



Stress Response Protein BolA Influences Fitness and Promotes *Salmonella enterica* Serovar Typhimurium Virulence

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ABSTRACT The intracellular pathogen *Salmonella enterica* serovar Typhimurium has emerged as a major cause of foodborne illness, representing a severe clinical and economic concern worldwide. The capacity of this pathogen to efficiently infect and survive inside the host depends on its ability to synchronize a complex network of virulence mechanisms. Therefore, the identification of new virulence determinants has become of paramount importance in the search of new targets for drug development. BolA-like proteins are widely conserved in all kingdoms of life. In *Escherichia coli*, this transcription factor has a critical regulatory role in several mechanisms that are tightly related to bacterial virulence. Therefore, in the present work we used the well-established infection model *Galleria mellonella* to evaluate the role of BolA protein in *S. Typhimurium* virulence. We have shown that BolA is an important player in *S. Typhimurium* pathogenesis. Specifically, the absence of BolA leads to a defective virulence capacity that is most likely related to the remarkable effect of this protein on *S. Typhimurium* evasion of the cellular response. Furthermore, it was demonstrated that BolA has a critical role in bacterial survival under harsh conditions since BolA conferred protection against acidic and oxidative stress. Hence, we provide evidence that BolA is a determining factor in the ability of *Salmonella* to survive and overcome host defense mechanisms, and this is an important step in progress to an understanding of the pathways underlying bacterial virulence.

IMPORTANCE BolA has been described as an important protein for survival in the late stages of bacterial growth and under harsh environmental conditions. High levels of BolA in stationary phase and under stresses have been connected with a plethora of phenotypes, strongly suggesting its important role as a master regulator. Here, we show that BolA is a determining factor in the ability of *Salmonella* to survive and overcome host defense mechanisms, and this is an important step in progress to an understanding of the pathways underlying bacterial virulence. This work constitutes a relevant step toward an understanding of the role of BolA protein and may have an important impact on future studies in other organisms. Therefore, this study is of utmost importance for understanding the genetic and molecular bases involved in the regulation of *Salmonella* virulence and may contribute to future industrial and public health care applications.

KEYWORDS BolA, *Galleria mellonella*, *Salmonella*, acidic stress, oxidative stress, pathogenesis, virulence determinants

Received 21 December 2017 Accepted 1 February 2018

Accepted manuscript posted online 9 February 2018

Citation Mil-Homens D, Barahona S, Moreira RN, Silva IJ, Pinto SN, Fialho AM, Arraiano CM. 2018. Stress response protein BolA influences fitness and promotes *Salmonella enterica* serovar Typhimurium virulence. *Appl Environ Microbiol* 84:e02850-17. <https://doi.org/10.1128/AEM.02850-17>.

Editor Volker Müller, Goethe University Frankfurt am Main

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Foodborne diseases represent severe clinical and economic challenges worldwide. One of the most common etiological agents of food poisoning is the pathogenic bacteria *Salmonella*. Hence, members of the genus *Salmonella* have been extensively used as model organisms to establish new concepts in pathogenesis. Particularly, nontyphoidal serovars, such as *Salmonella enterica* serovar Typhimurium, have become a hot research topic due to their serious morbidity and mortality rates in both developed and developing countries. This intracellular pathogen causes various clinical symptoms ranging from self-limiting enteritis to life-threatening systemic infections (1).

The effectiveness of the infection depends on the ability of *S. Typhimurium* to synchronize several physiological adaptations in order to overcome host defense mechanisms. Bacterial virulence factors are molecules that enable microorganisms to replicate and disseminate within a host (2). Among the wide range of virulence factors, *Salmonella* pathogenicity islands (SPIs) play a main role in host-pathogen interactions. The accurate coordination of a huge plethora of virulence effectors is also crucial for bacterial invasion and proliferation during infection (reviewed in reference 3). Transcriptional regulatory networks play a central role in the control of the hierarchical expression and cross talk of virulence effectors, ensuring a progressive and efficient bacterial response (4). Several studies have already highlighted the critical contribution of transcriptional regulators in bacterial adaptation and survival (5–9). These studies underline the importance of the identification of new effector proteins to understand and overcome *Salmonella* pathogenesis strategies. Interestingly, some of our previous work has indicated that one of the potential effectors could be the transcription factor BolA, a protein that affects different pathways directly related to virulence (10, 11).

BolA, initially characterized in *Escherichia coli* (12), has received increasing attention in both prokaryotic and eukaryotic organisms. Particularly, in prokaryotic organisms this protein stands out because of its pleiotropic effects. In Gram-negative bacteria, *bolA* expression is induced at the onset of the stationary phase or in response to several stresses, including sudden carbon starvation and heat, acidic, or oxidative shock (13). Characterized as a transcription factor, BolA has an essential role in the regulation of cell morphology, outer membrane permeability, and the transition from a planktonic to sessile lifestyle (11, 14, 15). Moreover, very recently we have shown that BolA establishes an important cross talk with the second messenger c-di-GMP in the regulation of biofilm development (16). All of these adaptation processes confer specific advantages for bacterial survival and are often associated with virulence (3).

In the present work, our main goal was to unravel the role of BolA protein in the virulence of *S. Typhimurium*. For this purpose, we have chosen the greater wax moth *Galleria mellonella* as the infection model. *G. mellonella* has been extensively used as a model organism for a wide range of bacterial species (reviewed in reference 17), and it has already been shown to be an appropriate host to study *S. Typhimurium* pathogenicity (18, 19). The *G. mellonella* larvae offer great benefits as hosts, including cost-effectiveness and easy handling. Additionally, this system allows the study of many stages of infection at 37°C, the human body core temperature, and possesses remarkable similarities with the innate immune response of vertebrates (20).

In the manuscript, we have uncovered the importance of BolA for the success of *S. Typhimurium* infection, providing new clues to the pathogenesis strategies of this organism.

RESULTS

Identification of *E. coli bolA* homologs in *S. Typhimurium*. To search for BolA homologs among *S. Typhimurium* genomes, the *E. coli* protein sequence (GenBank accession no. [APC50728.1](#)) was used. A protein from *Salmonella enterica* serovar Typhimurium LT2 was identified (GenBank accession no. [NP_459442](#)). BolA homologs of both LT2 and SL1344 strains showed an overall identity of 99% (data not shown). The alignment of both homologs from *S. Typhimurium* and *E. coli* strains revealed high sequence conservation, with 91% identity and 95% similarity (Fig. 1). Moreover, a synteny analysis of the genomic regions in the neighborhood of *bolA* indicated the

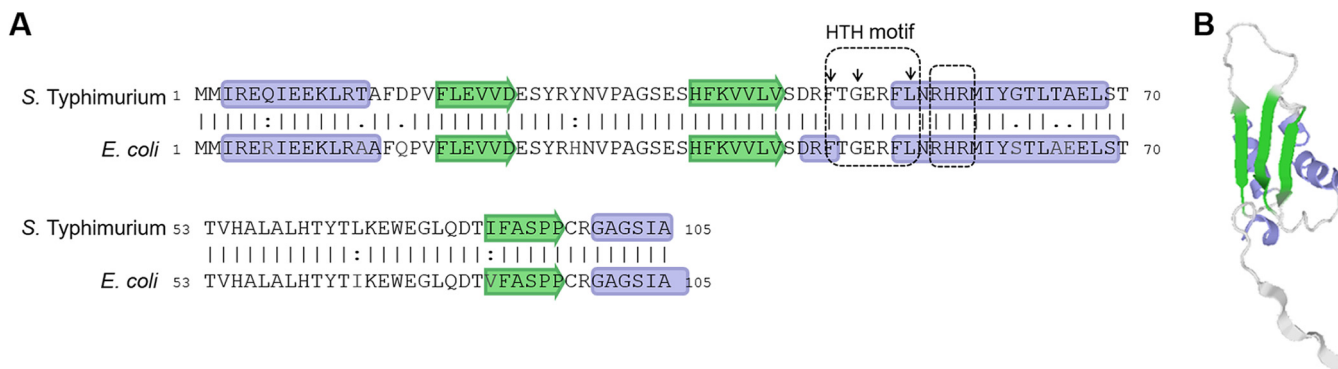


FIG 1 *In silico* analysis of BolA protein from *S. Typhimurium*. (A) Pairwise sequence alignment of BolA from *E. coli* K-12 W3110 and a BolA homolog from *S. Typhimurium* LT2 obtained using EMBOSS Needle, version 6.6.0. The secondary structure prediction for both BolA proteins is presented; in green are the β -sheets, and in blue are the α -helices. The small arrows above the sequence indicate the amino acids of the characteristic helix-turn-helix (HTH) signature FXGXXXL sequence. (B) Computer model of the 3D structure predicted for the *S. Typhimurium* LT2 BolA protein based on the crystal structure of BolA from *E. coli* K-12 W3110 (PDB accession number 2DHM).

conservation of the neighboring genes across genera. Given such high levels of sequence conservation, it was not surprising to find that an *S. Typhimurium* BolA structure prediction matched the structure of the BolA protein from *E. coli* (PDB accession number 2DHM) as the most similar. Using this structure as the template, we obtained a three-dimensional (3D) model prediction (Fig. 1B) with a global model quality score of 0.9 in a maximum of 1. The predicted structure of *S. Typhimurium* BolA consists of an $\alpha 1$ - $\beta 1$ - $\beta 2$ - $\alpha 2$ - $\beta 3$ - $\alpha 3$ topology, with $\beta 1$ and $\beta 2$ as antiparallel sheets and $\beta 3$ parallel to the $\beta 2$ sheet, similar to the topology observed in other BolA-like proteins (21). The helix-turn-helix (HTH) signature FXGXXXL sequence, characteristic of BolA proteins (22) and typically implicated in protein-DNA interaction, is also present (Fig. 1B). Moreover, within the second helix we observed a highly conserved RHR signature sequence that is present in 41% of BolA sequences (23).

BolA protects *S. Typhimurium* cells against acidic and oxidative stresses. The *S. Typhimurium* LT2 strain presents an attenuated level of virulence due to a suboptimal translation of RpoS (σ^S) (24). Therefore, we have used a virulent *S. Typhimurium* strain (SL1344) to construct a BolA mutant strain. The deletion of BolA in *E. coli* was reported to slightly increase growth rates compared to the wild-type rate (10). However, in *S. Typhimurium* SL1344, BolA deletion did not significantly affect the growth rate relative to that of the wild-type strain, and the BolA mutant showed only a slight increase in the optical density at 600 nm (OD_{600}) in stationary phase (Fig. 2A). Complementation of the SL1344 BolA mutant was achieved using a pRMA04 plasmid expressing BolA *in trans* (strain CMA821). The growth curve of the complemented strain was identical to that of the wild type (Fig. 2A). For growth under static conditions, a similar minor increase in the OD_{600} of the BolA mutant was registered (data not shown).

In *E. coli*, BolA is established as a general stress response transcription factor whose expression increases under imposition of diverse severe conditions, conferring protection to the cells (reviewed in reference 10). Therefore, we were interested in understanding whether *S. Typhimurium* BolA confers protection under acidic and oxidative stresses. For both stresses, growth curves were monitored by measuring the OD_{600} during the course of the experiment (Fig. 2B and C, graphs i). To evaluate the BolA role in the acid tolerance response, the bacterial cultures were first subjected to a preacidic adaptive shock stage (pH 4.4) for 1 h, followed by a pH fall (pH 3.2) during the same period (Fig. 2B). Our results revealed that although the *bolA* deletion strain is able to produce an acid tolerance response, it was about 5-fold more susceptible than the wild-type strain in both acidic challenges (Fig. 2B, graph ii). Similarly, under oxidative stress conditions, the absence of BolA produced a 7-fold decrease in viability compared to that of the wild-type strain (Fig. 2C, graph ii). Under both stresses, the effect obtained in the *bolA* deletion strain was at least partially reversed and wild-type levels were

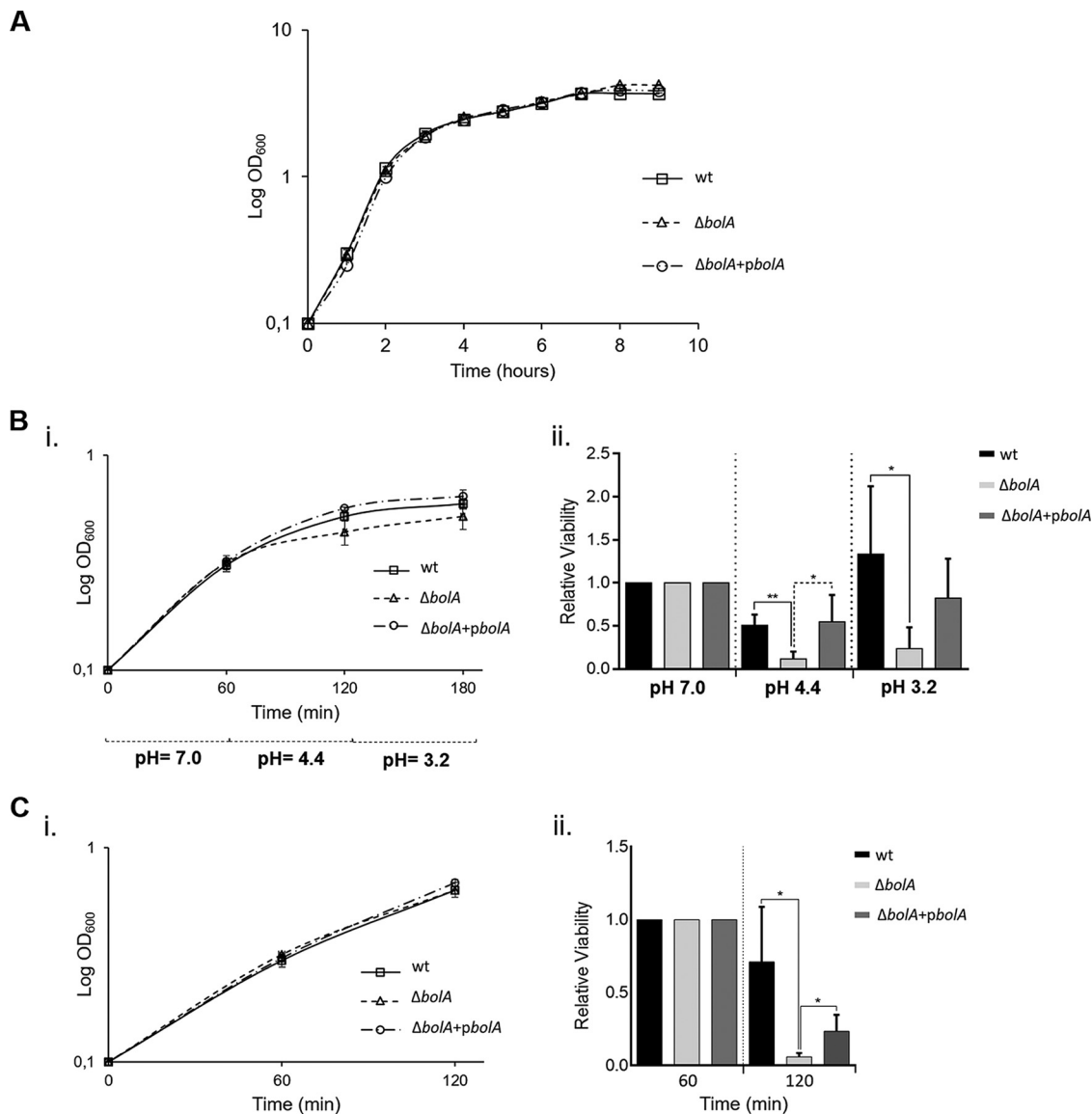


FIG 2 The role of BolA in *S. Typhimurium* growth and the stress response. (A) Growth curve of *S. Typhimurium* SL1344 strains. Growth characteristics of the *S. Typhimurium* wild-type (wt), BolA mutant (Δ *bolA*), and complemented (Δ *bolA*+*pbolA*) strains, obtained by following the OD₆₀₀ for 9 h. (B) Influence of BolA in the acid tolerance response of *S. Typhimurium*. Growth curves of *S. Typhimurium* SL1344 strains under acidic stress are shown (i). Cells were grown until they reached an optical density of 0.3 (corresponding to 60 min on the graph) in LB medium, pH 7, prior the adjustment to pH 4.4 for 1 h. The cells were then challenged by dropping the pH to 3.2 for 1 h. Viable cells were extrapolated by plating the appropriate dilutions on Luria agar plates (ii). (C) BolA impact on the *S. Typhimurium* oxidative response. Cultures were grown in LB medium and challenged for 1 h with 5 mM H₂O₂ at an optical density of 0.3 (corresponding to 60 min on the graph) (i). Viable cells were extrapolated by plating the appropriate dilutions on Luria agar plates (ii). The results were normalized and are presented relative to the number of viable cells obtained before stress induction (OD₆₀₀ of 0.3). The experiments were performed in triplicate, and the results are represented as mean \pm SD. *, $P < 0.05$; **, $P < 0.01$.

reached when a complemented strain was used, corroborating the previous results (Fig. 2B and C, graphs ii). These results indicate that *S. Typhimurium* BolA confers protection against acidic and oxidative stress.

The deletion of *bolA* decreases virulence of *S. Typhimurium* in a *Galleria mellonella* model of infection. To evaluate the involvement of BolA in virulence, we used the preestablished insect model for *Salmonella* infection, *G. mellonella*, the greater wax moth (18). *G. mellonella* larvae were inoculated with 3×10^3 CFU/larva of the *S. Typhimurium* SL1344 wild-type and BolA mutant strains and incubated at 37°C, and their survival rate was daily registered over a period of 72 h. The effects of infection by the wild-type strain were rapidly seen after the first 24 h of infection,

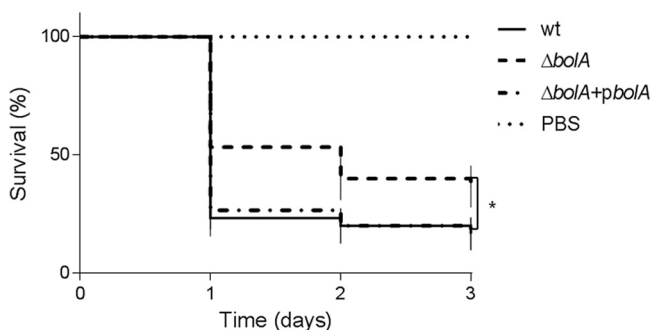


FIG 3 Survival of *G. mellonella* larvae following inoculation with *S. Typhimurium* strains. Kaplan-Meier survival curves represent larvae infected with 3×10^3 CFU/larva of the *S. Typhimurium* SL1344, BolA mutant ($\Delta bolA$), and complemented ($\Delta bolA+pbolA$) strains and PBS as a control. Larvae infected with the BolA mutant strain had enhanced survival compared to that of *S. Typhimurium* SL1344 or the complemented strain (*, $P < 0.05$). The data shown are means \pm standard errors of the means, and differences in survival were calculated using a log rank test (Mantel-Cox). wt, wild type.

with a reduction of about 80% of the initial larval population (Fig. 3). In contrast, the deletion of the *bolA* gene was found to increase by 25% the larval survival rate in the course of infection ($P < 0.05$) compared with the rate of the wild-type strain (Fig. 3). Complementation of the mutant with the *bolA* gene in *trans* was also evaluated and was shown to restore the wild-type survival rates (Fig. 3). These results clearly show the involvement of BolA in the pathogenesis of *S. Typhimurium* in the model host *G. mellonella*.

The bacterial load of the BolA mutant in hemolymph decreases during *G. mellonella* colonization. In order to evaluate the proliferation of the *S. Typhimurium* BolA mutant within the insect hemocoel, we determined the viable bacterial load within the hemolymph of larvae in the course of infection (at 1, 4, 8, 12, and 24 h). To that end, larvae were challenged with 3×10^3 CFU/larva of *S. Typhimurium* SL1344 wild-type and BolA mutant strains, hemolymph from three living larvae was extracted and pooled, and the number of CFU was determined (Fig. 4). At the start of the infection, the three strains presented similar bacterial loads and were able to persist and proliferate in the larval hemolymph up to 12 h postinfection. However, the BolA mutant showed lower levels of proliferation (Fig. 4). Nevertheless, with the progression of the infection, a decrease of bacterial levels was noticed, especially for the $\Delta bolA$ strain, which reached values similar to those observed during the first hours of

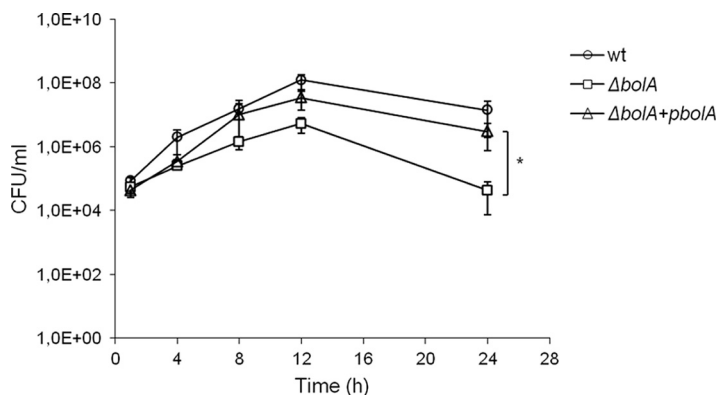


FIG 4 Hemolymph bacterial load in the course of *S. Typhimurium* infection. The viable bacterial load was determined in the hemolymph extracted from larvae infected with 3×10^3 CFU/larva of the *S. Typhimurium* SL1344, BolA mutant ($\Delta bolA$), and complemented ($\Delta bolA+pbolA$) strains during a period within the time course of infection. The bacterial load of larvae infected with the BolA mutant is significantly lower than the levels in larvae infected with wild-type (wt) strain and the complemented strain (*, $P < 0.05$; ANOVA and Tukey's test). Results (number of CFU per milliliter of hemolymph) represent means of three independent experiments.

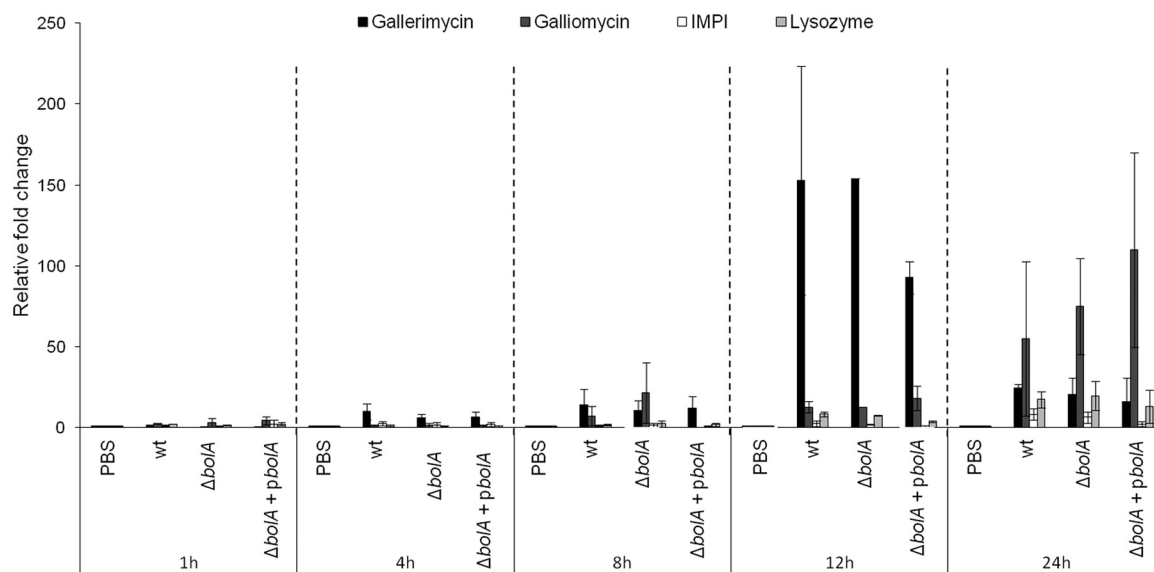


FIG 5 Immunogenic response of *G. mellonella* during infection with *S. Typhimurium* strains. Transcriptional activation of immune-responsive genes of *G. mellonella* was measured at 1, 4, 8, 12, and 24 h postinfection with 3×10^3 CFU/larva of the *S. Typhimurium* SL1344, *BolA* mutant ($\Delta bolA$), and complemented ($\Delta bolA + pbolA$) 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 PBS. No significant differences were observed between strains. Results were normalized to the expression of the housekeeping gene actin and represent three independent experiments.

infection. In fact, at 24 h postinfection, the bacterial load in hemolymph was significantly lower for the *bolA* mutant than for the wild-type strain ($P < 0.05$). When the complemented strain was analyzed, behavior similar to that of the wild-type strain was observed. These results are in accordance with the results of the survival rates of infected larvae (Fig. 3) since at 24 h postinfection the wild-type infection led to a higher level of larval mortality.

The effect of *BolA* on the immune response of *G. mellonella*. Host defense peptides are a crucial part of insect innate immunity, showing broad-spectrum microbicidal activity (17). In a previous study, we observed the induction of expression of four antimicrobial peptides upon infection of *G. mellonella* with *S. Typhimurium* SL1344 (18). In the present work, we examined whether the mutation in the *bolA* gene could either directly or indirectly interfere with the activation of the immune system of the insect host, leading to differences in the bacterial loads during infection. To evaluate this, larvae were infected with the wild-type, $\Delta bolA$, and complemented strains. Gene expression levels of four selected antimicrobial peptides, namely, gallerimycin, galliomycin, lysozyme, and an inducible metallo-proteinase inhibitor (IMPI), were analyzed at 1, 4, 8, 12, and 24 h postinfection. Gene expression was determined by quantitative real-time PCR (RT-PCR) of the total RNA extracted for each time point. As shown in Fig. 5, no significant differences in the expression levels of immune-related peptides was observed in a comparison of the wild type with the *BolA* mutant strain.

The deletion of *bolA* leads to lower levels of intracellular bacteria within hemocyte cultures. To further evaluate the effect of *S. Typhimurium bolA* deletion in the host-pathogen interaction in this insect model, hemocytes were extracted from larval hemolymph and used for *in vitro* cultures. The primary cell cultures of hemocytes were infected with the wild-type, *BolA* mutant, and complemented strains at a multiplicity of infection (MOI) of 50:1. Bacterial load was evaluated after 2, 4, and 8 h in a gentamicin protection assay and also visualized by fluorescence microscopy (Fig. 6). At 2 h, similar levels of phagocytic activity were observed for hemocytes infected with any of the three strains (Fig. 6A). These results were confirmed after 4 h of infection through the images of fluorescence microscopy in which only some hemocytes were infected with bacteria but the number of bacteria inside the hemocytes seemed similar for the

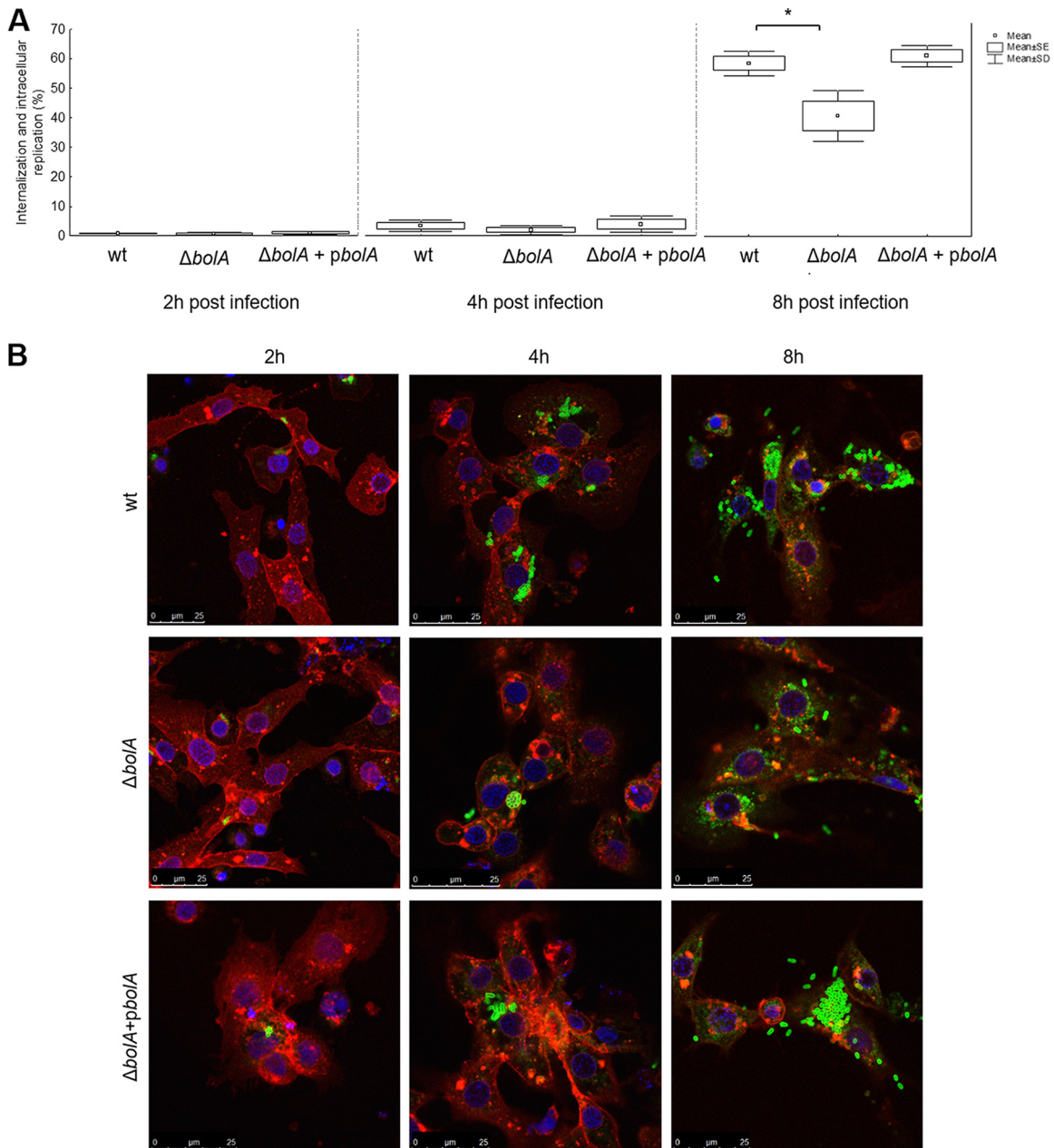


FIG 6 *In vitro* infection of hemocytes with *S. Typhimurium* strains. (A) Primary hemocytes cell cultures were infected with the *S. Typhimurium* SL1344 wild-type, *bolA* mutant ($\Delta bolA$), and complemented ($\Delta bolA + pbolA$) strains (MOI of 50:1). Bacterial load of hemocytes was assessed using a gentamicin protection assay, and values of internalization and intracellular replication were obtained at 2, 4, and 8 h. Bacterial load of hemocytes infected with the mutant was significantly lower than that of larvae infected with the wild-type strain or the complemented strain at 8 h postinfection (*, $P < 0.05$, ANOVA and Tukey's test). Results are shown as percentages of the bacterial input and represent means of three independent experiments. (B) Fluorescence confocal microscopy images of the hemocytes infected with the *S. Typhimurium* SL1344 wild-type, *bolA* mutant ($\Delta bolA$), and complemented ($\Delta bolA + pbolA$) strains at 2, 4, and 8 h postinfection. Hemocyte membranes were labeled with WGA conjugated with Alexa 633 (red), while bacteria were stained with *S. Typhimurium* LPS antibody and Alexa 488 (green), and Hoechst dye was used for staining the cell's nucleus (blue). Experiments were repeated at least three times, and one representative experiment is shown.

three strains (Fig. 6B). Within the next 2 h of infection, the number of intracellular wild-type bacteria increased 4-fold, while the mutant strain increased 3-fold compared to the level at the previous time point. Nevertheless, this difference was not statistically significant (data not shown). However, in the course of the experiment, at 8 h postinfection, the difference between the wild type and the mutant was noteworthy, with the wild type reaching nearly 60% of the input of infection and the mutant having only 40%

($P < 0.05$). Complementation was achieved, and the phenotype was restored (Fig. 6A). Fluorescence microscopy images taken at 8 h postinfection indicate that the difference observed was related to higher intracellular bacterial division in hemocytes infected with the wild-type and complemented strains (Fig. 6B). In fact, some hemocytes showed large agglomerates of bacteria that were not observed in the mutant strain. Despite initial cellular uptake of BolA mutant bacteria at a level similar to that of the wild type, the wild type seemed to resist infection better than the mutant.

DISCUSSION

Pathogenic bacteria have evolved a wide array of virulence mechanisms, essentially comprised by fine-tuned effectors that are critical for the success of infection. A proper coordination of these effectors is required for the efficiency of the virulence process. We have identified in *E. coli* a transcription factor, the BolA protein, that was shown to be involved in the regulation of several cellular processes related to virulence, such as membrane permeability, bacterial motility, and biofilm formation (11, 14–16). Moreover, we proposed that *E. coli* BolA is a general stress response transcription factor whose expression increases under diverse severe growth conditions, conferring protection to the cells (reviewed in reference 10). Homologs of this *E. coli* gene are found widely distributed across all cellular organisms. These facts prompted us to study the impact of BolA protein on the virulence capacity of *Salmonella* Typhimurium, one of the most common foodborne etiological agents.

When bacteria were cultured in a nutrient-rich medium (Luria-Bertani [LB]), the deletion of BolA in *E. coli* was reported to increase the growth rate compared to that of the wild-type strain (10). In contrast, in *S. Typhimurium* SL1344, BolA deletion did not significantly affect the growth rate relative to that of the wild-type strain, and only a slight increase in the OD₆₀₀ of the BolA mutant at stationary phase was observed (Fig. 2A). However, when *S. Typhimurium* with a deletion of *bolA* was exposed to low pH or hydrogen peroxide, we observed significantly reduced bacterial survival, similar to what was observed for the *E. coli* *bolA* homolog (Fig. 2B and C). Interestingly, both stresses are important since they are used as antimicrobial weapons by mammalian cells in order to rapidly clear bacterial pathogens (25, 26). All of these results point to a possible role of BolA in *S. Typhimurium* virulence capacity.

In order to study the effects of BolA on *S. Typhimurium* proliferation and host survival *in vivo*, we chose the wax moth *G. mellonella* as the infection host, a model organism that has several advantages and has been increasingly used to study virulence mechanisms. The *G. mellonella* larva has previously been established as a successful model for *S. Typhimurium* infection (18, 19). Infection of *G. mellonella* larvae with the *S. Typhimurium* BolA mutant showed an attenuation of about 25% in virulence compared to the wild-type level (Fig. 3). Importantly, the complementation of the *bolA* deletion perfectly restored the wild-type phenotype, showing that BolA contributes to the virulence of this pathogen.

Following infection, the host immune response displays a set of coordinated and complex mechanisms in order to successfully immobilize or kill the pathogen. Hemolymph, which functions analogously to mammalian blood, comprises an innate immune response with remarkable similarities to the response in vertebrates. Analysis of *S. Typhimurium* replication in *G. mellonella* by direct bacterial enumeration in hemolymph demonstrated that bacteria persisted and increased by 1,000-fold during the first 12 h of infection. This result is in accordance with results of our previous study (18, 19). Moreover, the level of *S. Typhimurium* SL1344 replication appears to be consistent with that of the mouse model, in which this strain exhibited a 10,000-fold increase in the mouse spleen within 3 days (27). Nevertheless, in the progression of the insect infection we could see a decrease in bacterial levels. More noteworthy was the attenuated replication of the BolA mutant strain that, despite an initial increase in bacterial numbers, had values similar to those observed in the early stages of infection after 24 h of infection (Fig. 4). This result is consistent with the pronounced survival of *G.*

TABLE 1 Strains and plasmids used in this work

Strain or plasmid	Description	Reference or source
Strains		
SL1344	<i>hisG rpsL xyl Str^r</i>	46
CMA820	SL1344 Δ <i>bolA</i> ::Cat ^r	This study
CMA821	CMA820 carrying the pRMA04 plasmid	This study
Plasmids		
pKD3	Plasmid encoding FRT-flanked Cat cassette; pANTS γ , Amp ^r Cat ^r	38
pKD46	Temperature-sensitive λ -Red recombinase expression plasmid; <i>oriR101</i> Amp ^r	38
pWKS29	Low-copy-number plasmid; pSC101, Amp ^r	47
pRMA04	pWSK29 carrying <i>bolA</i> , Amp ^r	This study

mellonella after 24 h of infection with the mutant bacterial strain and supports the involvement of BoIA in the successful colonization of *S. Typhimurium* (Fig. 3).

The innate immune responses of insects are of two major types, the cellular and the humoral. The cellular response is mediated by hemocytes that are involved in not only phagocytosis but also encapsulation and clotting (28, 29). The humoral response is composed of soluble effector molecules that immobilize or kill the pathogen and includes complement-like proteins, melanin, and antimicrobial peptides (30, 31). In order to deepen our understanding of the involvement of the humoral response in the differences between the survival rates of the BoIA mutant and the wild-type strain, we investigated the differences in the expression levels of immune-related peptides. As seen in Fig. 5, wild-type and BoIA mutant strains elicited analogous humoral responses, suggesting that this type of immune response is not relevant to the resolution of the infection. Nevertheless, it is worth noticing the inversion of the expression profiles in the course of infection. Up to 12 h of infection, gallerimycin had higher levels of expression; however, at 24 h mRNA levels of gallerimycin decreased, and those of galliomyacin increased. This response profile is commonly observed in response to different pathogens (32–35).

In contrast to what happens regarding the *G. mellonella* humoral response, BoIA seems to have a remarkable effect on *S. Typhimurium* evasion of cellular immunity. Despite the similar internalization capacities among the different strains, BoIA substantially improves the capacity of bacteria to replicate inside hemocytes in later stages of infection (Fig. 6). Hemocyte-mediated immunity constitutes one of the most effective components of the *G. mellonella* defense mechanisms. Upon phagocytosis, bacteria are exposed to a wide range of antimicrobial effectors that rapidly identify and kill foreign pathogens (36, 37). Consistent with this, BoIA promotes an increase of 5- and 7-fold in *S. Typhimurium* survival in response to oxidative stress and acidic burst. Therefore, we propose that BoIA is a relevant player in *S. Typhimurium* virulence, contributing to the survival of the pathogen in a hostile environment during the course of infection. In this regard, our work constitutes an important advance in the understanding of pathogenic strategies and indicates that BoIA contributes to *Salmonella* pathogenicity.

MATERIALS AND METHODS

Oligonucleotides, bacterial strains, and plasmids. All bacterial strains and plasmids used in this study are listed in Table 1. The oligonucleotides used in this work were synthesized by STAB Vida and are listed in Table 2. All *Salmonella* strains used are isogenic derivatives of the wild-type *S. Typhimurium* strain SL1344. Restriction enzymes, T4 DNA ligase, and Phusion DNA polymerase were purchased from Thermo Fisher Scientific and used according to the supplier's instructions.

The λ -Red-mediated mutagenesis method (38) was used to obtain the *bolA* deletion strain. Briefly, a PCR fragment was obtained by amplification of a pKD3 plasmid using the primer pair RNMS003/RNMS004 (Table 2). The resulting fragment, carrying the chloramphenicol (Cat) cassette flanked by 70-nucleotide (nt) homologous extension regions adjacent to the *bolA* gene, was transformed in *S. Typhimurium* SL1344 electrocompetent cells containing pKD46 to allow recombination with the bacterial chromosome. P22 HT105/1 *int-201* transduction was used to obtain the SL1344 Δ *bolA* strain (CMA820) in a fresh background (39).

The pRMA04 plasmid was constructed by inserting a PCR-amplified DNA fragment carrying the *bolA* coding sequence and promoter region (using primers RMNS011/RMNS012) (Table 2) into XbaI and KpnI sites of a pWSK29 vector. The resulting pRMA04 plasmid was transformed in CMA820 competent cells to

TABLE 2 Oligonucleotides used in this work

Primer name (target)	Sequence (5'–3')	Reference or source
RNMS003	AGGGATGGGTGACATCACAGCGTTGTCCGAGGAGATTTTCATGATGATACGTGAGCAA ATAGAAGAAAAAGTGTAGGCTGGAGCTGCTTC	This study
RNMS004	AACATACTGGAAAAGCGGAGGTAGTTGCAAATCCGTTTTGCTACGCGATGCTTCCCGCG CCGCGACAAGGCATATGAATATCCTCCTTAG	This study
RNMS011	GCTCTAGAGCGTCAACTCTACTCCGCAAGCAC	This study
RNMS012	AAAGGTACCTTTGTGGTCAACGCAGGTAAATTCG	This study
P1RT (gallerimycin)	CGCAATATCATTGGCCTTCT	48
P2RT (gallerimycin)	CCTGCAGTTAGCAATGCAC	48
P1RT (IMPI)	AGATGGCTATGCAAGGGATG	48
P2RT (IMPI)	AGGACCTGTGCAGCATTCT	48
P1RT (lysozyme)	TCCCAACTCTTGACCGACGA	48
P2RT (lysozyme)	AGTGGTTGCGCCATCCATAC	48
P1RT (actin)	ATCCTCACCTGAAGTACCC	48
P2RT (actin)	CCACACGCAGCTCATTGTA	48
P1RT (galliomycin)	TCGTATCGTCACCGCAAATG	49
P2RT (galliomycin)	GCCGCAATGACCACCTTTATA	49

obtain the complemented strain CMA821. All constructs were confirmed by DNA sequencing at STAB Vida.

Bacterial and insect growth conditions. *S. Typhimurium* strains were grown in Luria-Bertani (LB) broth at 37°C and 220 rpm. For *Galleria mellonella* infection experiments, cultures were grown overnight in LB medium at 37°C under static conditions. When appropriate, antibiotics were used at the following concentrations: 100 µg/ml ampicillin, 90 µg/ml streptomycin, and 25 µg/ml chloramphenicol.

Galleria mellonella larvae were reared in our laboratory, from egg to last-instar larvae, at 25°C in darkness, as previously described (18, 40). We used the natural foods beeswax and pollen grains, and last-instar larvae weighing 225 ± 25 mg were selected for the experiments.

Sequence analysis. The BolA homolog from *S. Typhimurium* was identified using the BLASTp tool at the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov>) with the BolA sequence from *E. coli* K-12 W3110 (GenBank accession no. [APC50728.1](https://pubmed.ncbi.nlm.nih.gov/100000000/)) as the query. Homologs were aligned using EMBOSS Needle, version 6.6.0, a pairwise sequence alignment tool at the European Bioinformatics Institute (EMBL-EBI) website (<http://www.ebi.ac.uk/>). Synteny analysis was performed using standard gene visualization tools at NCBI. Protein structural prediction was obtained with Swiss model (<https://swissmodel.expasy.org/>) (41), based on the crystal structure of BolA from *E. coli* K-12 W3110 (PDB entry 2DHM).

Acidic and oxidative stress assays. Bacterial cultures were diluted from overnight growth to an optical density of 0.1. The cultures were grown aerobically in LB medium (pH 7.0) at 37°C and 220 rpm for 1 h (corresponding to an optical density at 600 nm [OD₆₀₀] of 0.3) before challenge under the selected stress conditions. Acidic stress was accomplished by adding 30% (vol/vol) HCl. Specifically, the medium pH was adjusted to 4.4 for 1 h, followed by a challenge at pH 3.1 for an additional hour. For oxidative stress, cells were challenged for 1 h with 5 mM H₂O₂. To evaluate viabilities, cells were diluted in phosphate-buffered saline (PBS), serially diluted, and inoculated on LB agar plates. The number of viable cells was extrapolated directly from the number of colonies obtained after 16 h of growth at 37°C and was expressed as the number of CFU/milliliter. The results were normalized by the number of cells obtained before stress induction.

***Galleria mellonella* killing assays.** *G. mellonella* killing assays were performed based on a previously described method, with some changes (18). Briefly, cultures of *S. Typhimurium* were grown overnight in LB broth under static conditions at 37°C. The OD of the cultures was measured at 600 nm, and the appropriate volume was collected to ensure that all the strains had the same OD. Cells were then harvested by centrifugation and suspended in PBS. Tenfold serial dilutions were performed to obtain 3 × 10³ CFU per volume of injection. This concentration was confirmed by inoculating serial dilutions on LB agar plates. A micrometer was used to control the volume of a disposable hypodermic microsyringe, and a 3.5-µl aliquot of each bacterial dilution was injected into the larvae via the hindmost left proleg, which had been previously surface sanitized with 70% (vol/vol) ethanol. For each condition, we used 10 larvae, and as a control a set of larvae was injected with PBS. Following injection, larvae were placed in petri dishes and stored in the dark at 37°C.

Larval survival was followed over a period of 3 days, and caterpillars were considered dead when they displayed no movement in response to touch. Using results from three independent experiments, Kaplan-Meier survival curves were plotted, and differences in survival rates were calculated by using a log rank (Mantel-Cox) statistical test; all analyses were performed with GraphPad Prism, version 6, software.

Extraction of larval hemolymph. To determine the number of viable bacteria in the hemocoel during the course of infection, hemolymph of infected larvae was collected as previously described (18). Briefly, three living larvae were anesthetized on ice and surface sterilized with ethanol at 1, 4, 8, 12, and 24 h after injection. The larvae were bled, and the outflowing hemolymph was immediately transferred into a sterile microtube containing a few crystals of phenylthiourea to prevent melanization. Bacterial quantification was done by preparing 10-fold serial dilutions of hemolymph in PBS and inoculating the

mixture onto LB agar plates. Bacteria were enumerated by CFU counting after incubation at 37°C for 24 h, and results are presented as the number of CFU per milliliter of hemolymph.

In vitro cultivation of *G. mellonella* hemocytes. To isolate *G. mellonella* hemocytes, hemolymph was extracted from last-instar larvae, previously anesthetized on ice and surface sterilized with ethanol, by puncturing the larval abdomen with a sterile needle (42). Hemolymph was immediately collected into anticoagulant buffer (98 mM NaOH, 145 mM NaCl, 17 mM EDTA, and 41 mM citric acid; pH 4.5) in a 1:1 proportion. Hemocytes were spun in a centrifuge at $250 \times g$ for 10 min at 4°C, washed twice with PBS, and spun at $250 \times g$ for 5 min at 4°C. Finally, hemocytes were gently suspended in 1 ml of Grace's insect medium (GIM) (Thermo Fisher Scientific) supplemented with 10% (vol/vol) fetal bovine serum, 1% (wt/vol) glutamine, and 1% (wt/vol) antibiotic/antimycotic solution (10,000 units of penicillin G, 10 mg of 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 cell/ml. Monolayers of primary *Galleria* hemocytes were used for experiments the next day.

Gentamicin protection assay of hemocytes. To determine the intracellular bacterial load of *in vitro* hemocytes during the course of infection, a protocol based on the gentamicin protection assay was used (43). Prior to infection with bacteria, *Galleria* hemocyte monolayers were washed with PBS, and medium was replaced with GIM without antibiotics. Cultures of *S. Typhimurium* SL1344 were grown as described above, and the appropriate volume was collected to obtain 4×10^3 bacteria/ml in each well. After 1 h of infection at 37°C, the hemocytes were carefully washed twice with PBS, followed by the addition of GIM containing 100 mg/liter of gentamicin to kill extracellular bacteria. After 1 h of incubation, supernatants were inoculated in LB agar plates to confirm the effectiveness of antibiotic treatment, and medium was replaced with GIM containing 10 mg/liter of gentamicin. The quantification of viable intracellular bacteria was performed at 2, 4, and 8 h after infection. For that, infected cell monolayers were lysed with 0.5% (vol/vol) Triton X-100 for 20 min, and the number of CFU was determined by inoculating 10-fold dilutions of cell lysates on LB agar plates, followed by incubation at 37°C for 24 h.

Confocal microscopy of hemocytes. For microscopy analysis, *in vitro* hemocytes were isolated and infected as described above; however, glass coverslips were placed into the 24-well plates before hemocyte seeding. After 2, 4, and 8 h of infection, wells were washed with PBS, and samples were fixed with 3.7% (wt/vol) paraformaldehyde for 20 min, quenched with 50 mM NH_4Cl for 10 min, immersed in 0.2% (vol/vol) Triton X-100 for 5 min, and saturated with 5% (wt/vol) bovine serum albumin for 30 min. The bacterial immunostaining was performed using a monoclonal antibody to *S. Typhimurium* anti-lipopolysaccharide (LPS) (1:500; Abcam) followed by the secondary polyclonal goat anti-mouse serum coupled to Alexa 488 (1:500; Santa Cruz Biotechnology). Cells were stained using wheat germ agglutinin (WGA) conjugated with Alexa 633 (1:200; Thermo Fisher Scientific). Finally, coverslips were mounted in Vectashield (Vector Laboratories) containing Hoechst 33342 fluorescent dye (Thermo Fisher Scientific) for nuclear staining. All samples were examined on a Leica TCS SP5 (Leica Microsystems CMS GmbH) inverted microscope (model no. DMI6000) with a $63\times$ water apochromatic objective (44).

***G. mellonella* RNA extraction.** Sets of 20 larvae were infected with *S. Typhimurium* strains at a concentration of 3×10^3 CFU/larva, as previously described for the survival assays. At 1, 4, 8, 12, and 24 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 manufacturer's protocol. After extraction, RNA was treated with an RNase-free DNase set (Qiagen). The purified RNA was quantified spectrophotometrically (NanoDrop ND-1000).

Quantitative real-time PCR. The transcriptional levels of genes encoding the *G. mellonella* antimicrobial peptides gallerimycin, galliomyacin, inducible metalloproteinase inhibitor (IMPI), and lysozyme were determined with a Rotor-Gene 3000 (Corbett) system using a SensiFast SYBR kit (Bioline) according to the supplier's instructions. cDNA was synthesized from 1 μg of purified RNA with a SensiFast cDNA synthesis kit (Bioline). The primers used are listed in Table 2. All samples were analyzed in triplicate, and the amount of mRNA detected was normalized using the mRNA value of the housekeeping gene actin. Relative quantification of gene expression was calculated by using the $\Delta\Delta C_T$ (C_T is threshold cycle) method (45).

Statistical analysis. Data are expressed as mean values of a minimum of three independent experiments \pm standard deviations (SD). Statistical analysis was carried out using Statistica, version 7, software (StatSoft). One-way analysis of variance (ANOVA) and Tukey's multiple-comparison test with an unequal group sample size were performed to determine statistically significant differences. A *P* value of <0.05 was considered statistically significant.

ACKNOWLEDGMENTS

This work was supported by Fundação para a Ciência e Tecnologia (FCT), Portugal with fellowships to D. Mil-Homens (SFRH/BPD/91831/2012), S. Barahona (PD/BD/113983/2015), R. N. Moreira (SFRH/BPD/84080/2012), I. J. Silva (SFRH/BPD/84086/2012), and S. N. Pinto (SFRH/BPD/92409/2013). The work at Instituto de Tecnologia Química e Biológica was financially supported by Project PTDC/BIA-MIC/4046/2014, by Project LISBOA-01-0145-FEDER-007660 (Microbiologia Molecular, Estrutural e Celular) funded by FEDER funds through COMPETE2020, Programa Operacional Competitividade e Internacionalização. The work at Institute for Bioengineering and Biosciences was supported by FCT, Portugal UID/BIO/04565/2013, and Programa Operacional Regional de Lisboa 2020 (project no. 007317).

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