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The Virulence of *Salmonella enterica* Serovar Typhimurium in the Insect Model *Galleria mellonella* Is Impaired by Mutations in RNase E and RNase III

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***Salmonella enterica* serovar Typhimurium is a Gram-negative bacterium able to invade and replicate inside eukaryotic cells. To cope with the host defense mechanisms, the bacterium has to rapidly remodel its transcriptional status. Regulatory RNAs and ribonucleases are the factors that ultimately control the fate of mRNAs and final protein levels in the cell. There is growing evidence of the direct involvement of these factors in bacterial pathogenicity. In this report, we validate the use of a *Galleria mellonela* model in *S.* Typhimurium pathogenicity studies through the parallel analysis of a mutant with a mutation in *hfq*, a well-established *Salmonella* virulence gene. The results obtained with this mutant are similar to the ones reported in a mouse model. Through the use of this insect model, we demonstrate a role for the main endoribonucleases RNase E and RNase III in *Salmonella* virulence. These ribonuclease mutants show an attenuated virulence phenotype, impairment in motility, and reduced proliferation inside the host. Interestingly, the two mutants trigger a distinct immune response in the host, and the two mutations seem to have an impact on distinct bacterial functions.**

Salmonella infections are a serious medical and veterinary problem worldwide. This pathogenic bacterium is able to invade and replicate within eukaryotic host cells. For infection, *Salmonella* relies upon a range of laterally acquired virulence regions, the so-called *Salmonella* pathogenicity islands (SPIs). Of these, SPI-1 and SPI-2 contain genes that encode type III secretion systems (TTSS), which deliver effector proteins into host cells to facilitate either cellular invasion or intracellular survival, respectively (for a review, see reference 1). Hundreds of genes are upregulated during infection and play important roles in adaptation, survival, and proliferation within mammalian cells (2). Transcriptome analysis of *Salmonella enterica* serovar Typhimurium within epithelial cells and macrophages revealed distinct patterns of expression linked to the different stages of infection (3, 4).

Both the evolutionarily close relationship with *Escherichia coli* and the pathogen-specific aspects make *Salmonella* a very good model for studying the influence of RNA determinants in bacterial pathogenicity. In addition to transcriptional control, regulation of RNA decay has emerged as a major pathway in the fast adaptive process of bacteria to changes in the environment. RNAs may also act as regulatory molecules that can directly sense environmental clues and modulate the expression of target RNAs (for a review, see reference 5). The fate of RNA transcripts can be also controlled by proteins, including ribonucleases (RNases) and RNA chaperones.

RNases are enzymes that govern the maturation and degradation of RNA molecules. RNA decay in Gram-negative bacteria usually begins with an endonucleolytic cleavage at one or more internal sites on the RNA molecule. This cleavage is normally performed by RNase E and/or RNase III (6, 7). The single-stranded specific endoribonuclease RNase E is an essential enzyme involved in many aspects of RNA metabolism, including mRNA decay, tRNA processing, rRNA maturation, and small noncoding RNA (sRNA) processing and decay (6–9). The C terminus of the enzyme forms a scaffold for interactions with other proteins, which together form the degradosome, an important RNA degradation complex. RNase III is member of a highly conserved family of double-stranded RNA-specific enzymes with essential roles in RNA processing and decay (7). Bacterial RNase III is primarily known for its roles in rRNA maturation, mRNA degradation, and sRNA processing, turnover, and sRNA-dependent mRNA degradation (6, 10–13). Several genome-wide analyses reported that the absence of RNases E and III in *E. coli* (14) and RNase III in *Bacillus subtilis* (15) or *Staphylococcus aureus* (16, 17) affects the abundance of a high number of mRNAs and sRNAs. Namely, in *Salmonella* and in other bacteria, RNases E and III have roles in the control of a number of sRNAs implicated in the regulation of outer membrane proteins (18–20) and important virulence factors (11, 21, 22).

In the present work, it was our aim to determine the influence of these two main endoribonucleases on the virulence capacity of *S.* Typhimurium. For this, we have used the greater wax moth, *Galleria mellonella*, as a host model. The possibility of addressing many aspects of mammalian innate immunity in invertebrates has expanded their use as models to study human infections (23, 24). The human and insect immune systems demonstrate many similarities (24), with most insect species containing specialized cells, known as hemocytes, that phagocytose bacterial pathogens and

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TABLE 1 List of strains used in this work

Strain	Relevant marker(s) or genotype	Source or reference
E. coli		
DH5α	*recA1 endA1 gyrA96 thi-hsdR17 supE44 relA1* Δ(*lacZYA-argF*)*U169* ϕ80d*lacZ*ΔM15	New England Biolabs
MG1693	*thyA715*	79
S. Typhimurium		
SL1344	Str^r *hisG rpsL xyl*	54
CMA-537	SL1344 *rne-537* (Δ*rne*::Cm^r)	19
CMA-551	SL1344 *rnc-14*::ΔTn*10* (Tc^r)	20
JVS-0255	SL1344 *hfq* (Δ*hfq*::Cm^r)	45
CMA-700	CMA-537 carrying pSVA-8 plasmid	This study
CMA-701	CMA-551 carrying pSVA-7 plasmid	This study
CMA-702	JVO-0255 carrying pSVA-6 plasmid	This study

TABLE 2 List of plasmids used in this work

Plasmid	Description	Origin/marker	Reference
pWSK29	Low-copy-no. plasmid	pSC101/Amp^r	80
pSE420	IPTG-inducible plasmid	Amp^r	Invitrogen
pSVDA-01	pET-15b encoding His-RNase III	Amp^r	20
pSVA-6	pWSK29 expressing Hfq	pWSK29/Amp^r	This study
pSVA-7	pWSK29 expressing RNase III	pWSK29/Amp^r	This study
pSVA-8	pSE-420 expressing RNase E	pSE420/Amp^r	This study

form aggregates, to encapsulate and neutralize foreign microorganisms (25). The hemocyte-mediated response involves the trigger of a phenoloxidase melanization cascade and the synthesis of antimicrobial peptides by the insect's fat bodies (26). These molecules are rapidly released into the hemolymph, where they act synergistically against the microorganisms. A very good correlation between bacterial pathogenicity in *G. mellonela* and mammalian models of infection has been established (27–30), favoring the emergence of larvae of the greater wax moth *G. mellonella* as a reliable insect model host to study pathogenesis of a wide range of Gram-positive/negative bacteria (23, 27, 29, 30) and fungi (31, 32). *Galleria mellonella* combines the advantages of invertebrate host models with several other unique benefits, such as larger size, enabling easy manipulation and injection, low maintenance and breeding costs, status as an ethically acceptable animal model, and growth at 37°C. This is the temperature at which human pathogens are adapted and which is essential for synthesis and release of many pathogenicity factors (32, 33).

There is growing evidence of an effect of ribonucleases on bacterial virulence. We were interested in evaluating the role of two of those enzymes, RNases E and III, in the virulence of *S.* Typhimurium. In parallel we wanted to establish *G. mellonella* as a host model for *S.* Typhimurium pathogenicity studies, as already reported for other bacteria.

MATERIALS AND METHODS

Bacterial strains, insects, and growth conditions. All bacterial strains used in this study are listed in Table 1. All *Salmonella* strains used are isogenic derivatives of the wild-type *Salmonella enterica* serovar Typhimurium strain SL1344. Larvae were reared on their natural food, beeswax, and pollen grains at 25°C in darkness prior to use. Larvae weighing 250 ± 25 mg were used.

Bacteria were grown in Luria-Bertani (LB) broth at 37°C and 220 rpm, unless stated otherwise. SOC medium (Super Optimal Broth with catabolite repression) was used to recover *E. coli* and *Salmonella* transformants before plating. Growth medium was supplemented with the following antibiotics when appropriate: ampicillin (100 μg/ml), chloramphenicol (25 μg/ml), streptomycin (90 μg/ml), and tetracycline (25 μg/ml). For growth of the *E. coli* MG1693 strain, LB medium was supplemented with thymine (50 μg/ml).

For the *Galleria mellonella* infection experiments, cultures grown overnight in LB medium were diluted 1/100 (to an optical density at 600 nm [OD_{600}] of ~0.05) in 5 ml of high-salt LB medium (0.3 M NaCl) and further grown in 15-ml Falcon tubes with a tightly closed lid for 5 h at 37°C with shaking. To compare the growth behaviors of the *Salmonella* wild type and mutants, each diluted culture (OD_{600} of ~0.05) was split into 12 aliquots and further grown, under the aforementioned conditions, for 12 h. Each aliquot was opened only once to measure the OD_{600} every 60 min.

Plasmids. All plasmids used in this study are listed in Table 2. For construction of the pWSK29*hfq* plasmid (pSVA-6) expressing *hfq*, a PCR fragment containing the entire *hfq* sequence was amplified from the SL1344 chromosome, digested with the enzymes HindIII and XbaI, and ligated into plasmid pWSK29 digested with the same enzymes. For the construction of plasmid pWSK29*rnc* (pSVA-7), the pSVDA-01 plasmid expressing His-tagged RNase III (20) was digested with BamHI and XbaI to obtain the *rnc* fragment, which was ligated with pWSK29 digested with the same enzymes. In the case of plasmid pSVA-8 expressing RNase E, a PCR fragment containing the entire *rne* sequence was amplified from SL1344 chromosome and was cloned into the XbaI and EcoRI sites of the pSE420 vector (Invitrogen). Competent *E. coli* DH5α cells (New England BioLabs) were used for cloning procedures during plasmid construction. The selected clones were sequenced (at STAB Vida, Portugal) to confirm the presence of the correct gene sequence and transferred to the respective SL1344 derivative strain. Primers used for gene amplifications are presented in Table S1 in the supplemental material.

***Galleria mellonella* killing assay.** Cultures of *S.* Typhimurium and *E. coli* were grown under the aforementioned conditions for 5 h. The optical density of the cultures was measured, and the appropriate volume was collected to contain all of the strains with the same OD value. Cells were then harvested by centrifugation and resuspended in 10 mM $MgSO_4$ in a series of 10-fold serial dilutions corresponding to the number of CFU per volume of injection. A micrometer was adapted to control the volume of a microsyringe and inject 3.5-μl aliquots of each dilution into *G. mellonella*, via the hindmost left proleg, which had been previously surface sterilized with 70% (vol/vol) ethanol. Control larvae were injected with the same volume of 10 mM $MgSO_4$ to monitor any problem associated with the injection process. Following injection, larvae were placed in glass petri dishes and stored in the dark at 37°C for 4 days. For each condition, we have used 10 larvae and followed its survival and appearance at 24-h intervals. Caterpillars were considered dead when they displayed no movement in response to touch.

CFU count of *S.* Typhimurium. Each bacterial suspension used for worm infection was serially diluted in 10 mM $MgSO_4$ and plated. This was done to verify that all samples injected had similar numbers of cells. To determine intracellular bacterial load, hemolymph was collected from three living larvae at 1, 5, and 16 h after injection by puncturing the larval abdomen with a sterile needle. The outflowing plasma was immediately transferred into a sterile microtube containing a few crystals of phenylthiourea to prevent melanization. The hemolymph collected was serially diluted in 10 mM $MgSO_4$ and plated. In both cases, dilutions were plated in duplicate in LB-agar plates with the respective antibiotics, and CFU were determined after incubation at 37°C for 24 h.

***G. mellonella* RNA extraction.** Briefly, sets of 20 larvae were infected with *S.* Typhimurium at 10^5 CFU/larva as described above. For each time point (1, 5, and 16 h after injection), three living larvae per set were cryopreserved, sliced, and homogenized in 1 ml of TRIzol reagent (Sigma-Aldrich). Whole-animal RNA was extracted according to the manufactur-

er's protocol. After extraction, RNA was treated with Turbo DNase (Ambion, Applied Biosystems). The purified RNA was quantified spectrophotometrically (NanoDrop ND-1000).

Quantitative RT-PCR. The transcriptional levels of genes encoding the *G. mellonella* antimicrobial peptides gallerimycin, galliomycin, inducible metalloproteinase inhibitor (IMPI), and lysozyme were determined with the 7500 real-time PCR (RT-PCR) system (Applied Biosystems), using Power SYBR green master mix (Applied Biosystems), cDNA synthesized from 200 ng of purified RNA with the TaqMan kit (Roche, Applied Biosystems), and specific primers (see Table S1 in the supplemental material) as described previously (34). All samples were analyzed in triplicate, and the amount of mRNA detected was normalized to control actin mRNA values. Relative quantification of gene expression was calculated by using the threshold cycle ($\Delta\Delta C_T$) method (35).

***In vitro* cultivation of hemocytes of *G. mellonella*.** To isolate *G. mellonella* hemocytes, hemolymph was collected from larvae previously anesthetized on ice and surface sterilized with ethanol by puncturing the larval abdomen with a sterile needle (36). The outflowing hemolymph was immediately transferred into a sterile microtube containing anticoagulant buffer (98 mM NaOH, 145 mM NaCl, 17 mM EDTA, 41 mM citric acid [pH 4.5]) in a 1:1 proportion. The hemolymph was centrifuged at 250 × *g* for 10 min at 4°C to pellet hemocytes. The supernatant was taken off, and the pellet was washed twice with 0.9% NaCl and centrifuged at 250 × *g* for 5 min at 4°C. The hemocyte pellet was then suspended gently in 1 ml of Grace insect medium (GIM) (Sigma) supplemented with 10% fetal bovine serum, 1% glutamine, and 1% antibiotic or antimycotic solution (10,000 U penicillin G, 10 mg streptomycin, 25 mg/liter amphotericin B). Suspended hemocytes were counted with a hemocytometer and incubated at 26°C in 24-well plates at a concentration of 2×10^5 cells/ml. Monolayers of primary *Galleria* hemocytes were used for experiments the next day. All preparations and assays were carried out under sterile conditions.

Determination of *in vitro* bacterial load of hemocytes. Cultures of *S.* Typhimurium cells were grown under the above-mentioned conditions for 5 h. The optical density of the cultures was measured, and the appropriate volume was collected to have 4×10^3 bacteria/ml in 0.9% NaCl. *Galleria* hemocyte monolayer medium was replaced with GIM without antibiotics, and then cells were infected with the bacterial suspensions. After 1 h of infection at 37°C, the hemocytes were carefully washed twice with cell culture medium, followed by the addition of GIM containing 100 mg/liter of gentamicin to kill the extracellular bacteria. After 1 h, supernatants were plated to confirm the effectiveness of antibiotic treatment and the medium was replaced with GIM containing 10 mg/liter of gentamicin. The quantification of viable intracellular bacteria was achieved 2, 4, and 20 h after infection. Cell monolayers were lysed with 0.5% Triton X-100, and CFU were determined by plating dilutions of cell lysates on LB-agar plates supplemented with the respective antibiotics followed by incubation at 37°C for 24 h.

Motility assays. Bacterial strains were grown for 5 h under the aforementioned conditions, the optical density of the cultures was measured, and the appropriate volume collected to contain all of the strains with the same OD value. The different strains were inoculated (3 μl) in motility agar plates, incubated at 37°C for the time specified on the respective figure, and photographed. The experiment was repeated more than 3 times with independent cultures. The swimming medium was composed of 10 g/liter tryptone, 5 g/liter NaCl, and 0.3% (wt/vol) agar. The swarming medium was composed of LB supplemented with 0.5% (wt/vol) agar and 0.5% glucose.

RESULTS

Comparison of the growth properties of *S.* Typhimurium wild-type and mutant strains. It was our main aim to evaluate the virulence potential of *Salmonella* mutant strains deficient in the endoribonucleases RNase E and RNase III. RNase E is encoded by the essential gene *rne*, and mutations in the N-terminal catalytic domain are lethal. An RNase E mutant with a deletion of the C-terminal scaffold of the enzyme (*rne-537* mutation) was used (19). A similar mutation in *E. coli* (*rne-131*) was reported to cause a significant mRNA stabilization (37), and the mutant is defective in both the assembly of a functional degradosome and the interaction with the RNA chaperone Hfq (38, 39). This mutation also affects processing and decay of several sRNAs in *E. coli* and *Salmonella* (19). In the case of RNase III, strain CMA-551 (20), which is an SL1344 equivalent of the *rnc-14* mutant of *E. coli* (40), was used. Loss of *Salmonella* RNase III function results in a defect in rRNA processing (41) and has an effect on sRNA and sRNA-dependent mRNA degradation (20).

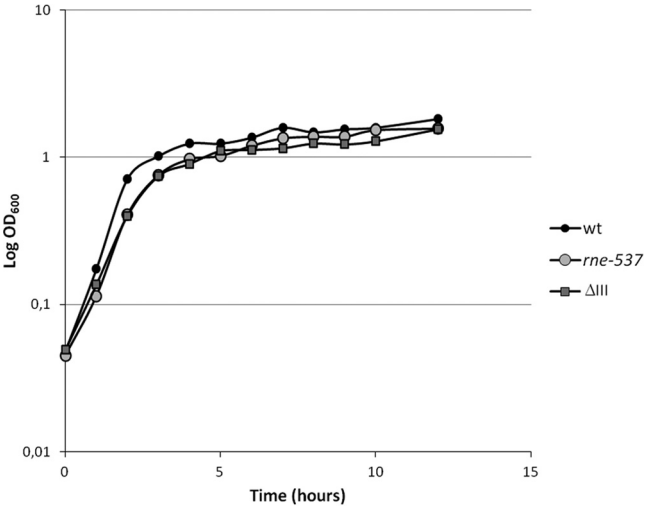

FIG 1 Growth characteristics of *S.* Typhimurium wild-type and mutant strains. To compare the growth behaviors of the *Salmonella* wild-type (wt) and *rne* (*rne-537*) and *rnc* (ΔIII) mutant strains under SPI-1-inducing conditions, the OD_{600} of the cultures was registered every 60 min until 12 h of growth.

The growth characteristics of the *S.* Typhimurium wild-type and mutant strains were analyzed under the experimental conditions used in this study. Invasive *Salmonella* strains control the expression of the proteins that stimulate entry into mammalian cells based upon environmental clues, which include osmolarity, oxygen levels in the growth medium, and the bacterial growth state. To mimic these conditions in the lab, cells were grown under SPI-1-inducing conditions—i.e., high osmolarity and low oxygen (42). The growth of the strains was monitored for a particular period; the results presented in Fig. 1 show that the growth profiles of the strains were similar. The growth of the wild-type strain is faster at the early stage, but after 5 h of growth (the time at which the bacteria were collected for injection), the optical densities of all strains were very similar. In addition, the viable counts of the bacterial samples were determined prior to injection for each survival assay. These numbers were always comparable between the strains.

Mortality of *Salmonella*-infected *G. mellonella* is dose dependent. An aim of this work was to evaluate the pathogenicity level of *S.* Typhimurium to *G. mellonella* larvae and to determine the optimal dosage for virulence studies with this insect model. For this, larvae were injected with 10^8, 10^6, 10^5, 10^4, 10^3, and 10^2 CFU of bacteria/larva and incubated at 37°C, and mortality was monitored daily. As a control, larvae were also injected with the $MgSO_4$ buffer alone. Infection of insects is accompanied by the

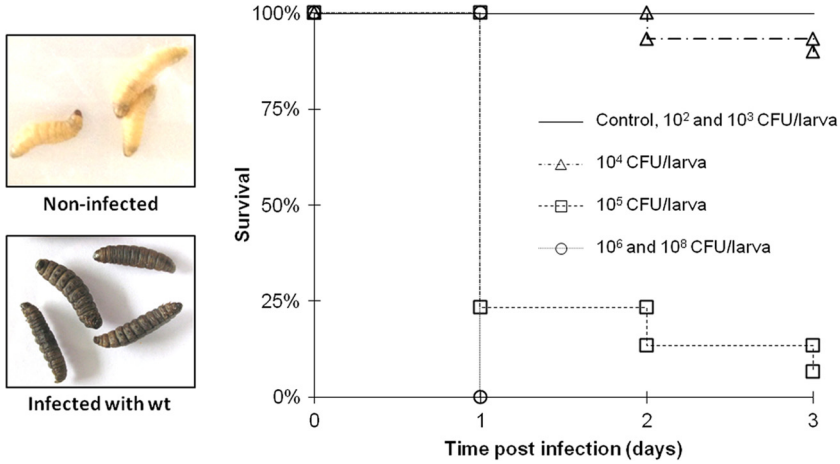

FIG 2 Dose-dependent survival of *Galleria mellonella* after *Salmonella* infection. Larvae were injected with 10^8, 10^6, 10^5, 10^4, 10^3, and 10^2 CFU/larva (CFU contained in 3.5 μl) of the wild-type *S.* Typhimurium strain and incubated at 37°C, and mortality was monitored daily. Kaplan-Meier survival curves were determined from three independent experiments. The control corresponds to the injection of $MgSO_4$ alone. "Non-infected" corresponds to the larvae injected with the $MgSO_4$ alone (control), and "Infected with wt" corresponds to the inoculation with 10^5 CFU/larva.

generation of melanin, which becomes deposited around pathogens (43), and as a consequence, infected *G. mellonella* larvae change from their normal cream color to a dark brown. As represented in Fig. 2, infection with *S.* Typhimurium causes a strong melanization of the larvae in comparison with the noninfected ones. At doses of *S.* Typhimurium higher than 10^5 CFU/larva, all wax moth larvae were killed after 24 h. At doses lower than 10^5, the mortality was null or near zero. Since the dose of 10^5 CFU/larva induces an intermediate level of virulence, it should allow us to discern the differences in the virulence potentials of the different strains and was chosen as the inoculation dose for the subsequent assays in this study. No deaths were recorded when larvae were injected with 10^5 CFU/larva of the nonpathogenic *E. coli* K-12 derivative strain MG1693.

Correlation between *Galleria mellonella* and mouse models of *Salmonella* infection. To establish *G. mellonella* as a host model for *Salmonella* infection, the effect of the deletion of a gene known to have a marked influence on *Salmonella* virulence was tested. Hfq acts as a pleiotropic regulator of *Salmonella* gene expression, controlling the expression of nearly one-fifth of its genes (44). Deletion of the *hfq* gene attenuates the ability of *S.* Typhymurium to infect mice, to invade epithelial cells, to secrete virulence factors, and to survive inside cultured macrophages (45). Therefore, the *hfq* mutant strain was included in the present study to confirm the reduced virulence phenotype of this mutant in our model.

The larvae of the wax great moth *G. mellonella* were inoculated with 10^5 CFU/larva of wild-type and *hfq* mutant strains and incubated at 37°C, and mortality was monitored daily. The effects of infection by the wild-type *Salmonella* strain were rapidly seen after the first 24 h of infection, with strong melanization of the caterpillars (Fig. 3A) and a reduction of survival to about 20% of the initial number of larvae (Fig. 3B). After 48 h of infection, all of the wild-type-infected larvae were dead. In contrast, the larvae injected with the *hfq* mutant show very weak signs of melanization, and after 4 days of infection, the survival of *G. mellonella* injected with this mutant was over 95% (Fig. 3B). Complementation of the mutant with the *hfq* gene in *trans* restores the wild-type phenotype (Fig. 3). These results confirm the prominent virulence attenuation phenotype seen before with this mutant in a murine model (45). The agreement of these observations with the previous data validates *G. mellonella* as a good host model of infection for *S.* Typhimurium pathogenicity studies. The *hfq* mutant was used henceforth as a positive control in our assays.

Mutations in endoribonucleases RNase E and RNase III reduce *Salmonella* virulence capacity. In order to analyze the influence of endoribonucleases RNase E and RNase III on *S.* Typhimurium virulence in the host model *Galleria mellonella*, larvae were injected with 10^5 CFU/larva (Fig. 2) of the wild-type, RNase E (*rne-537*) and RNase III (ΔIII) mutant strains, incubated at 37°C, and their mortality was monitored daily. The larvae infected with the mutant strains show modest signs of melanization, and more than 90% remained alive after 24 h of injection (Fig. 3). The survival of the larvae was monitored for 4 days after inoculation, and in the end, there were survival rates of about 60 and 70% for larvae injected with the RNase III and RNase E mutants, respectively. To corroborate that the endoribonuclease mutations were the main cause of the attenuated virulence phenotype of the mutants, it was tested whether it could be complemented by the expression of the respective genes in *trans*. A strong melanization of both complemented strains is evident after 24 h of infection (Fig. 3A), and there was a marked reduction of host survival to levels comparable to those observed with the wild-type strain (Fig. 3B).

The bacterial load of endoribonuclease mutants remains constant during *G. mellonella* colonization. In order to evaluate the proliferation of *S.* Typhimurium within the insect hemocoel, the viable bacterial load within the hemolymph of larvae was determined at three distinct time points in the course of infection. A total of 10^5 CFU/larva of the *S.* Typhimurium wild-type and RNase E, RNase III, and Hfq mutant strains were used to promote infection, and at the time points indicated in Fig. 4A, hemolymph from three living larvae was collected and pooled, and the number of CFU was determined. All three mutants were able to persist in larval hemolymph to the point of 16 h postinfection; however, they did not show signs of proliferation. On the contrary, infection of larvae with the wild-type strain resulted in a 100-fold increase of

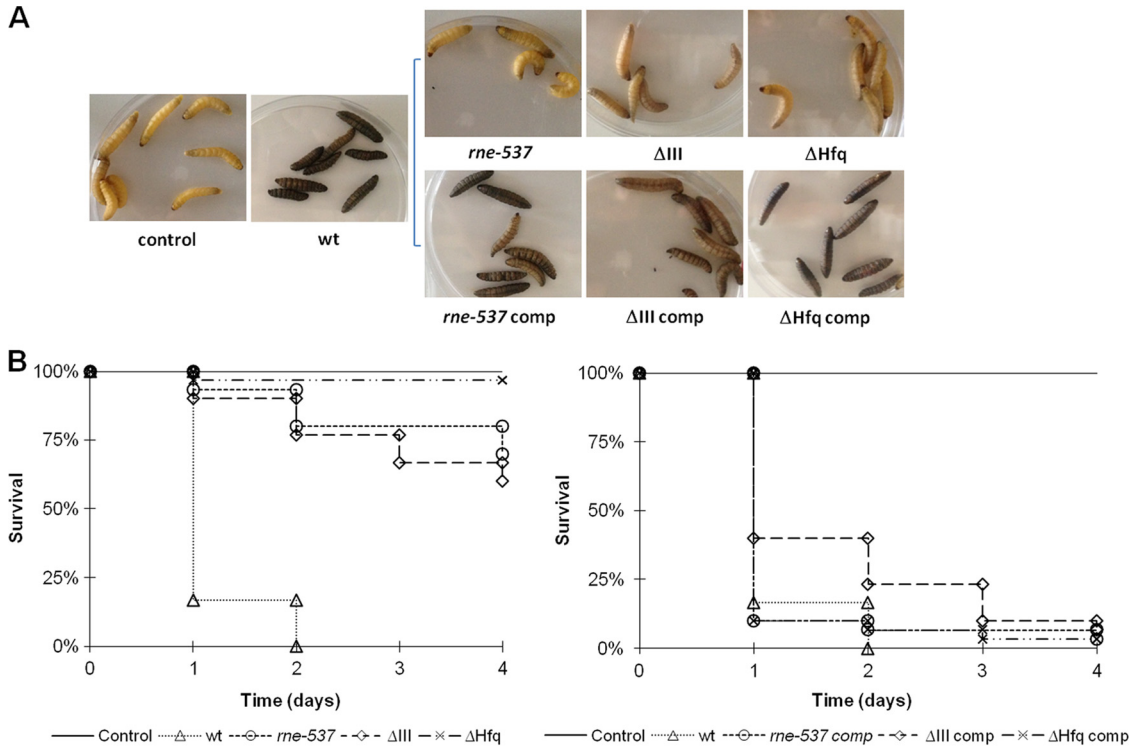

FIG 3 Effects of *Galleria mellonella* infection by different *Salmonella* strains. Survival of larvae injected with 10^5 CFU/larvae of *S.* Typhimurium wild-type, RNase E (*rne-537*), RNase III (ΔIII), and Hfq (ΔHfq) mutant strains. The control corresponds to the injection of $MgSO_4$ alone. (A) Larvae after 24 h of infection. (B) Kaplan-Meier survival curves of larvae after *Salmonella* infection. The results represent three independent experiments.

CFU for the same period of time, indicating that *S.* Typhimurium is able to replicate inside *G. mellonella*. These results are in accordance with the results of the survival of infected larvae (Fig. 3B), since at 48 h postinfection all infected insects were killed by the wild-type strain but infection with the mutants led to the insect's death in only 10 to 25%.

***In vitro* infection of hemocytes.** To further analyze the host-pathogen interaction in this insect model, hemocytes were extracted from *G. mellonella* hemolymph and used for *in vitro* cultures. The cultured hemocytes were infected with the *S.* Typhimurium wild type and the endonuclease mutant strains at a multiplicity of infection (MOI) of 50:1 (bacteria to hemocytes). The bacterial load of hemocytes was evaluated after 2, 4, and 20 h. The Hfq mutant strain was analyzed under the same conditions and functioned as a control. To evaluate the effectiveness of gentamicin treatment, we plated the supernatant before lysis, and no significant CFU were obtained.

At 2 h postinfection, there were ~5-fold fewer intracellular bacteria in hemocytes infected with the mutant strains compared with the wild type, likely reflecting the reduced invasion rate of these strains (Fig. 4B).

Within the next 2 h of infection, the number of wild-type bacteria increased 5-fold compared to the previous time point, whereas the number of mutant bacteria remained almost unchanged. This suggests an intracellular growth defect in addition to an invasion defect. At 20 h postinfection, the number of wild-type bacteria reached nearly 100% of the input of infection. Despite the initial reduction of cellular uptake of the mutants compared to the wild type, the increase in intracellular CFU of the RNase III mutant was comparable. In contrast, slower intracellular replication was noticed with the RNase E mutant (Fig. 4B). The defect in invasion and intracellular replication of the Hfq mutant, previously observed for epithelial and macrophage cell lines (45), was confirmed in the present study. The same assays were performed with mutants defective in SPI-1 and SPI-2 expression (see Fig. S2 in the supplemental material). The SPI mutant showed a strong invasion defect. In the SPI-2-defective strain, the cellular uptake was lower than that for the wild type and the intracellular replication was also defective. However, analysis of survival of the larvae with both strains showed only a modest attenuation of virulence for the SPI-1-defective mutant (see Fig. S1 in the supplemental material).

Mutations in endoribonucleases E and III trigger a different immune response by *G. mellonella*. Antimicrobial peptides play a crucial role in insect innate immunity against invading pathogens. In fact, *G. mellonella* comprises a remarkable antimicrobial peptide arsenal that is released into the hemolymph, where it attacks elements of the bacterial or fungal cell wall (46). Therefore, it is expected that antimicrobial peptides are induced in *Galleria* upon *S.* Typhimurium infection. To check this, larvae were infected with this bacterium and the gene expression of four selected antimicrobial peptides was monitored 1, 5, and 16 h postinfection. The selected genes coded for the cysteine-rich antifungal peptide gallerimycin (47), a defensin called galliomycin (48), lysozyme, and an inducible metalloproteinase inhibitor (IMPI) (47). Gene expression was determined by quantitative RT-PCR analysis of the total RNA extracted for each postinfection time point. The results showed that infection with the wild-type *S.* Typhimurium

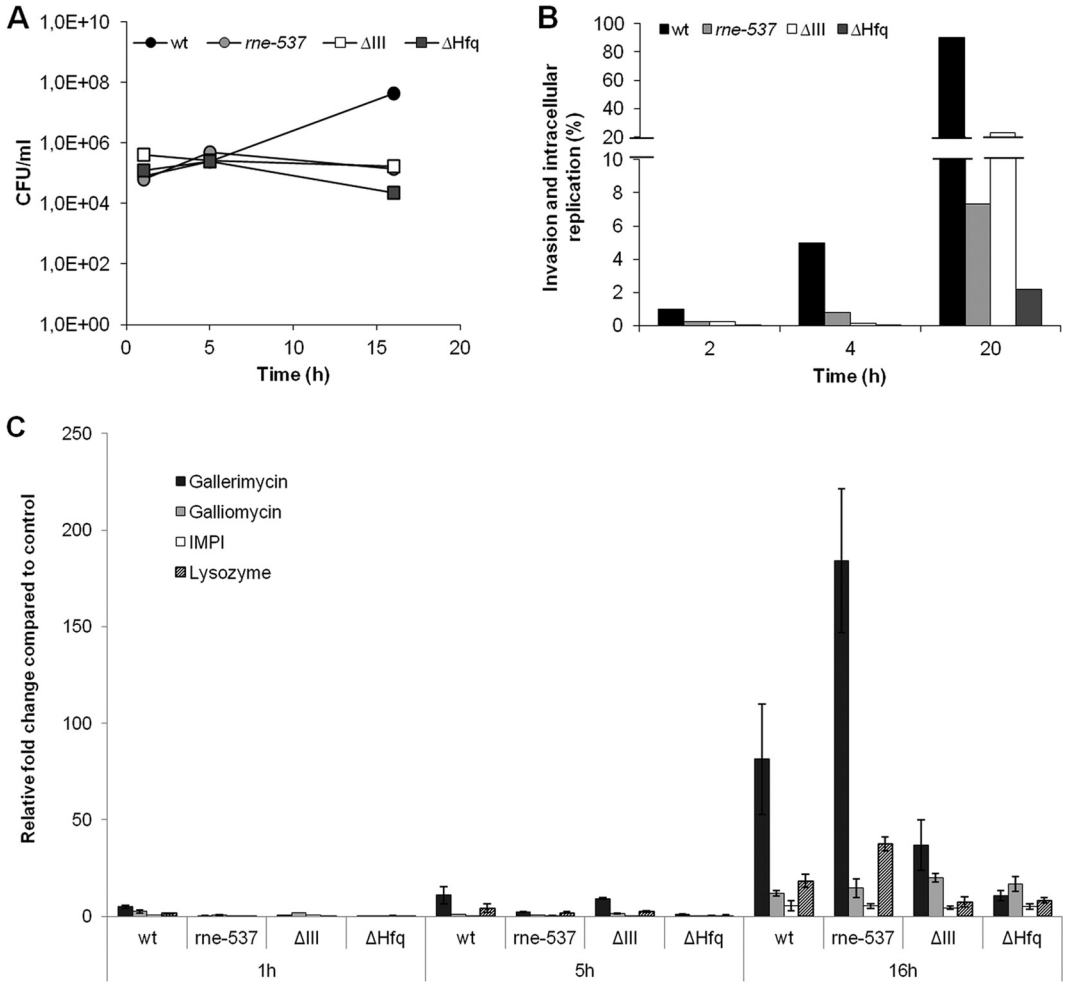

FIG 4 Repercussion of *Salmonella* infection inside the *G. mellonella* model. (A) The viable bacterial load was determined in the hemolymph of larvae infected with 10^5 CFU/larva of the *S.* Typhimurium wild-type strain or RNase E (*rne-537*), RNase III, and Hfq mutant strains, during a period within the time course of infection. (B) *In vitro* infection of hemocytes with *S.* Typhimurium wild-type and mutant strains (MOI, 50:1). The results are shown as percentages of the bacterial input. Experiments were repeated at least three times, and one representative experiment is shown. (C) Transcriptional activation of immune-responsive genes of *G. mellonella* at 1, 5, and 16 h postinfection with 10^5 CFU/larva of *S.* Typhimurium wild-type, RNase E (*rne-537*), RNase III (ΔIII), and Hfq (ΔHfq) mutant strains. The transcriptional levels of gallerimycin, galliomycin, IMPI, and lysozyme were determined by quantitative RT-PCR analysis and are shown relative to the expression levels in noninfected larvae injected with 10 mM $MgSO_4$. Results were normalized to the expression of the housekeeping actin gene.

strain lead to upregulation of the immune-related peptides tested compared to the case in control larvae injected with 10 mM $MgSO_4$ (Fig. 4C), and the induction is more pronounced at 16 h after injection. This result is in agreement with the 100-fold increase in bacterial load observed in the hemolymph of insects infected with wild-type *Salmonella* for the same length of infection.

To analyze the possible effect of the endoribonucleases RNase E and RNase III in the immune response of the larvae against *S.* Typhimurium infection, the same experiment was carried out using the RNase E, RNase III, and Hfq (as a control) mutant strains. At 16 h postinfection, all of the mutants exhibited increased expression levels of the four immune-related peptides analyzed, compared to the uninfected larvae (Fig. 4C). Galliomycin and IMPI levels are identical for the wild-type and mutant strains. In contrast, the expression levels of gallerimycin and lysozyme in the infections with RNase III and Hfq mutants were lower than with the wild-type strain. Surprisingly, infection with the RNase E mutant lead to a stronger increase in the expression levels of these two peptides (more of gallerimycin) than infection with the wild-type strain.

RNase mutants are less motile. The ability to move is a main advantage of bacteria, namely, to obtain nutrients, disperse more effectively and avoid unfavorable environments. Not surprisingly, motility constitutes a virulence factor for many pathogenic bacteria, namely, *Salmonella* (49–51). Two types of bacterial movement were evaluated in the *Salmonella* mutants studied. Swimming represents the individual movement of cells in aqueous or semisolid environments and is dependent on flagella (52). Swarming consists of the mechanism of propagation of a group of cells in a coordinated form on semisolid surfaces, which is dependent both on the flagella and on the production of specific extracellular compounds that reduce the superficial tension and allow the organism's motility (52). The medium's requirements for swarming

motility depend on the organism being considered. *Salmonella enterica* specifically requires the presence of glucose in an energy-rich semisolid medium to swarm, and swarmer cells become longer and hyperflagellated (53).

The results obtained show that both *rne* and *rnc* genes are important for both types of motility. The respective mutant strains present a reduced swimming and swarming motility (Fig. 5). The wild-type cells are motile in swimming plates, forming concentric motility rings around the point of inoculation that can be observed soon after 8 h of incubation at 37°C (Fig. 5A, upper panel). After 24 h of incubation, the wild-type cells have already covered all the plate (Fig. 5A, lower panel). Both RNase E and RNase III mutants displayed an impaired motility, by forming a barely detectable motility ring after 8 h of incubation that remained much smaller than the one formed by the wild type after 24 h of incubation. In the RNase III mutant, swarming is less affected than in the RNase E mutant. Complementation of the strains with the respective gene expressed from a plasmid restored the motility capabilities and characteristics of the wild-type strain.

It had been seen before that *Salmonella* Δ*hfq* cells are nonmotile in motility agar plates (45). Under the experimental conditions used, the Δ*hfq* cells showed a very limited motility on swimming and swarming plates that was fully restored in the complemented strain.

DISCUSSION

Salmonella is a facultative intracellular pathogen that can survive the host immune response and replicate inside host cells, most prominently in phagocytic cells, such as macrophages. The ease of genetic manipulation coupled with a detailed understanding of core metabolism has made *S.* Typhimurium an excellent model for studying host-pathogen interactions and intracellular survival.

Adequate infection models that approximate human disease are the key for the analysis of the molecular basis of bacterial pathogenesis. The possibility of addressing many aspects of mammalian innate immunity in invertebrates has expanded their use as models to study human infections (23, 24). One of the aims of this study was to examine the effectiveness of the larvae of the wax moth *G. mellonella* as a model of infection for the *S.* Typhimurium strain SL1344 (isolate virulent in mice) (54). There is only one very old report of the use of *Galleria* in a *Salmonella* study performed with the LT2 strain (not virulent in rats) (55). Therefore, we found it important to validate the use of this system with *Salmonella* model strain SL1344.

For this, a gene well known as being important for *Salmonella* virulence (*hfq*) was also included in our studies. The ubiquitous RNA-binding protein Hfq has been shown to be required for the fitness and virulence of an increasing number of bacterial pathogens (56–59). Transcriptome analysis suggests that Hfq regulates the expression of nearly a fifth of all *Salmonella* genes (44), and deletion of the *hfq* gene was shown to attenuate the ability of *S.* Typhimurium to infect mice, to invade epithelial cells, to secrete virulence factors, and to survive inside cultured macrophages. The present work confirms the results obtained before with the Hfq mutant, in a murine model, namely, the attenuated virulence phenotype and reduced proliferation inside the host cells. These results validate *G. mellonella* as a model for the identification of virulence genes.

These results enabled us to use this insect model to analyze the impact of two main endoribonuclease activities on *S.* Typhimu-

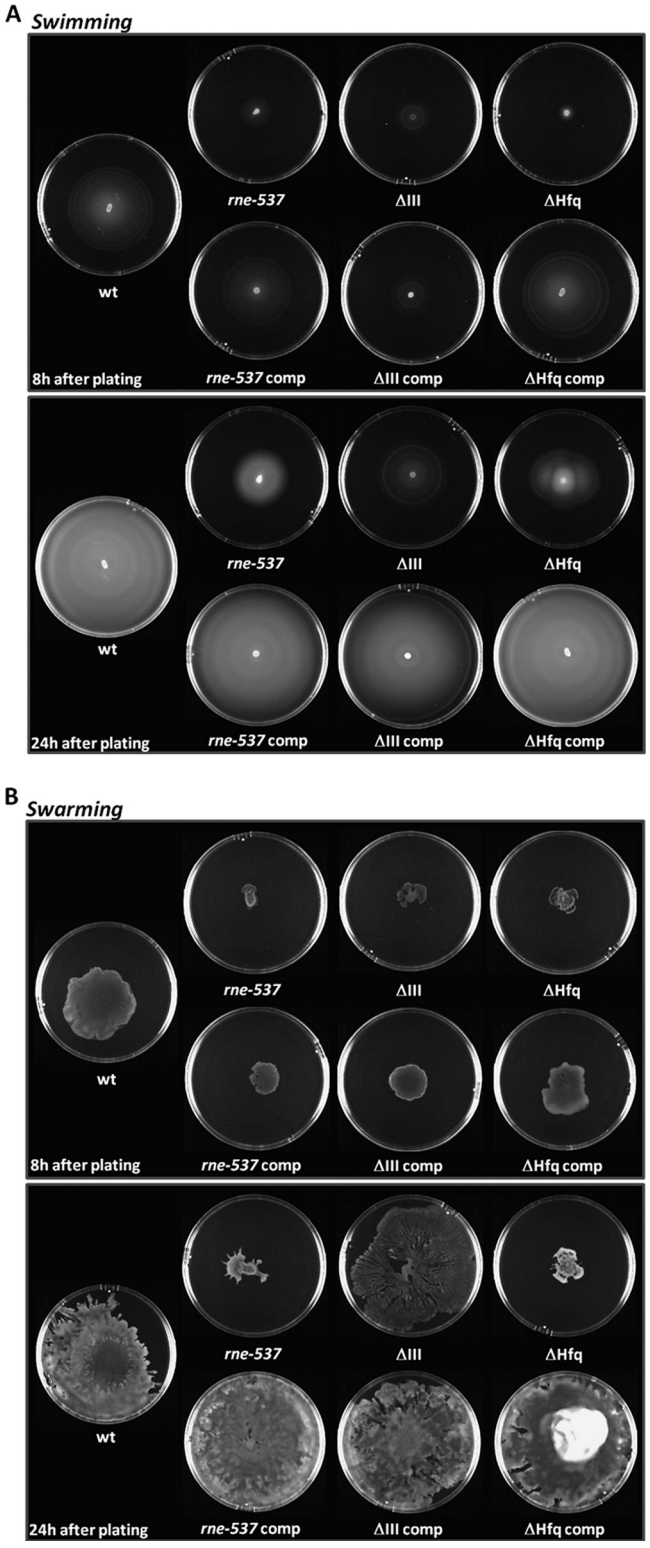

FIG 5 RNase E and RNase III mutant strains are impaired in motility. To measure motility, equal numbers of bacteria from each strain were inoculated onto swimming (A) and swarming (B) agar plates. The plates were incubated at 37°C for the time indicated on each figure and photographed.

rium SL1344 pathogenesis. There are already reports about the influence of ribonucleases, the key players in the RNA decay process, in the virulence of different pathogens (see reference 60 for a review). The influence of endoribonucleases RNase E and RNase III in *Salmonella* infection was investigated for the first time in this work, using *G. mellonella* as a host infection model. The level of infection of *G. mellonella* by the SL1344 wild-type strain is shown to be dose dependent and causes a high percentage of mortality, soon after 24 h of infection. The infection of insect larvae with RNase E and RNase III mutants is highly attenuated. Estimation of the proliferation of *Salmonella* strains inside the host hemolymph showed that wild-type bacteria persisted and replicated within the larvae, while the mutants did not replicate, despite remaining viable up to 16 h of infection. The assays with hemocyte cell lines also revealed that the endoribonuclease mutants are defective for invasion of the phagocytic cells compared to the wild-type bacteria.

Analysis of antimicrobial host immune response revealed that *Salmonella* infection of *G. mellonella* resulted in upregulation of the expression of the antimicrobial peptides tested compared to the uninfected control larvae. Interestingly, in studies of the pathogenic activity of *Listeria* (61), *Legionella pneumophila* (62), and *Burkholderia cenocepacia* (34) in *Galleria mellonella*, gallerimycin was also highly expressed in response to the infection by each of these bacteria, indicating a prominent role in antibacterial defense. These results show that *G. mellonella* develops an immune response to *Salmonella* that nonetheless is not effective in clearing wild-type bacteria, as shown by the proliferation of the bacteria inside the host and the high mortality rates. In comparison, the response of the host in terms of gallerimycin and lysozyme expression is much higher for the RNase E mutant (Fig. 4C). It could be observed that in the larva hemolymph, there is no significant replication of this mutant despite its persistence (Fig. 4A), and the reduced bacterial counts inside the hemocytes reveal defects in invasion and intracellular replication (Fig. 4B). All of these factors together seem to result in a more effective neutralization and clearance of the mutant reflected by a weaker effect on mortality in *Galleria* larvae (Fig. 3). In fact, in the case of RNase III mutant, for which the expression of antimicrobial peptides is much lower (Fig. 4C) and the replication inside the hemocytes is higher than for RNase E mutant (Fig. 4B), the virulence attenuation effect is lower (Fig. 3). RNase E was shown to regulate the expression of several outer membrane proteins through the action of sRNAs. Mutations in the enzyme may alter bacterial envelope composition, triggering a higher immune response from the host.

To enable pathogenesis, *Salmonella* has an array of specific virulence genes that are upregulated during infection and play important roles in adaptation, survival, and proliferation within mammalian cells. The analysis of the *Salmonella* transcriptome inside macrophages and epithelial cells revealed the differential regulation of at least one of the type three secretion systems (TTSS), SPI-1, SPI-2 and the flagellar systems (3, 4). The expression of a few randomly chosen representative genes of the systems known to be relevant for *Salmonella* pathogenicity was evaluated (see Fig. S3 in the supplemental material). Two growth conditions showing maximal *Salmonella* invasiveness have been defined: growth in LB with aeration to the early stationary phase (ESP) and growth in low-oxygen, high-salt medium (SPI-1 inducing). In fact, the expression of the genes analyzed was much lower at the late stationary phase (LSP) than under the other two conditions analyzed. Of note, it was observed that the expression of these genes changes according to the growth conditions and is affected by mutations in the RNase E and RNase III genes in different ways.

Among these were the SPI-1 genes *hilA* and *prgH*. HilA is the SPI-1 major transcriptional activator, which is responsible for most of the SPI-1 TTSS and effector gene expression (63); PrgH is required for the secretion of invasion effector proteins and is regulated by HilA (64, 65). The expression of both genes was differentially affected by RNase III mutation, depending on the growth condition (see Fig. S3 in the supplemental material). The expression of *ssav*, an SPI-2 gene needed for the secretion of SPI-2 effectors, and *gmm* (or *wcaH*), which is necessary for the production of the colanic acid capsule (66), was also affected by the RNase mutations. Overproduction of the capsule was reported to cause attenuation of *Salmonella* virulence in mice (67, 68); the higher expression of *gmm* (or *wcaH*) in the RNase mutants is in agreement with this observation.

Flagella are responsible for a number of bacterial behaviors, including motility, biofilm formation, and chemotaxis. On one hand, flagella provide a great advantage at the early stages of infection by increasing *Salmonella* invasiveness (69); on the other hand, flagellin monomers induce the host immune response (70, 71) to induce bacterial clearance from the host. Consequently, *Salmonella* cells tightly control their flagellum expression because flagella can mediate virulence. Expression of flagella is induced in response to a number of stimuli and is normally downregulated inside the host (72). There are reports showing that both the overexpression and downregulation of flagella can significantly attenuate *Salmonella* virulence (73–75). In *S.* Typhimurium, expression of flagella is controlled by phase variation, a mechanism by which the organism alternately expresses two different types of flagellin subunit proteins, FljB and FliC (76). The expression of these two genes was differentially affected by the endoribonuclease mutations under the different growth conditions tested (see Fig. S3 in the supplemental material): so was the expression of *csgD* gene, the transcriptional regulator which controls expression of biofilm-associated matrix compounds (77), mostly in the RNase III mutant. Altogether, these data show that both endoribonucleases have an effect on the expression of these virulence-related genes, as revealed by their differential expression in the mutants analyzed in comparison to the wild type. However, this effect is divergent and seems to indicate that these enzymes affect distinct bacterial functions.

Bacterial motility behavior has been reported to contribute to virulence in *Salmonella* and many other bacteria (49–51). This type of community motility seems to enable migration and expansion through sites of infection, contributing to facing host defenses. Therefore, the motility of the mutant strains was also evaluated. The nonmotile phenotype of the *Salmonella* Hfq mutant, previously shown to be associated with reduced expression of FliC flagellin (44, 45), was confirmed. In *E. coli*, it was already reported that RNase III was involved in motility (78). The present results show that RNase E and RNase III mutants have significantly reduced motility compared to wild-type *Salmonella*. The attenuated virulence phenotype of the endoribonuclease mutant strains studied was coincident with the reduced motility of these strains.

This study gives evidence that the function of endoribonucleases E and III is necessary for *Salmonella* virulence and sheds light on some of the functions that may underlie this fact. These RNases can act directly on virulence-related messages or impact their expression through sRNA regulation. Future genome-wide

analysis of these mutants should reveal the main *S.* Typhimurium genes affected, on the basis of the observed attenuated virulence phenotype.

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