

Salmonella Enteritidis antitoxin DinJ inhibits NLRP3-dependent canonical inflammasome activation in macrophages

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ABSTRACT The inflammasome is a pivotal component of the innate immune system, acting as a multiprotein complex that plays an essential role in detecting and responding to microbial infections. *Salmonella* Enteritidis have evolved multiple mechanisms to regulate inflammasome activation and evade host immune system clearance. Through screening *S. Enteritidis* C50336 Δ *fliC* transposon mutant library, we found that the insertion mutant of *dinJ* increased inflammasome activation. In this study, we demonstrated the genetic connection between the antitoxin DinJ and the toxin YafQ in *S. Enteritidis*, confirming their co-transcription. The deletion mutant Δ *fliC* Δ *dinJ* increased cell death and IL-1 β secretion in J774A.1 cells. Western blotting analysis further showed elevated cleaved Caspase-1 product (p10 subunits) and IL-1 β secretion in cells infected with Δ *fliC* Δ *dinJ* compared to cells infected with Δ *fliC*. DinJ was found to inhibit canonical inflammasome activation using primary bone marrow-derived macrophages (BMDMs) from *Casp1*^{-/-} C57BL/6 mice. Furthermore, DinJ specifically inhibited NLRP3 inflammasome activation, as demonstrated in BMDMs from *Nlrp3*^{-/-} and *Nlrc4*^{-/-} mice. Fluorescence resonance energy transfer (FRET) experiments confirmed the translocation of DinJ into host cells during infection. Finally, we revealed that DinJ could inhibit the secretion of IL-1 β and IL-18 *in vivo*, contributing to *S. Enteritidis* evading host immune clearance. In summary, our findings provide insights into the role of DinJ in modulating the inflammasome response during *S. Enteritidis* infection, highlighting its impact on inhibiting inflammasome activation and immune evasion.

KEYWORDS *Salmonella* Enteritidis, DinJ, NLRP3 inflammasome, immune evasion

The inflammasome is a multiprotein complex that plays a pivotal role in the innate immune system against bacterial infections. It operates as a molecular platform for sensing and responding to danger signals such as pathogenic microbes, cellular damage, or metabolic stress (1–4). Assembly of the inflammasome leads to the activation of Caspase-1, triggering the maturation and secretion of pro-inflammatory cytokines like IL-1 β and IL-18. These cytokines facilitate neutrophil migration to infected tissues and against pathogens (5). Additionally, activation of Caspase-1 leads to gasdermin D cleavage, resulting in cell rupture via pyroptosis, and inflammatory cell death mediated through the inflammasome (6). Various pathogens could activate the inflammasome, leading to the secretion of inflammatory cytokines (7–10). Moderate inflammation aids the host in pathogen clearance, while excessive inflammation could cause tissue damage (11). Pathogens have evolved multiple mechanisms to maintain the homeostasis of the inflammasome activation, balancing an effective immune response with the potential tissue damage caused by excessive inflammation.

Salmonella, a facultative intracellular pathogen, has the potential to cause severe illness in both humans and animals. The invasion of host cells by *Salmonella* is facilitated by a type III secretion system (T3SS) encoded by *Salmonella* pathogenicity island I (SPI-1) (12). Subsequently, *Salmonella* replicates within the host cell that is established by the

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second T3SS encoded by SPI-2 (13). *Salmonella* invasion into macrophages induces a form of cell death known as pyroptosis, and the T3SS could directly secrete the effectors into host cells to modulate the activation of the inflammasome (14, 15). This inflammatory form of cell death acts as a host defense mechanism, contributing to the elimination of *Salmonella*-infected cells and enhancing the immune response against the invading pathogen. *Salmonella* SopB effector could inhibit the NLRC4 inflammasome activation by specifically preventing apoptosis-associated speck-like protein containing a CARD (ASC) oligomerization (16). *Salmonella* AcnB could inhibit the NLRP3 inflammasome activation by mitochondrial reactive oxygen species (mtROS), contributing to *Salmonella* persistence infection *in vivo* (3). Furthermore, the *Salmonella* SiiD protein could inhibit NLRP3 inflammasome activation through mtROS-ASC signaling and plays an essential role in evading host immune clearance (17). Therefore, *Salmonella* have evolved mechanisms to inhibit or evade pyroptosis, aiding in their persistence within the host and establishment of infection.

The toxin and antitoxin (TA) systems are genetic modules commonly found in bacteria, playing a pivotal role in regulating cellular processes and responding to various stresses (18, 19). The TA systems consist of a pair of closely linked genes, one encodes a toxin protein and the other encodes an antitoxin to neutralize the toxin effects. Investigation into the *Salmonella* genome has revealed the presence of 24 TA loci, including five type I and 19 type II TA systems (20). DinJ-YafQ belongs to the type II TA system, is classified as a ribosome-dependent mRNA interferase, and is extensively studied in *Escherichia coli* (21, 22). The *dinJ-yafQ* genes form an operon with the antitoxin gene (*dinJ*) located upstream of the toxin gene (*yafQ*). The expression of this operon is directly repressed by the antitoxin DinJ or the DinJ-YafQ complex (23). Moreover, the DinJ-YafQ TA system has been reported to regulate persister formation by controlling indole production in *E. coli* (24). Similarly, the *Salmonella* Typhimurium TA system plays a role in mediating the phenotypic switch of persisters, contributing to persistent infections (25). However, the functions of *Salmonella* DinJ-YafQ TA system in the context of persistent infection and immune response remain poorly researched. Further exploration is needed to unravel its specific roles in *Salmonella* pathogenicity.

In our previous study, we identified that the insertion mutant of *dinJ* could cause an increase in the activation of inflammasome (26). However, the mechanisms by which DinJ inhibits inflammasome activation have not been thoroughly investigated. Therefore, we constructed *dinJ* deletion mutant and complemented strains, assessing inflammasome activation in both J774A.1 cells and bone marrow-derived macrophages (BMDMs). Significantly, the deletion of *dinJ* induced NLRP3-dependent inflammasome activation in *Salmonella*-infected macrophages. Furthermore, the deletion of *dinJ* also could increase the activation of inflammasome in the mice, contributing to the clearance of *Salmonella* by host immune systems. Collectively, our results suggest that the mediating activation of the NLRP3 inflammasome is beneficial for *Salmonella* infection.

RESULTS

Salmonella Enteritidis DinJ inhibits the activation of inflammasome in J774A.1 cells

Inhibition of the inflammasome activation could facilitate *Salmonella* to evade the clearance of the host immune system. In our previous studies, we used a transposon mutant library to screen the genes involved in inhibiting inflammasome activation and we found that DinJ insertion mutant strain increased the activation of inflammasome (26). In *S. Enteritidis*, we identified that the antitoxin *dinJ* and toxin *yafQ* were connected and showed 83% and 78% identity to the *E. coli* *dinJ* and *yafQ* genes, respectively (Fig. 1). RT-PCR yielded a product of the expected size with primers spanning the regions from *dinJ* to *yafQ*. No product was obtained using RNA as the template, while a product of the same size was observed when genomic DNA (gDNA) was used as the template (Fig. 1). This indicates that the *dinJ* and *yafQ* genes are co-transcribed in *S. Enteritidis*.

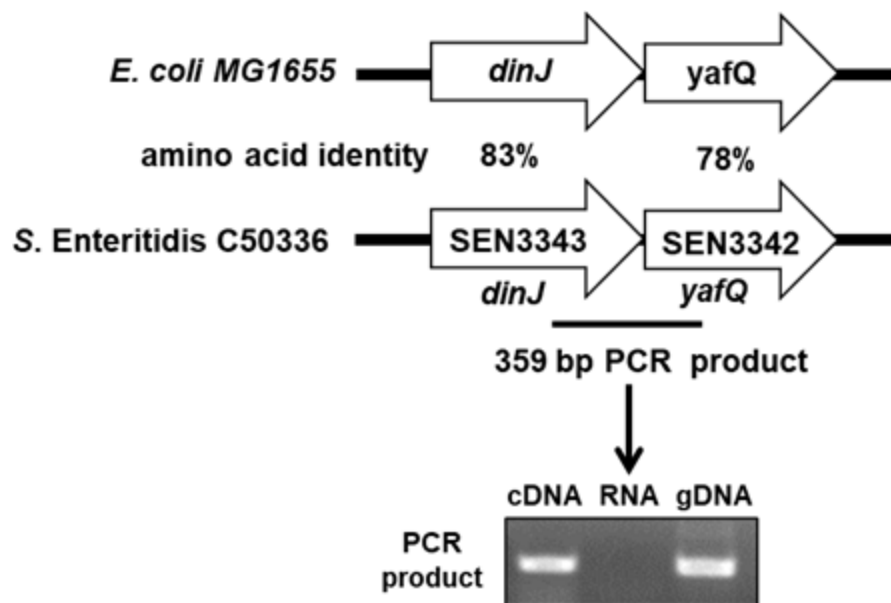


FIG 1 Co-transcription of *dinJ* and *yafQ*. A schematic representation of the predicted *dinJ-yafQ* genes in *S. Enteritidis*. Numbers represent the amino acid identity of the *S. Enteritidis* C50336 compared with *E. coli* MG1655. RT-PCR was used to determine the co-transcription of *dinJ* and *yafQ*.

Therefore, we constructed the *dinJ* deletion mutant and complemented strains to investigate cell death and the associated release of IL-1 β . As shown in Figure 2, cells infected with Δ *fliC* Δ *dinJ* released significantly increased levels of cell death (Fig. 2A) and IL-1 β secretion (Fig. 2B) compared to Δ *fliC*-infected cells, while the complemented strain was restored to the parental strain. The release of inflammasome-independent cytokine IL-6 showed no significant difference in J774A.1 cells infected with Δ *fliC* and its derived strains (Fig. 2C), indicating that DinJ specifically inhibited the release of inflammasome-dependent cytokines. Western blotting analysis also revealed a significant increase in the cleaved Caspase-1 product p10 subunits and IL-1 β in cells infected with Δ *fliC* Δ *dinJ* compared to Δ *fliC*-infected cells (Fig. 2D). Furthermore, growth curves showed no significant difference among Δ *fliC*, Δ *fliC* Δ *dinJ*, complemented, and empty vector-complemented strains (Fig. S1A). The deletion of *dinJ* did not affect the adhesion (Fig. S1B) and invasion (Fig. S1C) ability of *S. Enteritidis*, indicating that the inhibition of DinJ to inflammasome activation is unrelated to the growth, adhesion, and invasion ability of *Salmonella*. Taken together, these results indicated that DinJ inhibits the activation of inflammasome during *S. Enteritidis* infection.

Both NLRP3 and NLRC4 have been reported as the main inflammasomes involved in the host immune system against *Salmonella* infection (27, 28). Therefore, we measured the expression levels of NLRP3, NLRC4, and ASC to assess whether the inhibition of DinJ to inflammasome activation depended on the expression of these proteins. The expression levels of NLRP3, NLRC4, and ASC showed no significant differences among the cells infected with Δ *fliC*, Δ *fliC* Δ *dinJ*, complemented, and empty vector-complemented strains (Fig. 2D), suggesting that DinJ inhibits the activation of inflammasome independently of the expression levels of NLRP3, NLRC4, and ASC proteins.

DinJ inhibits the canonical inflammasome activation through Caspase-1

Primary BMDMs were obtained from wild-type (WT) C57BL/6 mice to confirm the inhibitory effect of DinJ on inflammasome activation. The cytotoxicity of BMDMs infected with Δ *fliC* Δ *dinJ* significantly increased compared to Δ *fliC*-infected BMDMs (Fig. 3A). Similar results were observed in IL-1 β secretion (Fig. 3B) and Caspase-1 activation (Fig. 3E). Furthermore, the expression levels of NLRP3 and NLRC4 showed no significant

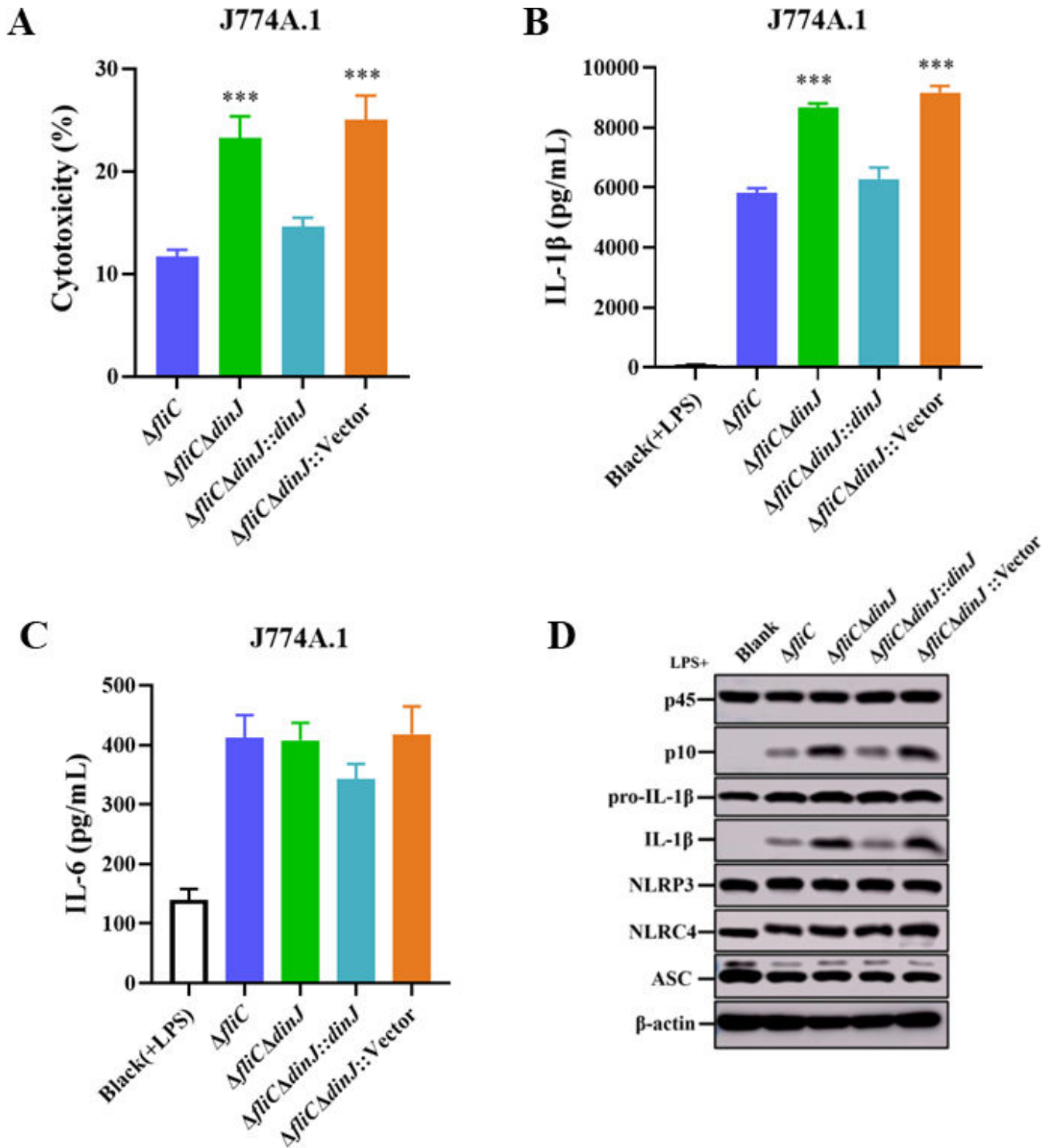


FIG 2 DinJ inhibits the activation of inflammasome in J774A.1 cells. (A) Evaluation of cytotoxicity in J774A.1 cells infected with Δ fliC, Δ fliC Δ dinJ, Δ fliC Δ dinJ::dinJ, or empty vector-complemented strain Δ fliC Δ dinJ::pMMB207 at an MOI of 100 for 4 h. (B and C) Enzyme-linked immunosorbent assay (ELISA) analysis assessing the secretion of IL-1 β (B) and IL-6 (C) in the infected cells. (D) Western blotting analysis of the cleavage of Caspase-1, secretion of IL-1 β , and the expression levels of NLRP3, NLRC4, and ASC in J774A.1 cells. β -actin was used as the loading control. Statistical significance was determined at *P* values of <0.001 (***) using the Student's *t*-test.

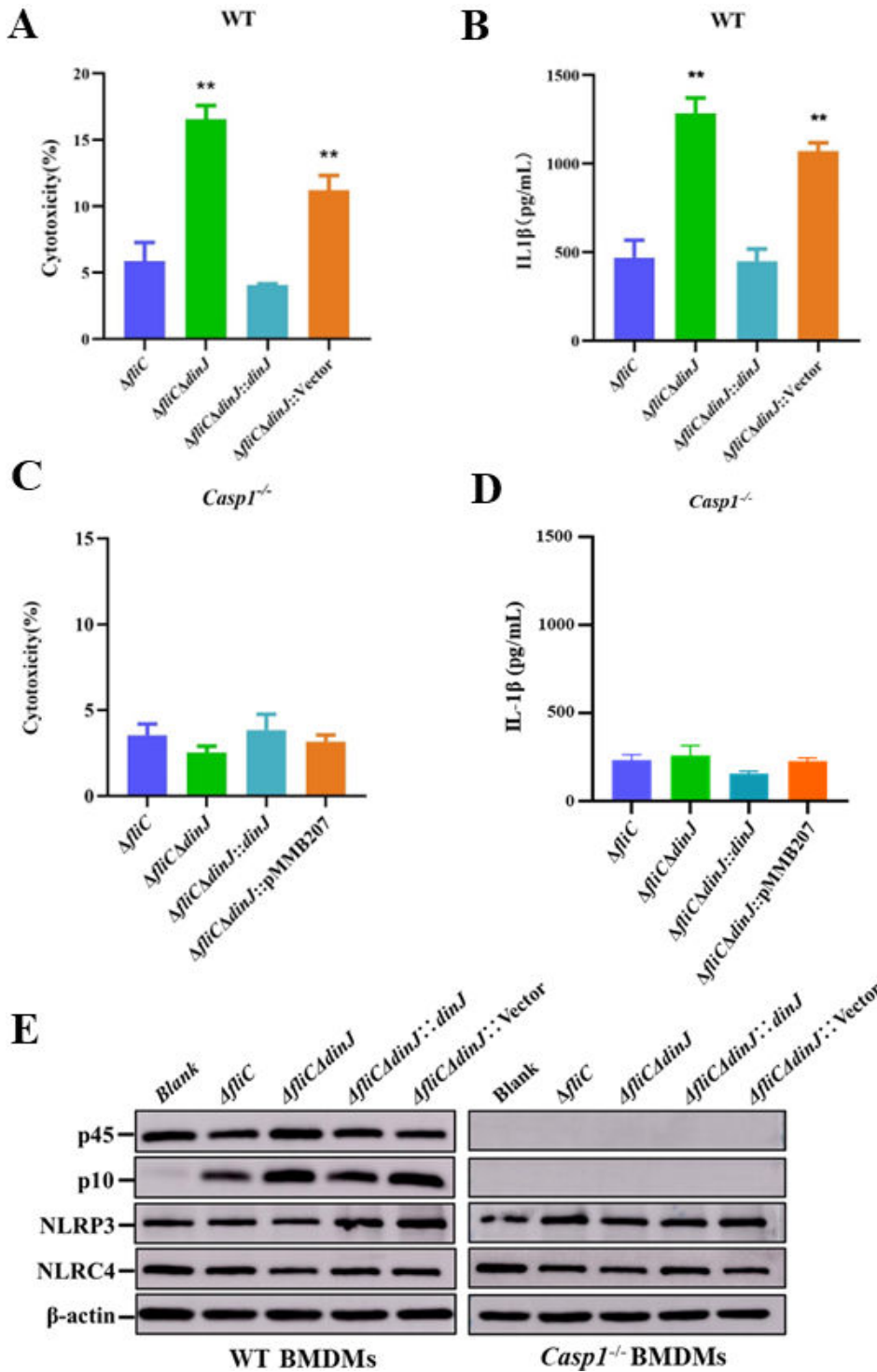


FIG 3 DinJ inhibits the canonical inflammasome activation through Caspase-1. (A and C) Cytotoxicity of the BMDMs obtained from WT (A) and *Casp1*^{-/-} (C) C57BL/6 mouse. (B and D) ELISA analysis measuring the secretion of IL-1β in BMDMs obtained from WT (B) and *Casp1*^{-/-} (D) C57BL/6 mouse. (E) Western blotting analysis evaluating the cleavage of Caspase-1 and the expression levels of NLRP3 and NLRC4. β-actin served as the loading control. Statistical significance was determined at *P* values of < 0.01 (**) using the Student's *t*-test.

difference in the BMDMs infected with the indicated strains (Fig. 3E). These results further confirm that the *S. Enteritidis* DinJ could inhibit the activation of inflammasome in primary macrophages from mice.

Caspase-1 is a key factor for the canonical inflammasome and essential for inflammasome-mediated inflammatory secretion. Therefore, the BMDMs obtained from *Casp-1* C57BL/6 mice were utilized to confirm whether DinJ inhibits inflammasome activation through Caspase-1. Cell death (Fig. 3C) and IL-1 β secretion (Fig. 3D) showed no significant differences among cells infected by the indicated strains. Western blotting failed to detect the expression of Caspase-1 and its cleaved subunit (Fig. 3E), further confirming the knockout of *casp1* gene. These results indicate that *S. Enteritidis* DinJ inhibits the canonical inflammasome activation.

DinJ specifically inhibits NLRP3-dependent inflammasome activation

To further confirm the role of DinJ in regulating inflammasome activation, we obtained the BMDMs from *Nlrp3*^{-/-} and *Nlrc4*^{-/-} C57BL/6 mice and infected them with Δ *fliC*, Δ *fliC* Δ *dinJ*, complemented, and empty vector-complemented strains. *Nlrc4*^{-/-}BMDMs infected with Δ *fliC* Δ *dinJ* and Δ *fliC* Δ *dinJ*::Vector strains lead to significantly increased cytotoxicity compared to the cells infected with Δ *fliC* (Fig. 4A). In contrast, *Nlrp3*^{-/-} BMDMs showed lower levels of cytotoxicity in response to infection by Δ *fliC* Δ *dinJ* and Δ *fliC* Δ *dinJ*::Vector strains, suggesting that the absence of *dinJ* induces activation of NLRP3 inflammasome. Furthermore, Δ *fliC* Δ *dinJ* and Δ *fliC* Δ *dinJ*::Vector strains induced significantly enhanced secretion of IL-1 β in *Nlrc4*^{-/-} BMDMs, while this phenotype was not observed in the *Nlrp3*^{-/-} BMDMs (Fig. 4D and E), indicating that NLRP3 inflammasome activation was essential for the increased secretion of IL-1 β . The secretion of inflammasome-independent cytokine IL-6 was not affected by DinJ in any of the BMDMs (Fig. 4C and F). Western blotting analysis also showed that the cleaved Caspase-1 subunit P10 and mature IL-1 β were increased in the Δ *fliC* Δ *dinJ*-infected WT and *Nlrc4*^{-/-} BMDMs compared to those infected by Δ *fliC*, but not in the *Nlrp3*^{-/-} BMDMs (Fig. 4G and H). All these observed phenotypes in BMDMs infected with the complemented strain were restored to Δ *fliC*-infected BMDMs. Taken together, these results indicate that *S. Enteritidis* DinJ protein specifically inhibits NLRP3-dependent inflammasome activation.

DinJ can be translocated into host cells

Salmonella secretion systems can deliver effectors into host cells to modulate inflammasome activation. Therefore, we employed fluorescence resonance energy transfer (FRET) to verify the translocation of DinJ protein. The DinJ protein was fused with the β -lactamase TEM-1, and the fused protein was confirmed by Western blotting (Fig. 5A). As shown in Fig. 5B, blue fluorescent cells were observed in the HeLa cells infected with Δ *fliC*-pCX340-*dinJ*, while no blue fluorescent cells were observed in the blank control and HeLa cells infected with Δ *fliC*-pCX340. Moreover, we randomly selected more than 600 cells to calculate the percentage of the cells exhibiting blue fluorescent, and the transfer efficiency was approximately 3.9% (Fig. 5C). These results demonstrate that DinJ can be translocated into host cells during infection.

DinJ enables *S. Enteritidis* evasion of the NLRP3 inflammasome *in vivo*

Several bacterial and viral pathogens can inhibit the activation of NLRP3 inflammasome to evade clearance by the host immune system. To confirm the function of DinJ in the virulence of *S. Enteritidis*, we investigated whether DinJ could inhibit inflammasome activation and antibacterial defense *in vivo*. The mice were pretreated with streptomycin before being orally infected with Δ *fliC* and Δ *fliC* Δ *dinJ*. The secretion of inflammasome-dependent cytokines IL-1 β and IL-18, as well as the inflammasome-independent cytokine IL-6, in the serum was assessed. Compared to Δ *fliC*-infected mice, IL-1 β (Fig. 6A) and IL-18 (Fig. 6B) were significantly increased in mice infected with Δ *fliC* Δ *dinJ*, while the secretion of IL-6 showed no significant difference in the infected mice (Fig. 6C).

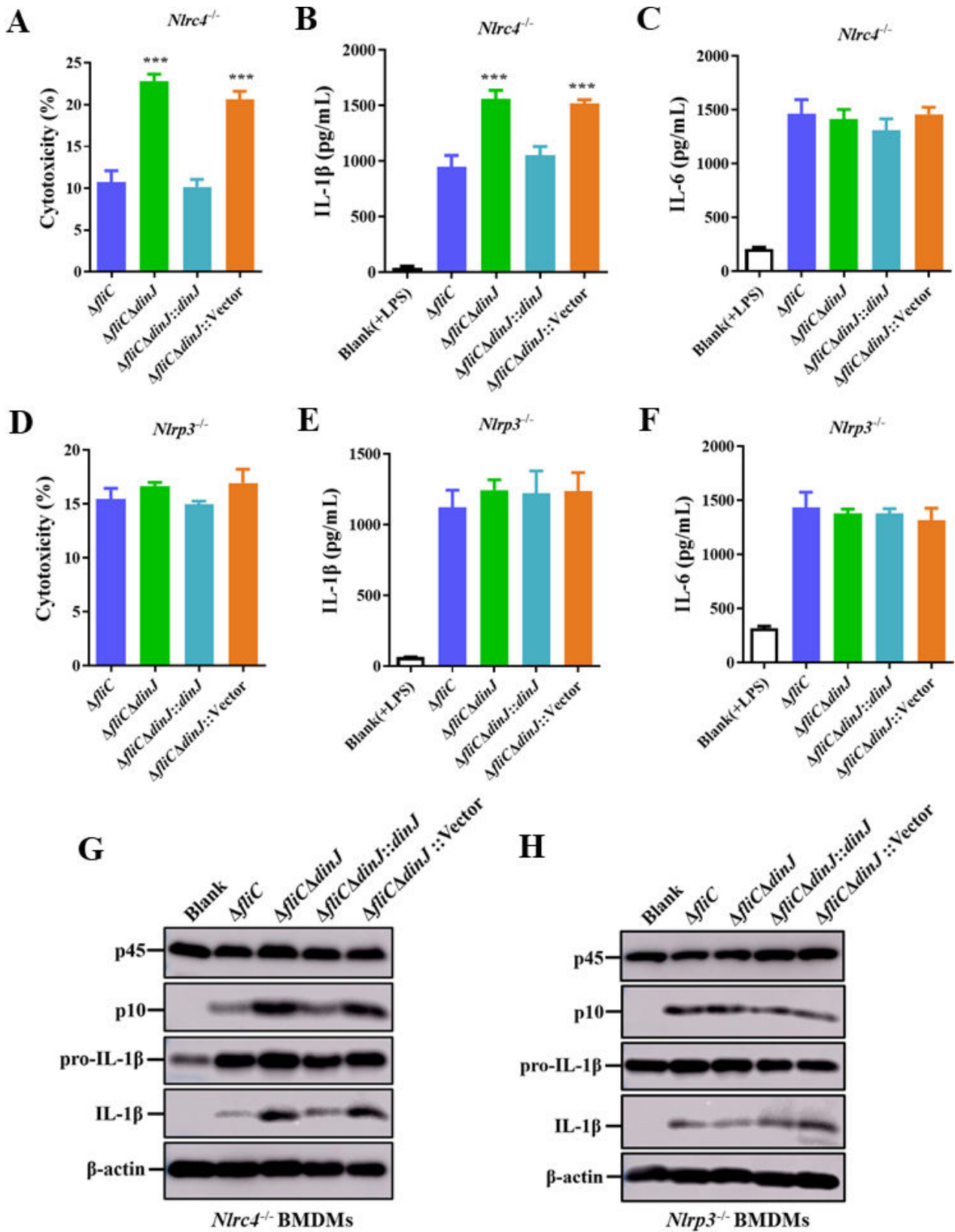


FIG 4 Deletion of *S. Enteritidis* *dinJ* induces NLRP3 inflammasome activation. (A–C) Evaluation of cytotoxicity (A), IL-1 β (B), and IL-6 (C) levels in the supernatants of *Nlrp4^{-/-}* BMDMs infected with the indicated strains. (D–F). Evaluation of cytotoxicity (D), IL-1 β (E), and IL-6 (F) levels in the supernatants of *Nlrp3^{-/-}* BMDMs infected with the indicated strains. (G and H) Western blotting analysis of Caspase-1 activation and IL-1 β secretion in *Nlrp4^{-/-}* BMDMs (G) and *Nlrp3^{-/-}* BMDMs (H) infected with the indicated strains. Statistical significance was determined at *P* values of < 0.01 (**). using the Student's *t*-test.

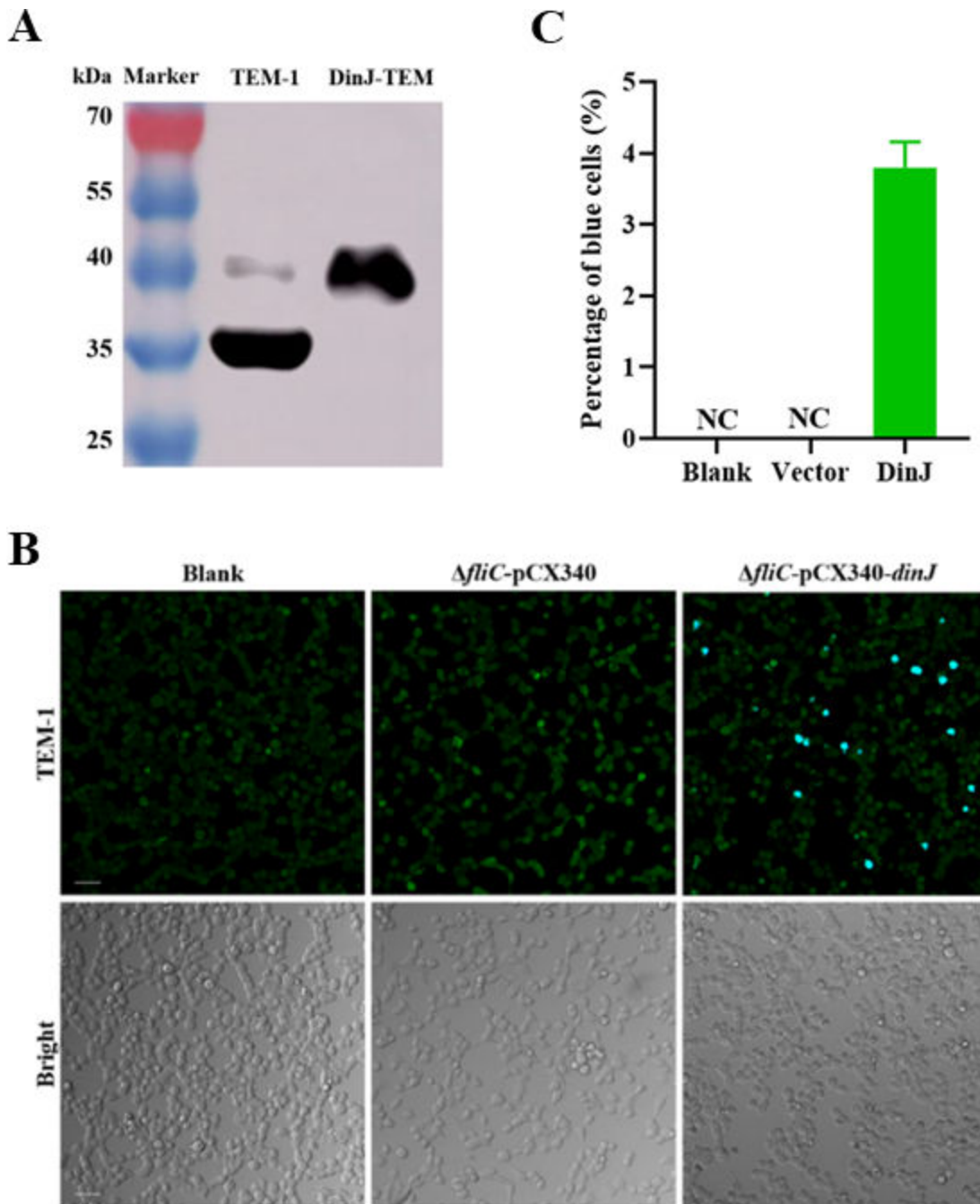


FIG 5 DinJ can be translocated into HeLa cells. (A) Western blotting analysis confirming the presence of DinJ-TEM-1 fusion protein. (B) HeLa cells were infected with *S. Enteritidis* $\Delta fliC$ strains carrying either pCX340 or pCX340-*dinJ*. Translocation of the DinJ-TEM-1 fusion protein into HeLa cells results in the cleavage of CCF2-AM, leading to the emission of blue fluorescence, while the uncleaved CCF2-AM emitted green fluorescence. Scale bar = 50 μ m. (C) The percentage of the blue fluorescent cells. Three images were captured per cell well, and approximately 600 cells were counted to calculate the percentage of the blue fluorescent cells.

Furthermore, bacterial burdens in the liver and spleen of the infected mice were also evaluated. The results showed that the bacterial CFUs of the liver (Fig. 6D) and spleen (Fig. 6E) in $\Delta fliC\Delta dinJ$ -infected mice were significantly lower than those in $\Delta fliC$ -infected mice. These results indicate that the *S. Enteritidis* DinJ protein inhibits NLRP3-dependent inflammasome activation and contributes to *Salmonella* evading the clearance of the host immune system *in vivo*.

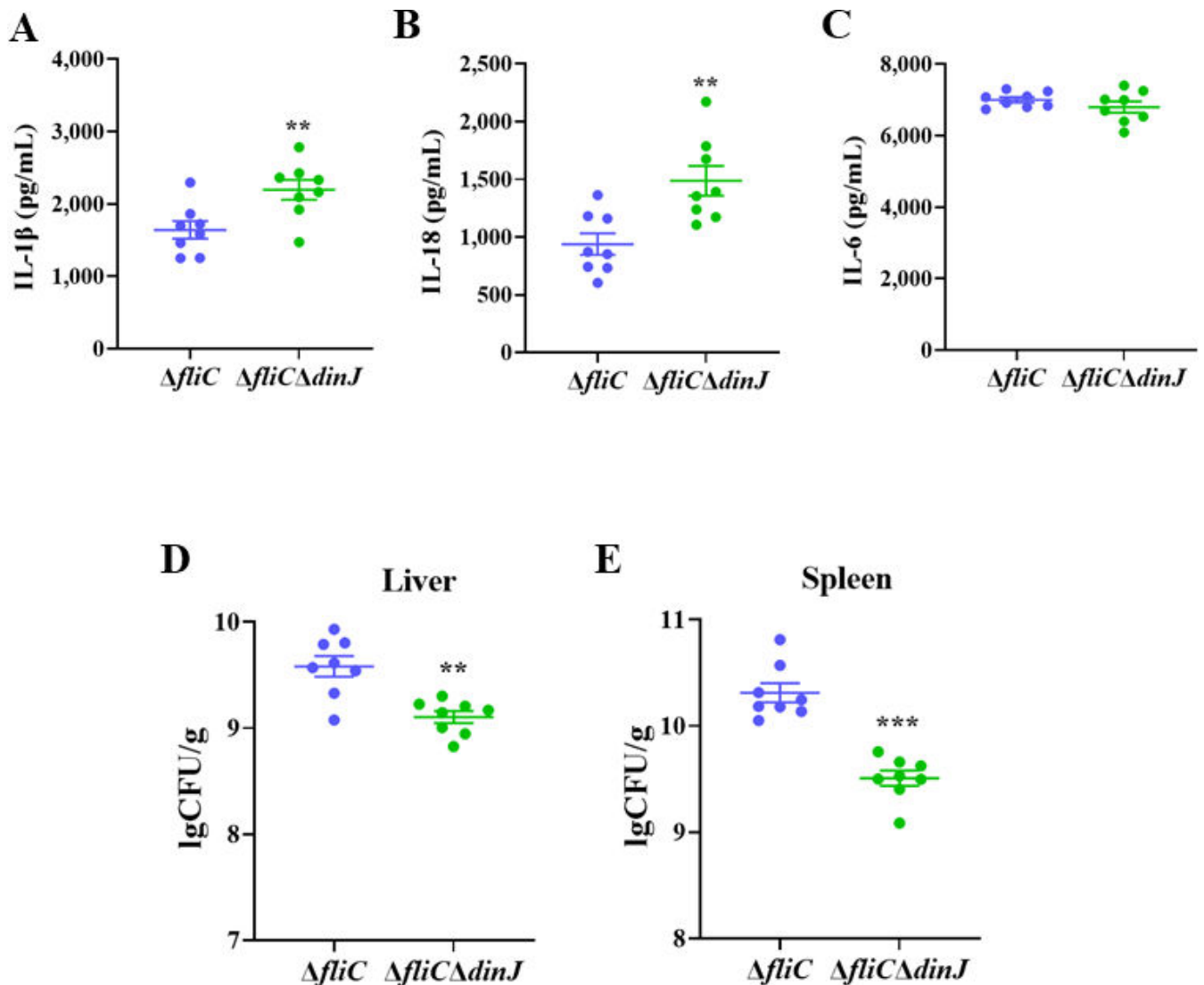


FIG 6 DinJ inhibits inflammasome activation to evade clearance of host immune system. (A–C) ELISA analysis of IL-1 β (A), IL-18 (B), and IL-6 (C) in the serum of WT mouse infected with $\Delta fliC$ or $\Delta fliC\Delta dinJ$. (D and E) Assessment of bacterial loading in the liver (D) and spleen (E) of WT mouse infected with $\Delta fliC$ or $\Delta fliC\Delta dinJ$. Statistical significance was determined at P values of <0.01 (**) or <0.001 (***) using the Student's t -test.

DISCUSSION

During infection, the host immune system recognizes *Salmonella* and activates both the NLR4 and NLRP3 inflammasome, contributing to the host defense against *Salmonella* infection (29, 30). The NLR4 inflammasome recognizes *Salmonella* flagellin, T3SS needle, and basal rod proteins, while the NLRP3 inflammasome responds to various structurally and chemically diverse signals (4, 31, 32). Deletion or downregulation of the flagellin gene impairs the ability to induce NLR4 inflammasome activation in *Salmonella*-infected macrophages (33, 34). Furthermore, purified flagellin could activate Caspase-1 in an NLR4-dependent manner (32, 34). Another study demonstrated that *Salmonella* flagellin induces NLR4 inflammasome activation in human macrophages (35). In the late logarithmic growth phase, flagellin genes are highly expressed, inducing robust inflammasome activation within 1 h *in vitro* (30). Therefore, in our previous studies, we used the *fliC* deletion mutant strain as the parental strain to construct the insertion mutant library and to screen for genes of *S. Enteritidis* involved in inhibiting inflammasome activation (17, 26). We have demonstrated that *S. Enteritidis* SiiD and GalE protein

could inhibit the activation of inflammasome in an NLRP3-dependent way (17, 26). In this study, we report for the first time that *S. Enteritidis* DinJ can inhibit NLRP3-dependent inflammasome activation. Notably, all of the *S. Enteritidis* SiiD, GalE, and DinJ proteins inhibit NLRP3-dependent canonical inflammasome activation, which is likely due to the deletion of the *fliC* gene, which could avoid the NLRC4 inflammasome activation. Therefore, we only screened for the proteins that inhibit NLRP3-dependent inflammasome activation.

The YafQ-DinJ system is classified as a type II toxin-antitoxin system and is recognized for its role in regulating persister formation. YafQ is an endoribonuclease-type toxin, exerting strict control on the expression of *tnaA*. Conversely, DinJ is an antitoxin protein, strongly inhibiting the effects of toxin (24). Under a stress condition, the antitoxin protein DinJ may undergo degradation by the protease, allowing the toxin protein YafQ to degrade the transcripts of target genes. However, there are fewer works to explore the contribution of YafQ-DinJ TA systems in *S. Enteritidis* infection. Our previous study found that the insertion mutant of *SEN3342* in *S. Enteritidis* caused the increased activation of the inflammasome, sharing 83% identity with the *dinJ* gene in *E. coli* (Fig. 1). Generally, the deletion of the antitoxin gene would increase the level of free toxin, leading to growth arrest or cell death (36). However, in our current study, the deletion mutant strain of antitoxin gene *dinJ* exhibited no significant difference in growth curves compared to the parent strain $\Delta fliC$ in Luria-Bertani broth (LB) medium (Fig. S1). Similar results were observed in other type II TA systems Hha-TomB of *S. Typhimurium* and *E. coli*, where the deletion of *hha* or *tomB* did not affect growth in the minimal or complex medium compared to the WT (37, 38). However, it's noteworthy that Hha was found to induce cell death under biofilm or acid stress conditions (39, 40). Previous findings also showed that DinJ-YafQ could be induced under a high temperature (24). Therefore, it is plausible that $\Delta fliC\Delta dinJ$ may exhibit a distinct growth curve under stress conditions compared to $\Delta fliC$.

Toxin-antitoxin systems and their impact on *Salmonella* infection have been studied extensively. *S. Typhimurium* type II TA systems Hha-TomB have emerged as a significant regulator of virulence and host immune response (41). In our study, the deletion of the antitoxin *dinJ* resulted in higher cytotoxicity in J774A.1 cells compared to the parent strain $\Delta fliC$ -infected cells; whereas, no attenuation was observed in mice infected with $\Delta fliC\Delta dinJ$. Moreover, the deletion of the toxin gene *yafQ* did not affect the cytotoxicity of the infected cells (Fig. S2), suggesting that the elevated cytotoxicity caused by $\Delta dinJ$ was not attributed to an increase in the expression of *yafQ*. Furthermore, our investigation revealed that the deletion of the *dinJ* gene did not influence the adhesion and invasion capabilities of *S. Enteritidis*. This observation contrasts with the impact of deleting the antitoxin gene *tomB*, which did not affect the adhesion rate but impaired the invasion rate in $\Delta tomB$ compared to the WT (41). These findings highlight the complex and multifaceted nature of TA systems in the context of host-pathogen interactions, in which different components may contribute differently to virulence and immune evasion mechanisms. Taken together, the higher cytotoxicity observed in $\Delta fliC\Delta dinJ$ -infected cells suggests a potential role for DinJ in modulating inflammasome activation. Further exploration of these mechanisms will provide valuable insights into the nuanced dynamics of bacterial pathogenesis and immune evasion.

To further elucidate the mechanisms associated with inflammasome activation and immune escape attributes of DinJ, we conducted *in vivo* analyses. The results showed a higher production of inflammatory cytokines IL-1 β (Fig. 6A) and IL-18 (Fig. 6B) in the serum of mice infected with $\Delta fliC\Delta dinJ$ as compared to $\Delta fliC$, indicating an increased inflammasome activation *in vivo*. Additionally, we observed a significantly lower bacterial load in the liver and spleen of mice infected with $\Delta fliC\Delta dinJ$ relative to $\Delta fliC$ (Fig. 6D and E). These findings align with previous studies suggesting that enhanced inflammasome activation and secretion of IL-1 β and IL-18 contribute to the clearance of *Salmonella* *in vivo* (17, 26). To promote host survival, bacteria have evolved various mechanisms to escape host inflammasome signaling and clearance. Previous studies have shown that Dam can increase inflammasome activation, while GalE and SiiD can inhibit NLRP3

inflammasome activation (17, 26, 42). Altogether, our results indicate that the deletion of *dinJ* induces the NLRP3 inflammasome activation and triggers the immune responses that facilitate the clearance of *S. Enteritidis*. What is the mechanism by which *dinJ*-deficient *S. Enteritidis* induces NLRP3 inflammasome activation? The expression levels of NLRP3 and NLRC4 showed no significant differences in the BMDMs infected with the $\Delta fliC$ or $\Delta fliC\Delta dinJ$ strains (Fig. 2D), indicating the inhibition of NLRP3 inflammasome activation by DinJ is independent of regulating its expression. *S. Typhimurium* AcnB and *S. Enteritidis* SiiD have been reported to inhibit NLRP3 inflammasome activation through mtROS (3, 17). Furthermore, the bacterial metabolites potentially serve as the signals that trigger inflammasome activation (3). Therefore, further studies will explore the mechanism of how DinJ inhibits NLRP3 inflammasome activation.

Our previous studies also showed that *S. Enteritidis* could translocate Dam, GalE, and SiiD proteins into the cells, and modulate the activation of inflammasome. In this study, we also found that DinJ could be translocated into the cells with a low translocation efficiency of 3.9%. TEM-1-based effector translocation assays identified nine candidate effectors of *Edwardsiella piscicida*, which also showed a low translocation efficiency of around 5% (43). The translocation efficiency of GalE was 4.3%, which also could inhibit the activation of inflammasome (26). TEM-1 assays rely on β -lactamase, which is a relatively large tag that may influence the translocation efficiency of the protein.

This study addressed a significant gap in previous research by investigating the role of toxin-antitoxin systems in NLRP3 inflammasome activation. Despite the well-documented influence of *Salmonella* flagellin, T3SS, and T4SS effector proteins, along with metabolic signaling pathways, on inflammasome activation (3, 4, 16, 17, 44), the exploration of toxin-antitoxin systems in inflammasome activation was limited. In summary, our studies revealed that the antitoxin protein DinJ, for the first time, can inhibit NLRP3-dependent inflammasome activation. Supported by *in vivo* experiments, the results uncovered the involvement of DinJ in assisting *Salmonella* to evade the host immune system by suppressing inflammasome activation. This newly established link between the inhibition of NLRP3 inflammasome activation and bacterial immune escape deepens our understanding of intricate host-pathogen interactions, confirming the significant roles of inflammasome activation in the persistence of *Salmonella*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The *S. Enteritidis* C50336 was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). *S. Enteritidis* C50336, *E. coli*, and derivatives were cultured at 37°C in LB medium with the appropriate antibiotics.

Construction of *dinJ* mutant and complemented strains

The deletion mutant strain C50336 $\Delta fliC\Delta dinJ$ was constructed by double exchange of homologous recombination with the plasmid pDM4 based on the parental strain C50336 $\Delta fliC$ (17, 26). Briefly, the primers listed in Table 2 were used to amplify the upstream and downstream fragments of *dinJ*. The products were cloned into the suicide plasmid pDM4 by the ClonExpress MultiS One Step Cloning Kit (Vazyme, Nanjing, China). The recombinant plasmid $\Delta dinJ::pDM4$ was transferred into C50336 $\Delta fliC$ by conjugation and was screened for the *dinJ* deletion mutant strain on LB plates with 15% sucrose. The complemented strain was constructed with the plasmid pMMB207. The PCR products of the *dinJ* gene were ligated into plasmid pCX340 with the restriction enzymes *Nde* I and *Kpn* I, and then the recombinant plasmids were electroporated into $\Delta fliC\Delta dinJ$ and $\Delta dinJ$ for FRET.

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source
<i>Escherichia coli</i> strains		
X7213λpir	Host for π requiring plasmids, conjugal donor	(17)
<i>Salmonella</i> Enteritidis strains		
C50336	Wild type	China Medical Culture Collection Center, National Institute for the Control of Pharmaceutical and Biological
C50336Δ <i>fliC</i>	C50336, in-frame deletion in <i>fliC</i> (parent strain)	(17)
Δ <i>fliC</i> Δ <i>dinJ</i>	Δ <i>fliC</i> , in-frame deletion in <i>dinJ</i>	This study
Δ <i>fliC</i> Δ <i>dinJ</i> :: <i>dinJ</i>	Δ <i>fliC</i> Δ <i>dinJ</i> with pMMB207 expressing the <i>dinJ</i> gene, Cm ^r	This study
Δ <i>fliC</i> Δ <i>dinJ</i> ::Vector	Δ <i>fliC</i> Δ <i>dinJ</i> with pMMB207, Cm ^r	This study
Δ <i>fliC</i> -pCX340	Δ <i>fliC</i> with pCX340, Tet ^r	This study
Δ <i>fliC</i> -pCX340- <i>dinJ</i>	Δ <i>fliC</i> with pCX340 expressing the <i>dinJ</i> gene, Tet ^r	This study
Plasmids		
pDM4	Suicide vector, <i>pir</i> dependent, R6K, <i>SacBR</i> , Cm ^r	(45, 46)
pMMB207	Expression vector, Cm ^r	(47)
pMMB207- <i>dinJ</i>	pMMB207 derivative containing <i>dinJ</i> , Cm ^r	This study
pCX340	pBR322 derivative, cloning vector used to fuse effectors to TEM-1-β-lactamase, Tet ^r	(33, 48)
pCX340- <i>dinJ</i>	pCX340 derivative containing <i>dinJ</i> , Cm ^r	This study

Mice and cell culture conditions

Wild-type (WT) specific pathogen-free (SPF) female C57BL/6 mice were purchased from Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). *Casp1*^{-/-}, *Nlrp3*^{-/-}, and *Nlrc4*^{-/-} mice were bred based on the C57BL/6 strain mice and were purchased from Shanghai Model Organisms Center, Inc (Shanghai, China).

J774A.1 and HeLa cells were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). The BMDMs were obtained from the femoral and tibial bone marrow of the mice, and their maturation was induced with 25 ng/mL macrophage colony-stimulating factor (M-CSF) (PeproTech, Rocky Hill, NJ, USA). All cells were grown in a cell incubator at 37°C with 5% CO₂ and were cultured in complete Dulbecco's

TABLE 2 Primers used in this study

Primer name	Primer sequence (5' to 3')	Target
<i>dinJ</i> -yafQ-F	AGGGGAAAAGGGTGGTGA	Co-transcribed
<i>dinJ</i> -yafQ-R	CGACGAAACGCTGAAGGA	Co-transcribed
<i>dinJ</i> -up-F	GAGCGGATAACAATTTGTGGAATCCCGGGATGGAGGTTACGCGCGGGACGCATAA	Deletion mutant
<i>dinJ</i> -up-R	CCCATAGTCACATCGATTAGTCTCTCTGTACTGTGT	Deletion mutant
<i>dinJ</i> -down-F	CTAATCGATGTGACTATGGGGCAAAGGGAAATGA	Deletion mutant
<i>dinJ</i> -down-R	AGCGGAGTGTATATCAAGCTTATCGATACCGCAGGTGTTAGGGCTTATCTATTCC	Deletion mutant
<i>dinJ</i> -in-F	ATGTCTGGCTGAAATGGGG	Deletion mutant
<i>dinJ</i> -in-R	ATCAACGCCAGCTTCGCTAT	Deletion mutant
<i>dinJ</i> -out-F	AGCCGAACGAAAGCGAAGAAAC	Deletion mutant
<i>dinJ</i> -out-R	CCGCAATCTGCTGTTACTCAATCA	Deletion mutant
pCX340-F	AGACAATCTGTGTGGGCACTCGACC	FRET
pCX340-R	TTCTGAGAATAGTGTATGCGGGCAGC	FRET
pCX340- <i>dinJ</i> -F	AATAAGGAGGAATAACATATGATGGCTGCAAATGCGCTTGTTCGTG	FRET
pCX340- <i>dinJ</i> -R	CGAATTCTCCGCGGAGGTACCGATCCCTAACTGGTCAAACAAA	FRET

modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

Salmonella infection

Overnight cultured *S. Enteritidis* and derivatives were diluted into fresh LB medium with a concentration of 1:100 and were cultured at 37°C for 3.5 h. The J774A.1 cells or BMDMs were seeded into 48-, 24-, or 12-well with a concentration of 1×10^5 , 2×10^5 , or 5×10^5 cells per well and were cultured at 37°C with 5% CO₂ for 24 h. The J774A.1 cells or BMDMs were pre-activated for 5 h with lipopolysaccharide (LPS) diluted in Opti-MEM reduced serum medium (Gibco Thermo Fisher Scientific, Waltham, MA, USA). After they were washed twice with phosphate-buffered saline (PBS), the bacteria strains were added to J774A.1 cells and BMDMs at a multiplicity of infection (MOI) of 100:1 and were incubated at 37°C for 1.5 h. The cell supernatant was discarded, and the cells were incubated in Opti-MEM with 50 µg/mL gentamicin at 37°C for another 3 h.

Adhesion and invasion assays

After infecting the J774A.1 cells with the indicated strains for 30 min, the cells were washed twice with PBS and were subsequently incubated in PBS with 0.2% Triton X-100 at 37°C for 15 min. The cell lysate was collected and diluted with PBS for the determination of bacterial adhesion ratio. For assessment of bacterial invasion, the J774A.1 cells were infected with the indicated strains for 30 min, washed twice with PBS, and then incubated in DMEM with 100 µg/mL gentamicin at 37°C for 1 h. Subsequently, the cells were incubated in PBS with 0.2% Triton X-100 at 37°C for 15 min. The resulting cell lysate was collected and diluted with PBS for determination of bacterial invasion ratio.

Cytotoxicity analysis

After the J774A.1 cells or BMDMs were infected with bacteria as described above, the supernatants were harvested for cytotoxicity analysis. Quantification of cytotoxicity was performed using the LDH Cytotoxicity Assay Kit according to the manufacturer's instructions (Beyotime, Haimen, China).

ELISA

After the J774A.1 cells or BMDMs were infected with bacteria as described above, the supernatants were harvested for quantification of cytokine. Expression of the cytokines IL-1β, IL-18, and IL-6 were quantified using Mouse IL-1 beta/IL-1F2 DuoSet ELISA, Mouse IL-18 DuoSet ELISA, and Mouse IL-6 DuoSet ELISA according to the manufacturer's manual (R&D Systems, Minneapolis, MN, USA).

Western blotting analysis

Following the infection of J774A.1 cell or BMDMs with bacteria as described above and harvesting of supernatants, the cells were lysed with cell lysis buffer for Western blotting. The supernatants and lysates were mixed, supplemented with a protease inhibitor, and subjected to centrifugation at 2,000 rpm for 5 min to remove the bacteria and cell debris. The cleared supernatants were then mixed with an equal volume of methanol and a 0.25 volume of chloroform. Following centrifugation at 12,000 rpm for 5 min, the upper aqueous phase was removed, and an additional equal volume of methanol was added. The supernatants were discarded after centrifugation, the remaining protein pellets were dried at 55°C for 10 min, and then resuspended in $1 \times$ SDS-PAGE loading buffer. Protein samples were boiled at 100°C for 10 min, separated by 12% SDS-PAGE, transferred onto digested nitrocellulose membranes, and blocked in PBS with 3% skim milk for 1.5 h. Membranes were then incubated with anti-Caspase-1 p10 antibody (AG-20B-0044-C100, AdipoGen, San Diego, CA, USA), anti-NLRP3 antibody (AG-20B-0014-C100, AdipoGen, San Diego, CA, USA), anti-NLRC4 antibody (ab201792, Abcam, Cambridge, UK), anti-IL-1β

antibody (12242S, Cell Signaling Technology, Danvers, MA, USA), and anti- β -actin antibody (A5441, Sigma-Aldrich, St. Louis, MO, USA). Following incubation, the membranes were exposed using Amersham Imager 600 Imaging System with enhanced chemiluminescence (ECL) substrate exposure.

FRET

The overnight cultured *S. Enteritidis* strains Δ *fliC*-pCX340 and Δ *fliC*-pCX340-*dinJ* were diluted into fresh LB medium with a concentration of 1:100 and were cultured for 3.5 h. Bacteria were washed twice with DMEM and infected HeLa cells with an MOI of 100:1. The bacteria were removed after infection for 3 h, and the HeLa cells were washed four times with DMEM before being subjected to another 4-h infection in DMEM. Following a single wash with DMEM, CCF2/AM was added, and the cells were incubated for 2 h in the dark. Cell images were acquired using Leica confocal microscope with excitation at 405 nm and emission at 450–470 nm and 520–540 nm.

Mouse infection

Female C57BL/6 mice (6–8 wk) were randomly divided into three groups, each consisting of eight mice. After a 4-h period of dietary restriction, each group orally received 7.5 mg of streptomycin, followed by a 20-h restoration of the regular diet. After another 4-h dietary restriction, each group was orally infected with 300 μ L of Δ *fliC* or Δ *fliC* Δ *dinJ* at a dose of 5×10^6 CFU per mouse. Control mice received 300 μ L PBS under the same conditions. After infection for 5 d, the mice were euthanized, and the serum, liver, and spleen samples were collected. The levels of IL-1 β , IL-18, and IL-6 in the serum were quantified using ELISA. The liver and spleen tissues were homogenized and diluted in PBS to calculate the bacterial load in the organs.

Statistical analysis

All experiments were conducted a minimum of three times. All results were expressed as the mean \pm SEM using GraphPad Prism 8.0 software. The statistical analysis used a Student's *t*-test; *** represents a *P* value < 0.001, ** is a *P* value < 0.01, and * is a *P* value < 0.05.

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ETHICS APPROVAL

All animal experiments were approved by the Animal Welfare and Ethics Committees of Yangzhou University and complied with the guideline of the Institutional Administrative Committee and Ethics Committee of Laboratory Animals (IACU license number: YZUEWLL-201811-001). All animals were handled with care to minimize distress during the experiments.

ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Fig. S1 (IAI00505-23-s0001.TIF). Impact of DinJ on *S. Enteritidis* growth, adhesion, and invasion.

Fig. S2 (IAI00505-23-s0002.TIF). Cytotoxicity of *S. Enteritidis* *yafQ* deletion mutant strain.

Supplemental legends (IAI00505-23-s0003.docx). Legends for Fig. S1 and S2.

REFERENCES

- Rathinam VAK, Fitzgerald KA. 2016. Inflammasome complexes: emerging mechanisms and effector functions. *Cell* 165:792–800. <https://doi.org/10.1016/j.cell.2016.03.046>
- Roberts TL, Idris A, Dunn JA, Kelly GM, Burnton CM, Hodgson S, Hardy LL, Garceau V, Sweet MJ, Ross IL, Hume DA, Stacey KJ. 2009. HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA. *Science* 323:1057–1060. <https://doi.org/10.1126/science.1169841>
- Wynosky-Dolfi MA, Snyder AG, Philip NH, Doonan PJ, Poffenberger MC, Avizonis D, Zwack EE, Riblett AM, Hu B, Strowig T, Flavell RA, Jones RG, Freedman BD, Brodsky IE. 2014. Oxidative metabolism enables *Salmonella* evasion of the NLRP3 inflammasome. *J Exp Med* 211:653–668. <https://doi.org/10.1084/jem.20130627>
- Zhao Y, Yang JL, Shi JJ, Gong YN, Lu QH, Xu H, Liu LP, Shao F. 2011. The NLR4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. *Nature* 477:596–600. <https://doi.org/10.1038/nature10510>
- Oliveira SHP, Canetti C, Ribeiro RA, Cunha FQ. 2008. Neutrophil migration induced by IL-1beta depends upon LTB4 released by macrophages and upon TNF-alpha and IL-1beta released by mast cells. *Inflammation* 31:36–46. <https://doi.org/10.1007/s10753-007-9047-x>
- Shi JJ, Zhao Y, Wang K, Shi XY, Wang Y, Huang HW, Zhuang YH, Cai T, Wang FC, Shao F. 2015. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature* 526:660–665. <https://doi.org/10.1038/nature15514>
- Higa N, Toma C, Koizumi Y, Nakasone N, Nohara T, Masumoto J, Kodama T, Iida T, Suzuki T. 2013. *Vibrio parahaemolyticus* effector proteins suppress inflammasome activation by interfering with host autophagy signaling. *PLoS Pathog* 9:e1003142. <https://doi.org/10.1371/journal.ppat.1003142>
- Ratner D, Orning MPA, Proulx MK, Wang D, Gavrillin MA, Wewers MD, Alnemri ES, Johnson PF, Lee B, Meccas J, Kayagaki N, Goguen JD, Lien E. 2016. The *Yersinia pestis* effector YopM inhibits pyrin inflammasome activation. *PLoS Pathog* 12:e1006035. <https://doi.org/10.1371/journal.ppat.1006035>
- Jiang JT, Wang WW, Sun F, Zhang YX, Liu Q, Yang DH. 2021. Bacterial infection reinforces host metabolic flux from arginine to spermine for NLRP3 inflammasome evasion. *Cell Rep* 34:108832. <https://doi.org/10.1016/j.celrep.2021.108832>

10. Bierschenk D, Boucher D, Schroder K. 2017. *Salmonella*-induced inflammasome activation in humans. *Mol Immunol* 86:38–43. <https://doi.org/10.1016/j.molimm.2016.11.009>
11. Crowley SM, Knodler LA, Vallance BA. 2016. *Salmonella* and the inflammasome: battle for intracellular dominance. *Curr Top Microbiol Immunol* 397:43–67. https://doi.org/10.1007/978-3-319-41171-2_3
12. Zhang K, Riba A, Nietschke M, Torow N, Repnik U, Pütz A, Fulde M, Dupont A, Hensel M, Hornef M. 2018. Minimal SPI1-T3SS effector requirement for *Salmonella* enterocyte invasion and intracellular proliferation *in vivo*. *PLoS Pathog* 14:e1006925. <https://doi.org/10.1371/journal.ppat.1006925>
13. Jiang L, Wang P, Song X, Zhang H, Ma S, Wang J, Li W, Lv R, Liu X, Ma S, Yan J, Zhou H, Huang D, Cheng Z, Yang C, Feng L, Wang L. 2021. *Salmonella* Typhimurium reprograms macrophage metabolism via T3SS effector SopE2 to promote intracellular replication and virulence. *Nat Commun* 12:879. <https://doi.org/10.1038/s41467-021-21186-4>
14. Bierschenk D, Monteleone M, Moghaddas F, Baker PJ, Masters SL, Boucher D, Schroder K. 2019. The *Salmonella* pathogenicity island-2 subverts human NLRP3 and NLRC4 inflammasome responses. *J Leukoc Biol* 105:401–410. <https://doi.org/10.1002/JLB.MA0318-112RR>
15. Naseer N, Egan MS, Reyes Ruiz VM, Scott WP, Hunter EN, Demissie T, Rauch I, Brodsky IE, Shin S. 2022. Human NAIP/NLRC4 and NLRP3 inflammasomes detect *Salmonella* type III secretion system activities to restrict intracellular bacterial replication. *PLoS Pathog* 18:e1009718. <https://doi.org/10.1371/journal.ppat.1009718>
16. Hu GQ, Song PX, Chen W, Qi S, Yu SX, Du CT, Deng XM, Ouyang HS, Yang YJ. 2017. Critical role for *Salmonella* effector SopB in regulating inflammasome activation. *Mol Immunol* 90:280–286. <https://doi.org/10.1016/j.molimm.2017.07.011>
17. Guo YX, Gu D, Huang TT, Li A, Zhou Y, Kang XL, Meng C, Xiong D, Song L, Jiao XA, Pan ZM. 2023. *Salmonella* Enteritidis T1SS protein SiiD inhibits NLRP3 inflammasome activation via repressing the mtROS-ASC dependent pathway. *PLoS Pathog* 19:e1011381. <https://doi.org/10.1371/journal.ppat.1011381>
18. Jurénas D, Fraikin N, Goormaghtigh F, Van Melderen L. 2022. Biology and evolution of bacterial toxin-antitoxin systems. *Nat Rev Microbiol* 20:335–350. <https://doi.org/10.1038/s41579-021-00661-1>
19. Hu Y, Benedik MJ, Wood TK. 2012. Antitoxin DinJ influences the general stress response through transcript stabilizer CspE. *Environ Microbiol* 14:669–679. <https://doi.org/10.1111/j.1462-2920.2011.02618.x>
20. Di Cesare A, Losasso C, Barco L, Eckert EM, Conficoni D, Sarasini G, Corno G, Ricci A. 2016. Diverse distribution of toxin-antitoxin II systems in *Salmonella enterica* serovars. *Sci Rep* 6:28759. <https://doi.org/10.1038/srep28759>
21. Motiejūnaite R, Armalyte J, Markuckas A, Suziedeliene E. 2007. *Escherichia coli* *dinJ-yafQ* genes act as a toxin-antitoxin module. *FEMS Microbiol Lett* 268:112–119. <https://doi.org/10.1111/j.1574-6968.2006.00563.x>
22. Ruangprasert A, Maehigashi T, Miles SJ, Dunham CM. 2017. Importance of the *E. coli* DinJ antitoxin carboxy terminus for toxin suppression and regulated proteolysis. *Mol Microbiol* 104:65–77. <https://doi.org/10.1111/mmi.13641>
23. Ruangprasert A, Maehigashi T, Miles SJ, Giridharan N, Liu JX, Dunham CM. 2014. Mechanisms of toxin inhibition and transcriptional repression by *Escherichia coli* DinJ-YafQ. *J Biol Chem* 289:20559–20569. <https://doi.org/10.1074/jbc.M114.573006>
24. Masuda Y, Sakamoto E, Honjoh KI, Miyamoto T. 2020. Role of toxin-antitoxin-regulated persister population and indole in bacterial heat tolerance. *Appl Environ Microbiol* 86:e00935-20. <https://doi.org/10.1128/AEM.00935-20>
25. Wiradiputra MRD, Khuntayaporn P, Thirapanmethee K, Chomnawang MT. 2022. Toxin-antitoxin systems: a key role on persister formation in *Salmonella enterica* serovar Typhimurium. *Infect Drug Resist* 15:5813–5829. <https://doi.org/10.2147/IDR.S378157>
26. Huang T, Gu D, Guo Y, Li A, Kang X, Jiao X, Pan Z. 2022. *Salmonella* Enteritidis GalE protein inhibits LPS-induced NLRP3 inflammasome activation. *Microorganisms* 10:911. <https://doi.org/10.3390/microorganisms10050911>
27. Pandeya A, Zhang Y, Cui J, Yang L, Li J, Zhang G, Wu C, Li Z, Wei Y. 2023. Inflammasome activation and pyroptosis mediate coagulopathy and inflammation in *Salmonella* systemic infection. *Microbiol Res* 275:127460. <https://doi.org/10.1016/j.micres.2023.127460>
28. Broz P, Newton K, Lamkanfi M, Mariathasan S, Dixit VM, Monack DM. 2010. Redundant roles for inflammasome receptors NLRP3 and NLRC4 in host defense against *Salmonella*. *J Exp Med* 207:1745–1755. <https://doi.org/10.1084/jem.20100257>
29. Franchi L. 2011. Role of inflammasomes in *Salmonella* infection. *Front Microbiol* 2:8. <https://doi.org/10.3389/fmicb.2011.00008>
30. Miao EA, Rajan JV. 2011. *Salmonella* and Caspase-1: a complex interplay of detection and evasion. *Front Microbiol* 2:85. <https://doi.org/10.3389/fmicb.2011.00085>
31. Yang J, Zhao Y, Shi J, Shao F. 2013. Human NAIP and mouse NAIP1 recognize bacterial type III secretion needle protein for inflammasome activation. *Proc Natl Acad Sci U S A* 110:14408–14413. <https://doi.org/10.1073/pnas.1306376110>
32. Miao EA, Alpuche-Aranda CM, Dors M, Clark AE, Bader MW, Miller SI, Aderem A. 2006. Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1 β via Ipaf. *Nat Immunol* 7:569–575. <https://doi.org/10.1038/ni1344>
33. Winter SE, Winter MG, Atluri V, Poon V, Romão EL, Tsolis RM, Bäumlér AJ. 2015. The flagellar regulator TviA reduces pyroptosis by *Salmonella enterica* serovar Typhi. *Infect Immun* 83:1546–1555. <https://doi.org/10.1128/IAI.02803-14>
34. Franchi L, Amer A, Body-Malapel M, Kanneganti TD, Ozören N, Jagirdar R, Inohara N, Vandenabeele P, Bertin J, Coyle A, Grant EP, Núñez G. 2006. Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1 β in *Salmonella*-infected macrophages. *Nat Immunol* 7:576–582. <https://doi.org/10.1038/ni1346>
35. Gram AM, Wright JA, Pickering RJ, Lam NL, Booty LM, Webster SJ, Bryant CE. 2021. *Salmonella* flagellin activates NAIP/NLRC4 and canonical NLRP3 inflammasomes in human macrophages. *J Immunol* 206:631–640. <https://doi.org/10.4049/jimmunol.2000382>
36. Sonika S, Singh S, Mishra S, Verma S. 2023. Toxin-antitoxin systems in bacterial pathogenesis. *Heliyon* 9:e14220. <https://doi.org/10.1016/j.heliyon.2023.e14220>
37. Jaiswal S, Paul P, Padhi C, Ray S, Ryan D, Dash S, Suar M. 2016. The Hha-TomB toxin-antitoxin system shows conditional toxicity and promotes persister cell formation by inhibiting apoptosis-like death in *S. Typhimurium*. *Sci Rep* 6:38204. <https://doi.org/10.1038/srep38204>
38. Barrios AFG, Zuo R, Ren D, Wood TK. 2006. Hha, YbaJ, and OmpA regulate *Escherichia coli* K12 biofilm formation and conjugation plasmids abolish motility. *Biotechnol Bioeng* 93:188–200. <https://doi.org/10.1002/bit.20681>
39. García-Contreras R, Zhang XS, Kim Y, Wood TK. 2008. Protein translation and cell death: the role of rare tRNAs in biofilm formation and in activating dormant phage killer genes. *PLoS One* 3:e2394. <https://doi.org/10.1371/journal.pone.0002394>
40. Ryan D, Pati NB, Ojha UK, Padhi C, Ray S, Jaiswal S, Singh GP, Mannala GK, Schultze T, Chakraborty T, Suar M. 2015. Global transcriptome and mutagenic analyses of the acid tolerance response of *Salmonella enterica* serovar Typhimurium. *Appl Environ Microbiol* 81:8054–8065. <https://doi.org/10.1128/AEM.02172-15>
41. Paul P, Patel P, Verma SK, Mishra P, Sahu BR, Panda PK, Kushwaha GS, Senapati S, Misra N, Suar M. 2022. The Hha-TomB toxin-antitoxin module in *Salmonella enterica* serovar Typhimurium limits its intracellular survival profile and regulates host immune response. *Cell Biol Toxicol* 38:111–127. <https://doi.org/10.1007/s10565-021-09587-z>
42. Guo Y, Gu D, Huang T, Cao L, Zhu X, Zhou Y, Wang K, Kang X, Meng C, Jiao X, Pan Z. 2020. Essential role of *Salmonella* Enteritidis DNA adenine methylase in modulating inflammasome activation. *BMC Microbiol* 20:226. <https://doi.org/10.1186/s12866-020-01919-z>
43. Liu Y, Zhao L, Yang M, Yin K, Zhou X, Leung KY, Liu Q, Zhang Y, Wang Q. 2017. Transcriptomic dissection of the horizontally acquired response regulator EsrB reveals its global regulatory roles in the physiological adaptation and activation of T3SS and the cognate effector repertoire in *Edwardsiella piscicida* during infection toward turbot. *Virulence* 8:1355–1377. <https://doi.org/10.1080/21505594.2017.1323157>
44. Kofoed EM, Vance RE. 2011. Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. *Nature* 477:592–595. <https://doi.org/10.1038/nature10394>

45. Wang SY, Lauritz J, Jass J, Milton DL. 2002. A ToxR homolog from *Vibrio anguillarum* serotype O1 regulates its own production, bile resistance, and biofilm formation. *J Bacteriol* 184:1630–1639. <https://doi.org/10.1128/JB.184.6.1630-1639.2002>
46. Milton DL, O'Toole R, Horstedt P, Wolf-Watz H. 1996. Flagellin A is essential for the virulence of *Vibrio anguillarum*. *J Bacteriol* 178:1310–1319. <https://doi.org/10.1128/jb.178.5.1310-1319.1996>
47. Morales VM, Bäckman A, Bagdasarian M. 1991. A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. *Gene* 97:39–47. [https://doi.org/10.1016/0378-1119\(91\)90007-x](https://doi.org/10.1016/0378-1119(91)90007-x)
48. Charpentier X, Oswald E. 2004. Identification of the secretion and translocation domain of the enteropathogenic and enterohemorrhagic *Escherichia coli* effector Cif, using TEM-1 beta-lactamase as a new fluorescence-based reporter. *J Bacteriol* 186:5486–5495. <https://doi.org/10.1128/JB.186.16.5486-5495.2004>