42 Miller units). These results indicate that transcription of the *sipC* gene was strongly affected in the 1009*yfgL*::*aphT* mutant and suggest a role of the *yfg-eng* locus in the transcriptional regulation of SPI-1 gene expression.

SPI-1 and flagellar genes are down-regulated in an *S. enterica* serovar Enteritidis *yfgL*::*aphT* mutant. In order to test whether the transcription of ORFs other than *sipC* could be altered in a *yfgL*::*aphT* mutant, we compared the expression pattern of total RNAs from an *S. enterica* serovar Enteritidis *yfgL*::*aphT* mutant with that of its parent strain under SPI-1-inducing conditions. For this purpose, we decided to use the *S. enterica* serovar Enteritidis LA5 wild-type strain and its isogenic *yfgL*::*aphT* mutant because this strain is lethal in the mouse infection model, unlike the wild-type *S. enterica* serovar Enteritidis 1009 strain previously used (3), which could therefore have one or more virulence defects that could be detrimental in our study (data not shown). Prior to microarray analysis, we demonstrated that the LA5*yfgL*::*aphT* mutant had the same phenotypic characteristics as the 1009*yfgL*::*aphT* mutant, i.e., low invasion and low motility, and also had a similar growth curve to that of its parent at 37°C (data not shown).

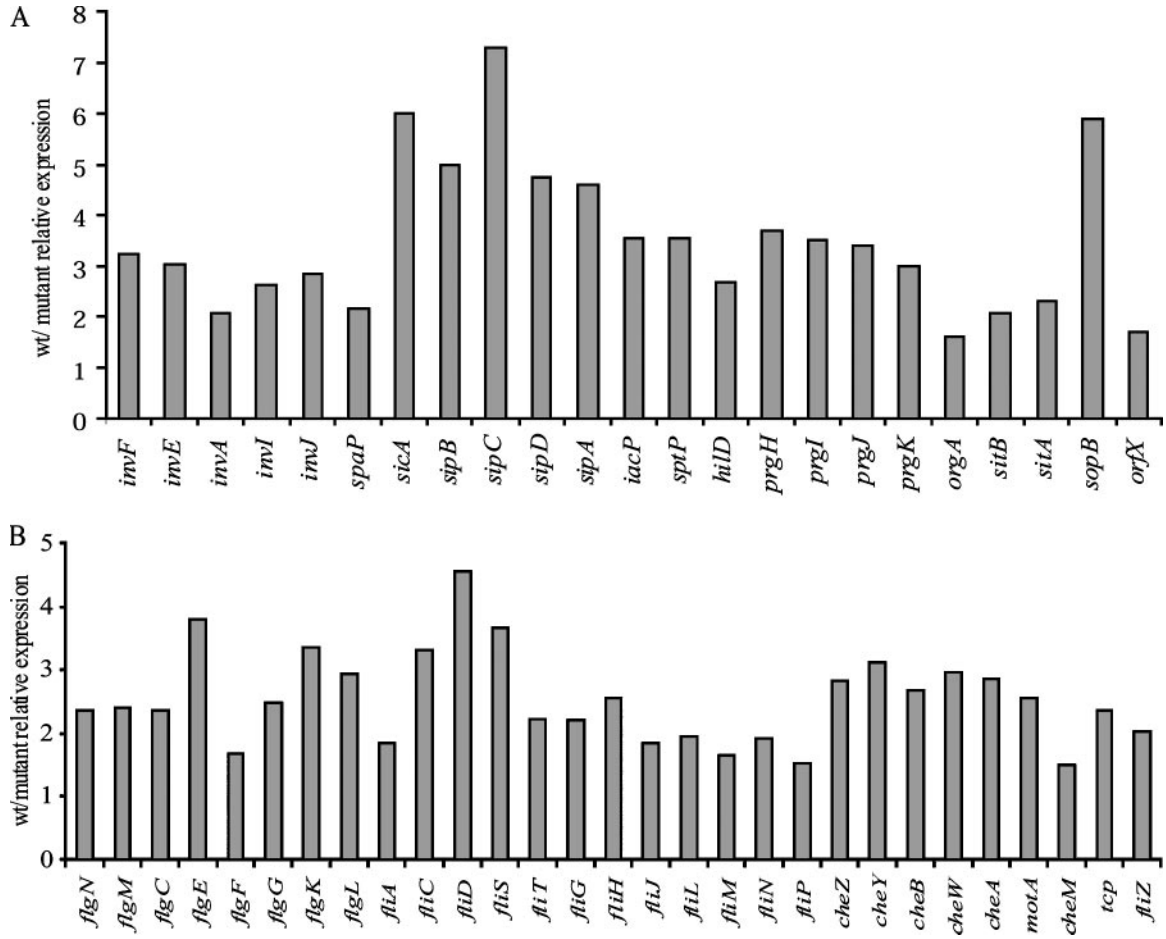

FIG. 2. SPI-1- and flagellum-related genes down-regulated in a *yfgL*::*aphT* mutant of *S. enterica* serovar Enteritidis. SPI1 (A)- and flagellum (B)-related genes which were found to be repressed in the LA5*yfgL*::*aphT* mutant compared to the wild-type strain after culture under conditions favorable for SPI-1 gene transcription are represented. These genes were considered significant when we admitted 11.64% of false-positive genes after statistical analysis. Relative expression corresponds to the ratio of wild-type to mutant gene expression.

Total RNAs of wild-type and mutant strains were extracted, reverse transcribed, and labeled. Equal amounts of the labeled probes from the two strains were mixed and hybridized to the DNA glass microarray. Sixty genes exhibited statistically significantly different expression levels between the wild-type LA5 strain and its isogenic LA5*yfgL*::*aphT* mutant ($P < 0.05$). All of these genes had their transcription down-regulated in the mutant. To obtain an overview of the microarray results, we classified genes according to the *S. enterica* serovar Typhi CT18 genome annotation (http://www.sanger.ac.uk/Projects/S_typhi/St_gene_list_hierarchical.shtml) and then calculated the percentages of differentially regulated genes in selected functional categories (Fig. 1). The complete list of genes identified by microarray analysis can be found in Table S1 in the

TABLE 3. Effect of *yfgL* deletion on transcription of genes involved in invasion, motility, and intracellular survival of *S. enterica* serovar Enteritidis

Strain	Log_{10} cDNA copy no. of target gene (mean ± SEM)[a]					
	sipA[b]	*invF*[b]	*hilA*[b]	*fliD*[b]	*sseA*[c]	*ssrB*[c]
LA5	8.33 ± 0.21	7.86 ± 0.08	6.89 ± 0.08	7.77 ± 0.30	6.41 ± 0.06	7.79 ± 0.24
LA5*yfgL*::*aphT*	7.12 ± 0.10	6.68 ± 0.08	5.95 ± 0.21	6.33 ± 0.22	ND	ND
LA5*yfgL*::*aphT*(pS*yfg-eng*)	7.56 ± 0.17	7.46 ± 0.13	6.26 ± 0.3	7.17 ± 0.27	ND	ND
LA5Δ*yfgL*	6.53 ± 0.22	ND	5.63 ± 0.20	6.37 ± 0.08	5.10 ± 0.25	6.97 ± 0.33
LA5Δ*yfgL*(pAC*yfgL*)	8.04 ± 0.14	ND	6.89 ± 0.11	7.09 ± 0.09	6.03 ± 0.17	7.56 ± 0.29

[a] The mRNA expression of *sipA*, *invF*, *hilA*, *fliD*, *sseA*, and *ssrB* was assessed by real-time RT-PCR. The expression levels of the indicated genes were normalized to the expression level of the housekeeping gene *tufA*. The results correspond to the means for at least three independent RNA extractions quantified in duplicate. ND, not done.

[b] Expression of the gene was analyzed from bacteria cultured in LB containing 0.3 M NaCl, corresponding to conditions allowing SPI-1 gene expression induction.

[c] Expression of the gene was analyzed from bacteria cultured in LPM, pH 5.8, corresponding to conditions allowing SPI-2 gene expression induction.

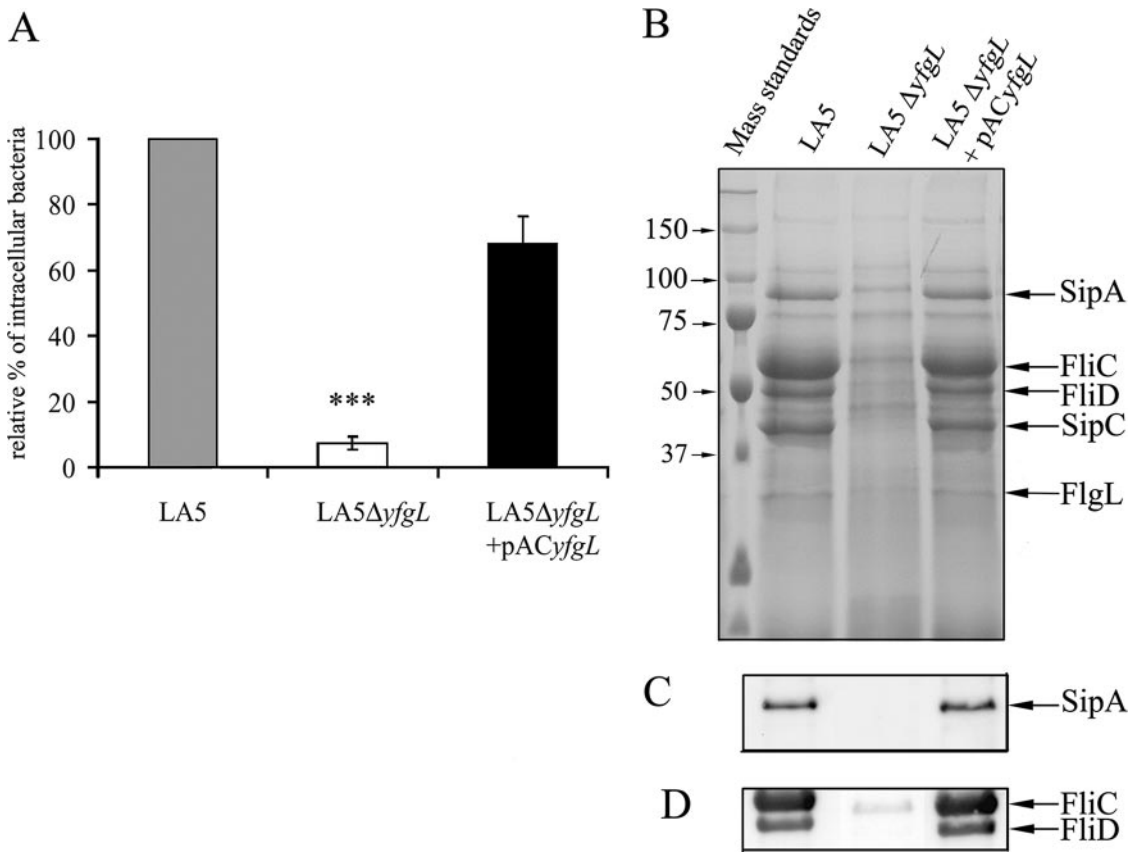

FIG. 3. Effect of *yfgL* deletion on the invasion process of *S. enterica* serovar Enteritidis. (A) The percentage of intracellular bacteria was determined after infection of human HT-29 epithelial cells and gentamicin treatment. Cells were infected with the *S. enterica* serovar Enteritidis wild-type strain LA5, the LA5Δ*yfgL* mutant, or the LA5Δ*yfgL* mutant complemented with pAC*yfgL* for 1 h (multiplicity of infection = 30). Results correspond to the means of three independent experiments carried out in duplicate. Statistical significance was calculated using one-way analysis of variance. Values were compared using the Student-Newman-Keuls test. ***, $P < 0.001$ for LA5 versus Δ*yfgL* mutant and for Δ*yfgL* mutant versus Δ*yfgL* mutant plus pAC*yfgL*. (B and C) Protein secretion by *S. enterica* serovar Enteritidis wild-type strain LA5, the LA5Δ*yfgL* mutant, and the LA5Δ*yfgL* mutant complemented with pAC*yfgL* grown in LB supplemented with 0.3 M NaCl. Proteins from culture supernatants were precipitated with trichloroacetic acid, separated by 10% SDS-PAGE, and stained with Coomassie brilliant blue (B) or transferred to a nitrocellulose membrane and probed with polyclonal antibodies raised against either SipA (C) or H:g,m flagellin (D). Proteins were revealed using peroxidase-labeled goat anti-rabbit antibody and the Uptilight enhanced chemiluminescence substrate.

supplemental material. The most affected functional categories were "pathogenicity" and those related to flagellar biosynthesis, i.e., "chemotaxis and motility" and "cell envelope."

A more detailed analysis revealed that genes down-regulated in the mutant were primarily distributed among the genes connected to the three TTSS of *Salmonella* (32 of 60 genes, corresponding to 53% of the affected genes). We found that 18 of 44 genes on SPI-1 and 12 of 50 genes involved in flagellum biosynthesis and chemotaxis had their transcription down-regulated in the mutant compared to that in the wild-type strain. If we admitted more false-positive genes ($P \leq 0.1164$), then the number of genes affected in the mutant increased to 21 for SPI-1 and 29 for the flagellum. Moreover, two genes carried on SPI-2 were significantly repressed in the mutant compared to the wild-type strain. The ratios of relative expression of wild-type to mutant genes related to TTSS-1 or flagellum biosynthesis are depicted in Fig. 2. Concerning SPI-1, the repressed genes were distributed throughout this pathogenicity island, except for the *mutS* proximal region carrying the *pig* ORFs, whose role in virulence is still unclear (39). The SPI-1 genes down-regulated in the mutant encode secretion apparatus structural proteins, effector proteins secreted by this TTSS, or chaperones of these proteins and proteins of the putative iron transport system SitABCD. Related to the inhibition of these genes, the *hilD* and *invF* genes, encoding transcriptional regulators of SPI-1 genes, were repressed in the mutant strain. We also observed decreased expression of two SPI-5-carried ORFs, namely, *sopB* and *orfX*. SopB is one of the effector proteins translocated in a TTSS-1-dependent manner (12). With regard to the flagellum, the majority of the genes involved in flagellation, motility, and chemotaxis displayed reduced transcriptional expression in the *yfgL*::*aphT* mutant compared to that in the wild-type strain. In *Salmonella* and *E. coli*, the flagellum and chemotaxis systems account for about 50 genes transcribed in at least 17 operons belonging to three temporal classes, namely, class 1, class 2, and class 3 (9). In the LA5*yfgL*::*aphT* mutant, we observed a decreased transcription of genes depending on class 2 and/or class 3 promoters, in particular the genes encoding FliA and FliZ, two essential flagellar regulatory proteins. In addition, even if culture con-

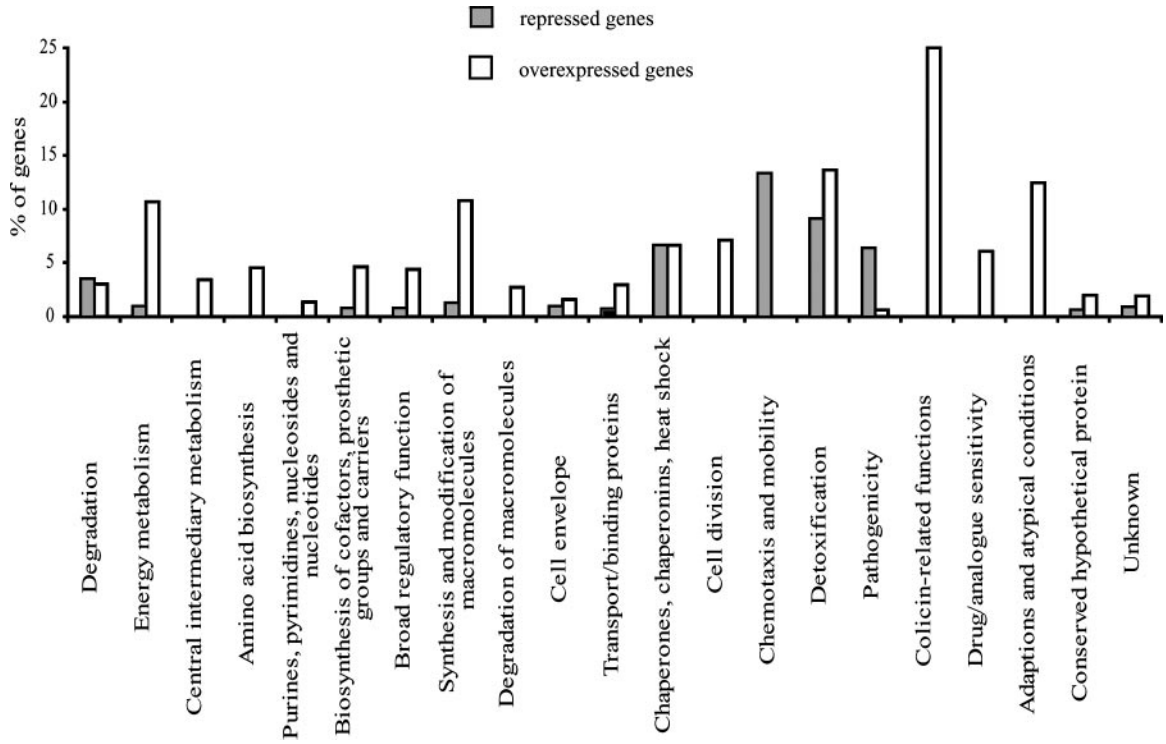

FIG. 4. Functional categories of genes affected by YfgL under SPI-2-inducing conditions. Strains were cultured under conditions favorable for SPI-2 gene transcription (LPM, pH 5.8). After statistical analysis of the microarrays, genes differentially expressed in the LA5Δ*yfgL* mutant compared to the wild-type strain were grouped into functional categories based on the *S. enterica* serovar Typhi genome annotation (http://www.sanger.ac.uk/Projects/S_typhi/St_gene_list_hierarchical.shtml). The histogram shows the percentage of genes affected in each category.

ditions were not favorable for TTSS-2 expression, the transcription of the *sseA* and *sseB* ORFs, encoding effector proteins of this TTSS, was lower in the mutant than in the wild-type strain (6.9-fold and 4.8-fold, respectively). SseA and SseB are essential for the creation of the unique intracellular compartment, called the *Salmonella*-containing vacuole, in which *Salmonella* survives and replicates (6). This result suggests that in addition to SPI-1 and flagellar operons, the transcription of genes carried on SPI-2 could also be down-regulated in the *yfgL*::*aphT* mutant.

Quantitative real-time RT-PCR was used to confirm the differential expression of ORFs. We analyzed ORFs related to TTSS-1 and flagella that had been identified by statistical analysis as differentially regulated. The RT-PCR data confirmed the observed down-regulation of *sipA* (16-fold; SPI-1), *fliD* (27-fold; flagella), and *invF* (15-fold; SPI-1) in the LA5*yfgL*::*aphT* mutant compared to the wild-type strain (Table 3). The decreased expression seen in the mutant was restored by plasmid complementation of LA5*yfgL*::*aphT* with the pS*yfg-eng* plasmid harboring the *yfgM*, *yfgL*, *engA*, and *yfgJ* ORFs (Table 3).

The *hilD* and *invF* genes displayed by microarray analysis

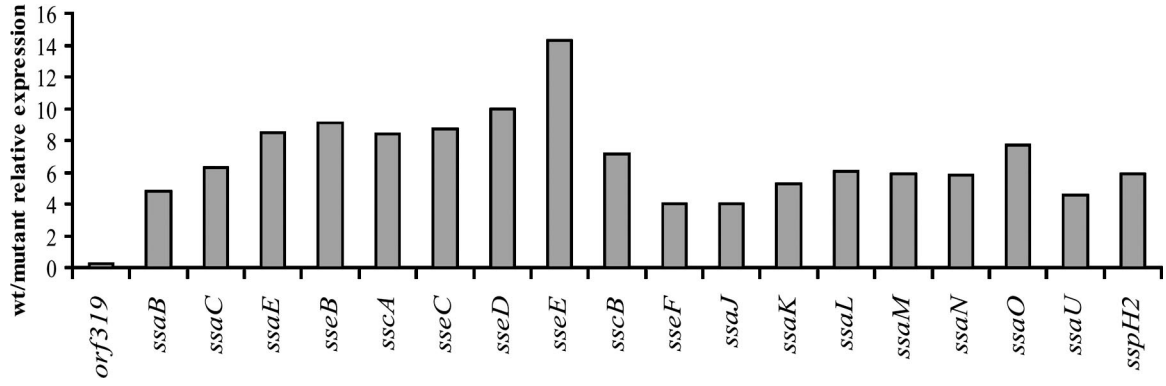

FIG. 5. SPI-2 genes affected by YfgL under SPI-2-inducing conditions. SPI-2 genes which were found to be significantly repressed in the LA5Δ*yfgL* mutant compared to the wild-type strain after culture under conditions favorable for SPI-2 gene transcription (LPM, pH 5.8) are represented. Relative expression corresponds to the ratio of wild-type to mutant gene expression.

reduced transcription in the LA5*yfgL*::*aphT* mutant compared to that in the wild-type strain. Since HilD activates *hilA* transcription, which in turn activates the transcription of the InvF transcriptional regulator (2), it was surprising that reduced transcription of the central regulator HilA was not detected by microarray analysis. To analyze this discrepancy, *hilA* transcription was assessed using real-time RT-PCR with the three RNA preparations used for microarray analysis and with two additional independent RNA extracts, all obtained from cultures grown under SPI-1-favorable conditions. Using this method, and in line with *hilD* and *invF* down-regulation, *hilA* was shown to be expressed approximately 10 times less in the LA5*yfgL*::*aphT* mutant than in the wild-type strain with all RNA preparations tested. This decreased expression was restored in the complemented strain (Table 3). We assume that the absence of *hilA* detection by microarray analysis was most likely due to a problem of spotting of the corresponding PCR product on the slides.

It should also be noted that *spvA*, which is an important virulence gene of *Salmonella* carried on the virulence plasmid, was found by microarray analysis to be significantly repressed in the mutant compared to the wild-type strain. However, we were unable to confirm this result by real-time RT-PCR either after bacterial culture in LB containing 0.3 M NaCl or after culture in LB medium until stationary phase (data not shown), which is known to favor *spv* gene expression (11).

YfgL is responsible for the transcriptional down-regulation of SPI-1 and flagellar genes observed in an *S. enterica* serovar Enteritidis *yfgL*::*aphT* mutant. RT-PCR experiments on RNAs extracted from the *S. enterica* serovar Enteritidis LA5 wild-type strain revealed the existence of *yfgL-engA* and *engA-yfgJ* cotranscripts (data not shown), suggesting a potential polar effect of the *yfgL*::*aphT* mutation on the downstream *engA* and *yfgJ* ORFs. Since Rolhion et al. (44) recently suggested that the YfgL protein plays a role in the virulence of an adherent-invasive *E. coli* strain, we decided to construct a Δ*yfgL* mutant of the LA5 strain (LA5Δ*yfgL*) in order to investigate the role of the *yfgL* ORF in the virulence of *S. enterica* serovar Enteritidis. The capacity of the LA5Δ*yfgL* mutant to invade HT-29 epithelial cells was significantly reduced compared to that of the wild-type strain ($P < 0.001$). The introduction of pAC*yfgL*, carrying only the *yfgL* ORF, allowed the mutant to recover its ability to invade cells (Fig. 3A). This reduced invasive ability of the mutant could be related to its lower secretion of the SPI-1 effector proteins SipA and SipC and the flagellar proteins FliC, FliD, and FlgL than that in the wild-type strain, as shown by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. The introduction of pAC*yfgL* into the LA5Δ*yfgL* mutant fully restored its secretion ability (Fig. 3B). These results were confirmed by Western blotting using sera raised against SipA or H:g,m flagellin (Fig. 3C and D). Finally, we demonstrated that, as previously shown for the LA5*yfgL*::*aphT* mutant, the transcription of the *sipA*, *fliD*, and *hilA* genes was significantly down-regulated in the Δ*yfgL* mutant compared to that in the wild-type strain (63-, 25-, and 18-fold, respectively) and was fully restored after complementation with plasmid pAC*yfgL* (Table 3). Overall, these results show that the Δ*yfgL* mutant has the same phenotypes as the *yfgL*::*aphT* mutant and demonstrate that YfgL is required for optimal expression of TTSS-1 and flagella by *S. enterica* serovar Enteritidis.

Transcription of SPI-2 genes is down-regulated in an *S. enterica* serovar Enteritidis Δ*yfgL* mutant. Since the transcription of the SPI-2 *sseA* and *sseB* genes was shown by microarray analysis to be down-regulated in the LA5*yfgL*::*aphT* mutant under SPI-1-inducing conditions, we performed microarray analyses under SPI-2-inducing conditions (LPM, pH 5.8) (10) to determine whether other *S. enterica* serovar Enteritidis genes, including other SPI-2 genes, could be repressed under these culture conditions. Analysis of RNAs extracted from the LA5 wild-type strain and from its isogenic LA5Δ*yfgL* mutant revealed that 63 genes were significantly repressed in the mutant compared to the wild-type strain, whereas 162 genes had their transcription significantly activated. These genes were classified as described above, according to the functional categories proposed by the *S. enterica* serovar Typhi CT18 genome annotation (Fig. 4). The complete list of genes identified under these culture conditions can be found in Tables S2 and S3 in the supplemental material. Concerning the down-regulated genes in the mutant, most of them belong to stress-related functional categories and, as observed under SPI-1-inducing conditions, to flagellar biosynthesis and pathogenicity categories. Moreover, it should be noted that 23 genes belonging to the "energy metabolism" category were up-regulated in the mutant. These genes encode enzymes mainly involved in glycolysis and the tricarboxylic acid cycle. These results and the fact that 33 genes involved in the synthesis and modification of macromolecules were up-regulated in the mutant suggest that the LA5Δ*yfgL* mutant has to adapt to the acid environment of LPM, pH 5.8, to a greater extent than does the wild-type strain.

Analysis of genes carried on SPI-2 revealed that 18 of 44 genes on this pathogenicity island had their transcription significantly modified in the mutant compared to the wild-type strain (i.e., 29% of the down-regulated genes). Seventeen genes were down-regulated (4- to 14-fold), and one gene, *orf319*, was up-regulated (4.5-fold) (Fig. 5). All of these genes encode proteins related to TTSS-2 (structural proteins, chaperones, and secreted proteins), and none belong to the *ttr* locus involved in tetrathionate respiration. Transcription of *sspH2*, whose product is translocated in a SPI-2-dependent manner, was also repressed in the mutant strain. The lower transcription of SPI-2 genes in the Δ*yfgL* mutant was confirmed by real-time RT-PCR for the *sseA* and *ssrB* ORFs (Table 3). According to this method, the transcription of these genes was down-regulated 20- and 7-fold, respectively, in the mutant compared to the wild-type strain. These genes were selected because they encode, respectively, a TTSS-2 translocated protein and the central transcriptional regulator of the SsrA/SsrB two-component system required for SPI-2 gene transcription. The expression of these genes was restored in the mutant harboring the *yfgL* ORF in *trans*. All of these results demonstrate that YfgL is required for optimal expression of TTSS-2 genes. Moreover, in line with the results presented above, three genes associated with TTSS-1 (*invG*, *spaO*, and *sptP*) and six genes related to flagellum biosynthesis (*flhC*, *fliA*, *fliT*, *fliZ*, *motB*, and *tsr*) were significantly repressed (3.5- to 5-fold) in the mutant strain, even though the SPI-2-inducing culture conditions used were not optimal for their expression.

It should also be noted that nine genes belonging to the σ^E regulon (42), including *rpoE*, encoding this alternative σ factor, were found to be significantly overexpressed in the mutant

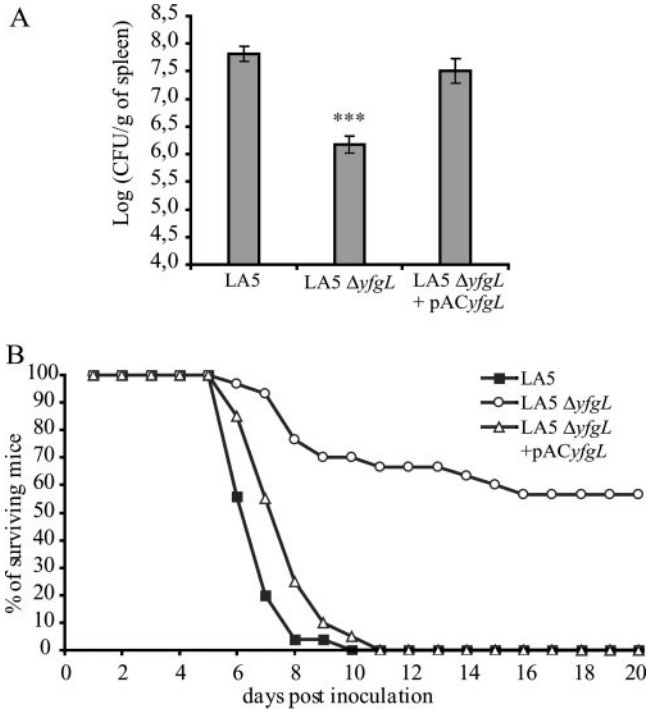

FIG. 6. Effect of *yfgL* deletion on *S. enterica* serovar Enteritidis virulence in mice. Groups of 10 6-week-old BALB/C mice were orally inoculated with about 5×10^8 CFU of the *S. enterica* serovar Enteritidis wild-type strain LA5, the LA5Δ*yfgL* mutant, or the LA5Δ*yfgL* mutant harboring the plasmid pAC*yfgL*. (A) Spleen colonization at 6 days postinoculation. Results were compared by analysis of variance and analyzed by the Bonferroni-Dunn test. ***, $P < 0.001$ for LA5 versus the Δ*yfgL* mutant and for the Δ*yfgL* mutant versus the Δ*yfgL* mutant plus pAC*yfgL*. (B) Kinetics of mouse survival over time. Results were compared by analysis of variance and analyzed by the Student *t* test ($P < 0.05$). Results of three independent experiments are shown for panels A and B.

compared to the wild-type strain under SPI-2-inducing conditions (see the supplemental material). This result confirmed the observation of Onufryk et al. that deletion of *yfgL* in *E. coli* increases σ^E activity (38).

YfgL is required for *S. enterica* serovar Enteritidis virulence in mice. The lower transcription of genes related to the three TTSS in the LA5Δ*yfgL* mutant suggested that YfgL is important for *S. enterica* serovar Enteritidis virulence. To test this hypothesis, BALB/c mice were inoculated by the oral route with the wild-type *S. enterica* serovar Enteritidis strain LA5, the LA5Δ*yfgL* mutant, or the LA5Δ*yfgL* mutant harboring pAC*yfgL*. At 6 days postinoculation, the LA5Δ*yfgL* mutant colonized the spleens of mice 45 times less than the wild type or the complemented strain ($P < 0.001$) (Fig. 6A). Moreover, when we followed mouse survival over time, the Δ*yfgL* mutation clearly attenuated virulence, since 60% of mice survived for at least 20 days postinoculation, whereas all mice inoculated with the LA5 strain died between days 6 and 10 postinoculation (Fig. 6B). The difference observed is significant from day 7 postinoculation until the end of the kinetics ($P < 0.05$). This effect was related only to the *yfgL* deletion, since the introduction of the pAC*yfgL* plasmid fully restored the virulence of the mutant, with all infected mice dying between 6 and 11 days after inoculation (Fig. 6B).

TABLE 4. Effect of *yfgL* deletion on antibiotic sensitivity of *S. enterica* serovar Enteritidis

Strain	Inhibition zone (mm)[a]				
	Van	Bac	Ery	Rif	Nov
LA5	ND	ND	8	11	ND
LA5Δ*yfgL*	13	9	14	21	ND

[a] Sensitivity to vancomycin (Van), bacitracin (Bac), erythromycin (Ery), rifampin (Rif), and novobiocin (Nov) was determined by a disk diffusion assay. Readings were performed after an incubation time of 20 h. ND, no detectable zone of growth inhibition.

YfgL is involved in *S. enterica* serovar Enteritidis outer membrane biogenesis. Recently, YfgL was demonstrated to play a role in membrane permeability in *E. coli* (46, 56). To determine whether YfgL could play a similar role in *S. enterica* serovar Enteritidis, we analyzed the LA5Δ*yfgL* mutant's sensitivity to various antibiotics, using a disk diffusion assay. We observed that the mutant was more sensitive than the wild-type strain to vancomycin, rifampin, bacitracin, and erythromycin but not to novobiocin (Table 4). These compounds need to cross the bacterial membrane to reach their target. Thus, the increased LA5Δ*yfgL* mutant sensitivity to most of these molecules reflects an increase in the membrane permeability of this strain, suggesting similar roles of YfgL in *S. enterica* serovar Enteritidis and *E. coli*.

In *E. coli*, the increase in membrane permeability was related to reduced amounts of β-barrel proteins, such as OmpA and OmpC/F, in the outer membrane of the Δ*yfgL* mutant (38, 46, 56). We thus analyzed the membrane protein profile of the *S. enterica* serovar Enteritidis LA5Δ*yfgL* mutant and its wild-type parental LA5 strain under conditions of TTSS-1 and TTSS-2 expression. We observed a marked reduction of the OmpC/F level and a slight decrease of the OmpA level in the mutant compared to those in the LA5 strain when bacteria were cultured in LB containing 0.3 M NaCl (Fig. 7). Moreover, in agreement with the reduced transcription of the *fliC* gene and the reduced synthesis of the corresponding protein observed above, the FliC protein level was lower in the LA5Δ*yfgL* mutant than in the wild-type strain. In LPM, pH 5.8, reduced OmpC/F and OmpA levels were observed, but to a lesser extent than in LB containing 0.3 M NaCl (Fig. 7). We conclude that, as in *E. coli*, YfgL plays a role in outer membrane biogenesis in *S. enterica* serovar Enteritidis.

A Δ*yfgL* mutant is able to assemble functional TTSS-1 in its membrane when HilA is overexpressed. To determine whether a Δ*yfgL* mutant can assemble functional TTSS-1 in the bacterial membrane, we forced the transcription of SPI-1 genes by introducing a recombinant plasmid expressing the *hilA* gene (pAC*hilA*) constitutively into the Δ*yfgL* mutant of *S. enterica* serovar Enteritidis 1009 or LA5. These strains were able to transcribe *hilA* but also *sipA*, whose transcription is at the end of the HilA transcriptional regulatory cascade (2), at an intermediate level between those of the wild-type strains and their respective mutants (data not shown). We also observed by Western blotting that expression of HilA partially restored the ability of the 1009Δ*yfgL* mutant to synthesize and secrete SipA

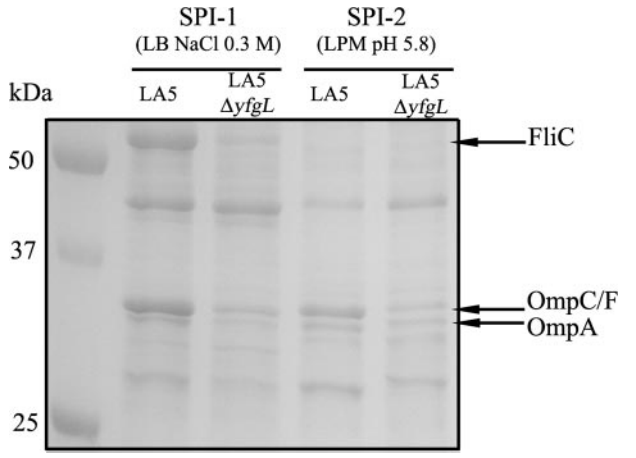

FIG. 7. Effect of *yfgL* deletion on outer membrane protein level of *S. enterica* serovar Enteritidis. Total membrane protein profiles of *S. enterica* serovar Enteritidis wild-type strain LA5 and the LA5Δ*yfgL* mutant grown in LB broth supplemented with 0.3 M NaCl or in LPM at pH 5.8 were analyzed. Membrane proteins were obtained as described in Materials and Methods, analyzed by 12% SDS-PAGE, and stained with Coomassie brilliant blue. The positions of FliC, OmpC/F, and OmpA are indicated.

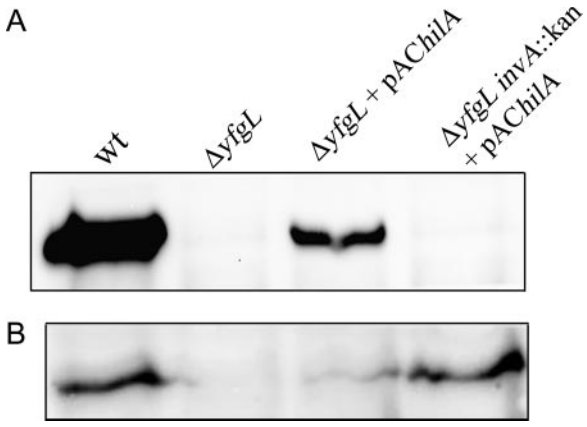

FIG. 8. Effect of *hilA* overexpression in a Δ*yfgL* mutant of *S. enterica* serovar Enteritidis. Protein secretion by *S. enterica* serovar Enteritidis wild-type (WT) strain 1009, the 1009Δ*yfgL* mutant, the 1009Δ*yfgL* mutant complemented with pAC*hilA*, and the Δ*yfgL invA*::*aphT* mutant complemented with pAC*hilA* grown in LB supplemented with 0.3 M NaCl was analyzed. (A) Proteins from culture supernatants were precipitated with trichloroacetic acid. Intracellular proteins (B) were extracted directly with Laemmli sample buffer. Proteins were separated using 10% SDS-PAGE, transferred to a nitrocellulose membrane, probed with polyclonal antibodies raised against SipA, and finally revealed with peroxidase-labeled goat anti-rabbit antibody and the Uptilight enhanced chemiluminescence substrate.

under conditions allowing TTSS-1 expression. Introduction of an *invA* mutation into this strain, which prevents the correct assembly of a functional TTSS-1, abolished SipA secretion, demonstrating that the secretion of SipA observed was TTSS-1 dependent (Fig. 8). Analysis of total bacterial extracts confirmed this result, since an intracytoplasmic accumulation of SipA was observed in the 1009Δ*yfgL invA*::*kan* strain harboring pAC*hilA* (Fig. 8). Similar results were obtained with the LA5 strain, except that secretion of SipA was lower in the mutant harboring pAC*hilA* than that observed for the corresponding strain in the *S. enterica* serovar Enteritidis 1009 background (data not shown). This is probably related to the lower secretion ability of strain LA5 than of strain 1009 under the culture conditions used (data not shown). These results demonstrate that YfgL is not required for TTSS-1 assembly and that the perturbation of the *Salmonella* membrane associated with the *yfgL* mutation does not prevent the assembly of a functional TTSS-1 in a Δ*yfgL* mutant.

Absence of TTSS-1 assembly in *S. enterica* serovar Enteritidis does not lead to feedback control of SPI-1 gene transcription. Even if the Δ*yfgL* mutant was able to "artificially" assemble TTSS-1 in its membrane, one could hypothesize that the absence of assembly of this TTSS in the mutant could lead to feedback control of SPI-1 gene transcription. Sukhan et al. (51) previously showed that transcription of the *prgI* and *prgJ* genes was not reduced in an *invA* mutant of *S. enterica* serovar Typhimurium which is unable to assemble a functional TTSS-1 in its membrane. In order to confirm this result with *S. enterica* serovar Enteritidis, we constructed *invA* mutants of strains 1009 and LA5. As expected, these mutants were unable to secrete SipA (data not shown). In addition, no difference in *sipA* transcription relative to the expression of the housekeeping gene *tufA* was observed using real-time RT-PCR for the *invA* mutants ($\log_{10}$ relative expression, 5.55 ± 0.11) compared to their respective wild-type strains ($\log_{10}$ relative expression, 5.47 ± 0.07). Therefore, the absence of a functional TTSS-1 in the membrane does not lead to reduced transcription of SPI-1 genes in *S. enterica* serovar Enteritidis, indicating that no feedback control occurs.

DISCUSSION

In a previous study, we showed that the *yfg-eng* locus, harboring the *yfgM*, *yfgL*, *engA*, and *yfgJ* ORFs, is involved in cell invasion and motility of *S. enterica* serovar Enteritidis. Moreover, we demonstrated that a *yfgL*::*aphT* mutant of *S. enterica* serovar Enteritidis secreted smaller amounts of SPI-1-encoded effectors and flagellar proteins than did the wild-type strain, although no intracytoplasmic accumulation of these proteins could be observed (3). One hypothesis was that the *yfg-eng* locus could alter the synthesis of these proteins. In the first part of this paper, we demonstrate that the deletion of the *yfgL* ORF results in the transcriptional down-regulation of most (if not all) genes encoding proteins of the three secretion apparatuses and secreted effector proteins of these TTSS in *Salmonella*. In accordance with these results, we show that YfgL is necessary for *S. enterica* serovar Enteritidis virulence in mice.

YfgL is most likely the only protein encoded by the *yfg-eng* locus which is involved in the regulation of TTSS expression and in the virulence of *S. enterica* serovar Enteritidis. Indeed, we identified cotranscripts between *yfgL* and *engA* and between *engA* and *yfgJ*, suggesting a potential polar effect of the *aphT* cassette in the LA5*yfgL*::*aphT* strain. However, we were able to restore the virulence of the LA5*yfgL*::*aphT* mutant in mice by introducing the plasmid pAC*yfgL*, harboring only the *yfgL* ORF (unpublished results). This result and the fact that the LA5Δ*yfgL* mutant presented similar phenotypes to those of the LA5*yfgL*::*aphT* mutant strongly suggest the absence of a role

for *engA* or *yfgJ* in the virulence of *S. enterica* serovar Enteritidis.

Complex transcriptional regulatory circuits are involved in *Salmonella* TTSS expression. All of these circuits include central regulators encoded in the genetic region involved in the biosynthesis of these TTSS (HilA for TTSS-1, SsrB for TTSS-2, and $FlhD_2C_2$ for flagella). Moreover, regulators outside these regions allow tight regulation of TTSS expression depending on the environmental conditions encountered by the bacteria, such as osmolarity, pH, and oxygen tension as well as the Mg^{2+}, Ca^{2+}, or bile concentration (2, 25, 41). Through genome-wide transcriptional profiling and/or real-time RT-PCR, this paper shows that *yfgL* ORF deletion from the *S. enterica* serovar Enteritidis genome leads to the transcriptional down-regulation of genes related to TTSS-1 (21/44 genes, including the *hilA*, *invF*, and *sopB* genes and *inv*/*spa*, *prg*, and *sic*/*sip* operons), flagella (29/50 genes, including middle and late class operons), and TTSS-2 (*sseA* and *sseB* genes) under SPI-1-inducing conditions (Fig. 2; see the supplemental material). This down-regulation of the genes involved in the biosynthesis of the three TTSS of *Salmonella* was also observed after bacterial culture under SPI-2-inducing conditions, except that, as expected, most down-regulated genes were related to TTSS-2 (Fig. 5; see the supplemental material). Moreover, we observed a reduced transcription of two genes (*sopB* and *sspH2*) located outside SPI-1 and SPI-2 but which encode effector proteins secreted by TTSS-1 and TTSS-2, respectively. Interestingly, genes encoding the central regulators HilA, SsrB, and FlhC were shown to be repressed in strains unable to express YfgL. HilA is encoded on SPI-1 and plays a central role in the regulatory cascade controlling the expression of TTSS-1 by *Salmonella* (2). It is a member of the OmpR/ToxR family that activates the SPI-1 *inv*/*spa* and *prg* operons, encoding components of the TTSS-1 secretion apparatus (5, 32). Moreover, HilA indirectly regulates the expression of secreted proteins by activating the transcription of the SPI-1 *invF* gene, encoding an AraC family transcriptional regulator. Indeed, InvF controls the expression of SPI-1 *sip* (also called *ssp*) genes and also that of at least one TTSS-1 secreted effector protein, SopB, encoded on SPI-5 (12, 13, 28). Similar to the central role of HilA in SPI-1 gene transcription, the expression of flagellar operons depends on the master transcriptional regulator $FlhD_2C_2$, which is at the beginning of the regulatory cascade leading to hierarchical activation of all genes required for flagellum biosynthesis. $FlhD_2C_2$ activates the transcription of class 2 operons, including genes whose products are required for basal body and hook completion and also the flagellum-specific σ^{28} factor *fliA* gene. FliA, which was also down-regulated in the mutant strain, is necessary for the transcription of genes depending on class 3 promoters, including the *fliC* flagellin gene (49). Less is known about regulators encoded on SPI-2, with SsrB being the only transcriptional regulator characterized so far. SsrB is part of a two-component regulatory system, SpiR-SsrB, required for the transcription of genes encoding structural components and secreted effector proteins of TTSS-2 (15, 54, 55). Taking all these data into account, our results demonstrate that deletion of YfgL leads to the down-regulation of a regulatory cascade of genes involved in the biosynthesis of the three TTSS identified in *Salmonella.* We assume that all genes related to the TTSS are under this regulation, since the reduced transcription observed in the Δ*yfgL* mutant of *S. enterica* serovar Enteritidis is the consequence of the transcriptional down-regulation of the central regulators HilA, SsrB, and $FlhD_2C_2$. These results explain the reduced motility, invasiveness, and in vivo behavior of the mutant in mice and chicks (this paper and reference 3). As far as we know, YfgL is the first outer membrane protein whose deletion leads to reduced expression of all TTSS.

YfgL is an outer membrane lipoprotein associated in a complex that has recently been shown to be involved in the assembly of outer membrane β-barrel proteins in *E. coli* (56). In this bacterium, the complex is composed of the outer membrane protein YaeT and three lipoproteins—YfiO, YfgL, and NlpB—all of which have been implicated in membrane permeability (38, 56). Our results on the antibiotic sensitivity of the Δ*yfgL* mutant showed that the membrane permeability of the mutant was greater than that of the wild type. Moreover, analysis of the outer membrane proteins of this mutant revealed a reduced amount of β-barrel proteins, confirming the role of YfgL in outer membrane biogenesis in *S. enterica* serovar Enteritidis. Based on these characteristics, three main hypotheses could be proposed to explain the TTSS transcriptional down-regulation and the low virulence of the Δ*yfgL* mutant. The first one is that this down-regulation could be due to the inability of the mutant to assemble these TTSS in an altered membrane. If that were the case, then the decreased transcription observed could result from feedback regulation due to the absence of TTSS assembly. However, we demonstrated that functional TTSS-1 was assembled in the membrane of *S. enterica* serovar Enteritidis in the absence of YfgL when transcription of SPI-1 genes was forced by expressing HilA constitutively (Fig. 8). Moreover, using an *invA* mutant, we confirmed that no transcriptional feedback regulation was observed when TTSS assembly defects occurred in *S. enterica* serovar Enteritidis. These results demonstrate that the YfgL protein is not required for TTSS assembly and that the down-regulation of TTSS expression observed in the Δ*yfgL* mutant is not a direct consequence of TTSS unassembly. The second hypothesis is that the virulence-related phenotypes of the Δ*yfgL* mutant could be a direct consequence of a modification of expression of the σ^E or Cpx envelope stress response due to alteration of the membrane structure. Indeed, the inactivation of one of these regulatory systems reduces the virulence of *S. enterica* serovar Typhimurium (21, 22, 36, 45). However, σ^E is most likely not responsible for the low-virulence phenotype of the Δ*yfgL* mutant, as we observed increased transcription of *rpoE* in this mutant. Concerning the Cpx system, CpxA remains a potential candidate to explain our results. Indeed, CpxA seems to be involved in *Salmonella* invasion and mouse virulence, even if its role remains unclear (21, 36). Finally, one could hypothesize that YfgL, in addition to its already published role with YaeT, YfiO, and NlpB in membrane biogenesis, could be part of a specific "regulatory" cascade leading to the activation of TTSS expression in *S. enterica* serovar Enteritidis. This hypothesis of an additional and specific role for the proteins of the YaeT-YfiO-YfgL-NlpB complex is supported by the fact that NlpB has been shown to down-regulate swarming motility in *Serratia marcescens* (48) and therefore has an opposite effect on motility to that of YfgL in *S. enterica* serovar

Enteritidis. The last two hypotheses are under investigation in our lab.

YfgL plays a key role in the virulence of at least *Salmonella* and *E. coli* (this paper and reference 44). In *E. coli*, this protein has been shown to be important for the invasion of the Int-407 cell line and for type 1 pilus expression by enteroinvasive *E. coli* strains (44). Since the *yfgL* ORF is present in the genomes of many gram-negative pathogenic bacteria, with most of them using TTSS for host infection, it is tempting to suggest that YfgL plays a preponderant role in the virulence of other pathogenic bacteria. In that case, the outer membrane lipoprotein YfgL could be a privileged target for fighting bacterial infections either for the design of new therapeutics or for the development of new vaccine strains.

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