Inhibition of phagocytic killing of Escherichia coli in Drosophila Hemocytes by RNA Chaperone Hfq

Inhibition of Phagocytic Killing of *Escherichia coli* in *Drosophila* Hemocytes by RNA Chaperone Hfq[1]

Running title: ROLE OF RNA CHAPERONE IN BACTERIAL EVASION OF HOST IMMUNITY

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Abstract

An RNA chaperone of *Escherichia coli*, called host factor required for phage Qβ RNA replication (Hfq), forms a complex with small non-coding RNAs to facilitate their binding to target mRNA for the alteration of translation efficiency and stability. Although the role of Hfq in the virulence and drug resistance of bacteria has been suggested, how this RNA chaperone controls the infectious state remains unknown. In the present study, we addressed this issue using *Drosophila melanogaster* as a host for bacterial infection. When adult flies abdominally infected with *E. coli* were analyzed, an *E. coli* strain with mutation in *hfq* was eliminated earlier, while flies survived longer, compared to infection with a parental strain. The same was true with flies deficient in humoral responses, but the mutant phenotypes were not observed when a fly line with impaired hemocyte phagocytosis was infected. The results from an assay for phagocytosis *in vitro* revealed that Hfq inhibits the killing of *E. coli* by *Drosophila* phagocytes after engulfment. Furthermore, Hfq seemed to exert this action partly through enhancing the expression of σ^{38}, a stress-responsive sigma factor that was previously shown to be involved in the inhibition of phagocytic killing of *E. coli*, by a post-transcriptional mechanism. Our study indicates that the RNA chaperone Hfq contributes to the persistent infection of *E. coli* by maintaining the expression of bacterial genes, including one coding for σ^{38}, that help bacteria evade host immunity.

Introduction

Under hostile environmental conditions, bacteria alter their gene expression pattern for adaptation and survival. One such mechanism involves the action of small non-coding RNAs that bind mRNAs and change their translational efficacy and stability (1–6). A protein called 'host factor required for phage Qβ RNA replication' (Hfq) forms a complex with a variety of small non-coding RNAs and facilitates their binding to target mRNAs (7–13). Hfq of *Escherichia coli* is a protein of 102 amino-acid residues, and its orthologues exist in a number of Gram-negative and -positive bacteria constituting the Hfq family of proteins (14–17). Hfq has been shown to play a role in the virulence of uropathogenic *E. coli* (18) and other bacterial species (19–35). However, the mechanism involved in Hfq-mediated changes in the virulence of bacteria remains unclear.

We approached this issue using *Drosophila melanogaster* as a host for bacterial infection. This insect has been used as an animal model to clarify the mechanisms of a wide variety of biological events occurring in higher animal species, including humans (36, 37). As for its immune system, *Drosophila* has only innate immunity, which closely resembles that of humans (38–40). Bacteria that have invaded the hemolymph of *Drosophila* are recognized by innate immune receptors, which exist in a soluble form or at the surface of immune cells called hemocytes. These receptors bind substances that are secreted from bacteria or present as components of the cell wall and cell membrane of bacteria (38–40). Once bound by bacterial substances, the receptors activate signaling pathways in immune cells to induce antibacterial reactions, including the production of antimicrobial peptides and the phagocytosis of bacteria (38–40). *E. coli* is generally considered to be non-pathogenic in *Drosophila*, but our previous study showed that a high load of *E. coli* may kill flies (41). Under such conditions, the Imd pathway, which is responsible for the production of antimicrobial peptides, and phagocytosis by hemocytes are required for the long survival of adult flies after infection with *E. coli* (41), suggesting the involvement of both humoral

and cellular immune responses in the protection of *Drosophila* from pathogenic infection with *E. coli*.

Phagocytosis is a major cellular response among the self-defense strategies of *Drosophila*, which are employed to fight against infection with pathogenic bacteria, and its mechanism has been investigated in adult flies abdominally injected with bacteria, a surrogate procedure for septic infection (41–47). We previously reported that σ^{38}, a stress-responsive sigma factor of *E. coli* (48–52), is involved in the bacterial evasion of phagocytic killing in *Drosophila* (41). RNA polymerase of *E. coli* is a multi-subunit enzyme consisting of a complex of four 'core' subunits, $\alpha_2\beta\beta'\omega$, and a promoter-recognizing subunit named σ (53–55). There are seven sigma factors in *E. coli*, and σ^{38} forms a complex with the core subunits of RNA polymerase (54, 56–58) when *E. coli* encounters hostile conditions (50–54). σ^{38}-encoding *rpoS* is included in the genes, the expression of which is controlled by Hfq (13, 14, 51, 59, 60). The expression of *rpoS* is transiently augmented when *E. coli* abdominally infects adult *Drosophila* (41). Anticipating the involvement of Hfq in σ^{38} actions to modulate the susceptibility of *E. coli* to phagocytosis, we conducted a series of biochemical and genetic experiments. The outcome suggested a link between Hfq and σ^{38} in controlling the phagocytic killing of *E. coli* in *Drosophila*.

Materials and Methods

Fly stocks, bacterial strains, and cell cultures

The following fly lines were used in this study: *Oregon R* (Kyorin-Fly, Kyorin University, Tokyo, Japan) as a wild-type line, w^{1118}, y^{1} w^{1118}, *da-GAL4*, *pxn-GAL4 8.1* (a gift from Michael J. Galko), *UAS-rpr* (Bloomington Drosophila Stock Center, Indiana University, Bloomington, IN; stock number 5824), imd^{1} (61) (a gift from Bruno Lemaitre), and $PGRP\text{-}LE^{112}$;$PGRP\text{-}LC^{7454}$ (62) (a gift from Shoichiro Kurata). Some of the fly lines were used after changing balancers. The *E. coli* strains IT1568 (parental) and TM589 (deficient in *hfq* expression) (*Δhfq*) (63) were used throughout the study. *Δhfq* was transfected with the plasmid pTWV-Hfq-HIS_6 for the expression of His-tagged Hfq. To construct pTWV-Hfq-HIS_6, the DNA fragment coding for the entire Hfq, fused to the His tag at the C-terminus, was prepared by PCR using the chromosomal DNA of W3110*mlc* (IT1568) as a template and the DNA oligomers 5'–CCCAAGCTTTAAAACTTTAACGGAACTGA–3' and 5'–CGCGAATTCTTAGTGATGGTGATGGTGATGCGATCCTTCGGTTTCTTCGCTG–3' as primers, and inserted into the plasmid pTWV228 (Takara Bio Inc., Ohtsu, Japan) after digestion with *Hin*dIII and *Eco*RI. We have confirmed the functional complementation of the defect observed with *Δhfq* by the expression of His-tagged Hfq. The *E. coli* gene *katG* was forcedly expressed in *Δhfq* using the plasmid pCA24N and T5-*lac* promoter, which was obtained from ASKA clone (National BioResource Project: National Institute of Genetics, Mishima, Japan). Bacteria were cultured with Luria-Bertani liquid medium, supplemented with antibiotics if necessary, at 37˚C until the cultures reached the stationary phase of cell growth, after which they were harvested by centrifugation, washed with PBS, and used in the experiments. Hemocytes were isolated from third-instar larvae according to a standard procedure (64) with slight modifications (45). l(2)mbn, a larval hemocyte-derived cell line, was maintained with Schneider's Drosophila medium (Life Technologies Japan, Tokyo, Japan) containing 10% (v/v) heat-inactivated

FBS at 25˚C, as described previously (45).

Bacterial infection and assays for colony-forming bacteria and phagocytosis

All procedures were performed as described previously (41, 45). In brief, male adult flies, 3~7 days after eclosion (15~20 flies per vial, 1~3 vials in each experiment), were anesthetized with CO_2 and abdominally injected with 50 nl of PBS containing a given number of bacteria (stated in each figure) with the aid of a nitrogen gas-operated microinjector (Narishige, Tokyo, Japan). We varied the number of bacteria when infecting flies so that about 80% of the flies that succumbed most severely to infection died in 3-7 days. Flies that had received the injection were maintained at 29˚C until analysis. To determine the level of persistent infection in flies with bacteria, five randomly chosen viable flies were homogenized, inoculated onto agar-solidified Luria-Bertani medium, and incubated at 37˚C, followed by the determination of the number of colonies. Under these conditions, lysates prepared from uninfected flies did not give rise to colonies (data not shown), indicating that endogenous bacteria, commensal or environmental, do not influence this assay. To determine the level of phagocytosis, larval hemocytes were incubated with FITC-labeled bacteria (phagocytes:bacteria = 1:500) at 25˚C for 10 min, supplemented with trypan blue to extinguish the fluorescence from unengulfed bacteria, and microscopically examined for hemocytes containing engulfed bacteria (41). For the determination of bacterial deaths in phagocytes, l(2)mbn cells were incubated with unlabeled bacteria as described above, washed with serum-free medium to remove unengulfed bacteria, maintained in culture up to 1.5 h, osmotically lysed with water, and subjected to an assay for colony-forming bacteria. We were unable to use larval hemocytes for this experiment because they died when cultured longer than 30 min (41). For the inhibition of hemocyte phagocytosis of bacteria, flies were induced to undergo hemocyte apoptosis by the expression of the pro-apoptotic gene *reaper* using the GAL4-UAS system with the hemocyte-specific GAL4 driver *pxn-GAL4 8.1*, the activity of which was confirmed in the hemocytes of adult flies using the fly line *pxn-GAL4 UAS-GFP* (see Fig. 4A), or the administration

of latex beads (2 μm in diameter; Life Technologies Japan) in the abdomen before the injection of bacteria (41).

Western blotting and RT-PCR

All procedures were performed as described previously (41). Briefly, flies, l(2)mbn cells, and bacteria were lyzed in a buffer containing detergents, separated by SDS-PAGE (5 μg of proteins were analyzed unless otherwise stated in the corresponding figure legends), followed by Western blotting analysis with anti-Hfq rabbit antiserum (63), anti-σ^{38} rabbit antiserum (a gift from Akira Ishihama), or anti-GFP mouse monoclonal IgG (mFX75: Wako Pure Chemical Industries, Osaka, Japan), in which signals were visualized by a chemiluminescence reaction. For RT-PCR and RT-qPCR analyses of flies, cultured cells, and *E. coli*, total RNA was prepared using the acid-phenol method with TRIzol (Life Technologies Japan), and used as a template for RT with random primers, and the resulting cDNA was subjected to conventional semi-quantitative PCR or qPCR using THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan) and Mx5005p (Agilent, CA). The DNA oligomers used as the primers in PCR were: 5'–ATGCAGGGTTCTGTGACAGA–3' (forward) and 5'–AGAATACGGCGCAGTGCGTT–3' (reverse) for the mRNA of RNA polymerase α subunit (RpoA); 5'-GATGAGAACGGAGTTGAGGT-3' (forward) and 5'-ACGCAAGTTACTCTCGATCAT-3' (reverse) for σ^{38} mRNA; 5'–GTTAACCACCGCTAATTGAT–3' (forward) and 5'–GACTACACACAGCAATTCGT–3' (reverse) for RpoS regulator RNA A (RprA); 5'–GCTGCGCAATCGCTTCTACT–3' (forward) and 5'–TGGTGGAGTGGGCTTCATG–3' (reverse) for diptericin-A mRNA; 5'–CCCGGAGTGAAGGATG–3' (forward) and 5'–GTTGCTGTGCGTCAAG–3' (reverse) for the mRNAs of attacin-A and attacin-B; and 5'–GACGCTTCAAGGGACAGTATCTG–3' (forward) and 5'–AAACGCGGTTCTGCATGAG–3' (reverse) for the mRNA of ribosomal protein 49 (Rp49). The levels of bacterial and fly mRNA are shown relative to those of RpoA and Rp49, respectively, which were analyzed as internal

controls.

Data processing and statistical analysis

The results from quantitative analyses are expressed as the mean ± SD of the data from at least three independent experiments, unless otherwise stated in the corresponding figure legends. Statistical analyses were performed using the log-rank test (Kaplan-Meier method) or two-tailed Student's *t* test. A p value < 0.05 was considered significant.

Results

Hfq-mediated persistent infection of E. coli *in* Drosophila

As the first step toward clarifying how Hfq participates in controlling the persistent infection and pathogenicity of *E. coli* in *Drosophila*, we examined how an *E. coli* strain deficient in the expression of *hfq* (Δ*hfq*) behaves, in comparison with its parental strain, when infecting adult flies. When these bacteria were maintained in Luria-Bertani liquid medium, Δ*hfq* showed a lower growth rate with almost the same level of culture density at the stationary phase compared with the parental strain (Fig. 1A). We then abdominally infected adult flies with these bacterial strains at two different doses and examined the rate of fly death and the bacterial load. We first analyzed the pathogenic effect of bacteria. Under conditions where the parental strain killed nearly 70% of flies in 2 days, about 80% of flies were still alive even 4 days after the injection with Δ*hfq* (Fig. 1B). For the amount of bacteria present in flies, we homogenized infected flies and inoculated the resulting lysates onto agar-solidified culture medium to determine the number of colony-formable bacteria. The data showed that Δ*hfq* was eliminated more rapidly from flies than the parental strain (Fig. 1C). These results suggest the involvement of Hfq in the persistent infection of *E. coli* in flies. To confirm that the lowered persistence observed in the mutant strain was solely due to the loss of Hfq, we expressed *hfq* in Δ*hfq* as a protein fused to ‘His tag’ (Fig. 2A) and examined its effect on the two phenotypes. The growth rate of Δ*hfq* in a liquid medium became almost the same as that of the parental strain after expression of His-tagged Hfq (Fig. 2B), suggesting that the tagged Hfq was functional in *E. coli*. We found that the expression of His-tagged Hfq increased the rate of fly deaths after infection with Δ*hfq* (Fig. 2C) and augmented the level of persistent infection of Δ*hfq* in adult flies (Fig. 2D), to the levels comparable with the data obtained using the parental *E. coli* strain. From these findings, we concluded that Hfq is required for *E. coli* to achieve a high level of persistent infection in *Drosophila*.

Observation of hfq *phenotype in* Drosophila *deficient in humoral immune response*

We then investigated whether the function of Hfq, in terms of the control of bacterial persistence, is related to the humoral immunity of the host. Humoral immune responses in *Drosophila* against Gram-negative bacteria are induced through the recognition of invaders by peptidoglycan recognition proteins (PGRPs) and the subsequent activation of the Imd pathway leading to the production of antimicrobial peptides such as diptericin and attacin (61, 62, 65–68). First, we determined the level of mRNA of these peptides in flies infected with Δ*hfq* and the parental strain, but found no significant difference between flies infected with the two *E. coli* strains (Fig. 3A). Next, we used mutant *Drosophila* lines having a defect in the humoral response as a host for infection with *E. coli*. The fly line imd^1 that lacks Imd, a central component in the Imd pathway, succumbed to *E. coli* infection more severely than the control fly line (left panel in Fig. 3B), indicating a reduced level of immunity in those flies. We found that Δ*hfq* showed decreased levels of killing effect and persistent infection in this mutant fly line (middle and right panels in Fig. 3B, respectively), as observed in the experiment using wild-type flies (see Figs. 1B and 1C). To confirm this further, we used another mutant fly line deficient in the expression of both PGRP-LE and PGRP-LC, peptidoglycan-recognizing receptors responsible for the detection of *E. coli* and the subsequent induction of the Imd pathway (left panel in Fig. 3C), and again found that Δ*hfq* killed flies at a lower rate (middle panel in Fig. 3C) and was less persistent (right panel in Fig. 3C) than the parent strain. Collectively, these results indicate that Hfq is required for the host-killing effect and persistent infection of *E. coli* in flies deficient in the Imd pathway as observed using wild-type flies as a host. It is most probable that Hfq-mediated persistence of *E. coli* in adult flies is achieved independent of humoral immune responses.

Involvement of cellular immunity in the control of bacterial persistence in Drosophila *by Hfq*

Next, we asked if phagocytosis, a main cellular immune response, is related to the Hfq-mediated

control of the persistence of *E. coli*. To generate flies defective in phagocytosis, we expressed the pro-apoptotic gene *reaper* (69) specifically in hemocytes, the major phagocytes in *Drosophila*, using the GAL4-UAS system. Flies that possessed both *UAS-rpr* and hemocyte-specific *pxn-Gal4* contained a smaller number of hemocytes compared to control flies having only *pxn-Gal4* (left panel in Fig. 4A). Those flies were more vulnerable to infection with the parental *E. coli* strain than control flies (middle panel in Fig. 4A), suggesting impaired phagocytosis of bacteria. We found that these flies died at almost the same rate after infection with the parental and Δ*hfq* strains (right panel in Fig. 4A). Similar results were obtained with wild-type flies that had been pre-injected with latex beads (Fig. 4B), another method of inhibiting hemocyte phagocytosis (41, 70). Furthermore, Δ*hfq* no longer showed a lowered persistence phenotype when it infected the bead-injected flies (Fig. 4C). To further confirm the above findings, we used flies deficient in both the Imd pathway and hemocyte phagocytosis as a host for bacterial infection. imd^1 flies were injected with latex beads, abdominally infected with *E. coli*, and subjected to assays for fly survival and bacterial persistence. We found no difference in either assay between the parental and Δ*hfq E. coli* strains (Fig. 4D), as observed using flies with impaired phagocytosis. The data obtained so far indicate that Hfq is no longer required for the host-killing effect and persistent infection of *E. coli* in flies deficient in hemocyte phagocytosis. It is thus likely that Hfq-mediated persistence of *E. coli* in adult flies is related to phagocytosis.

A simple explanation for the above observations is that Hfq-lacking *E. coli* undergoes phagocytic killing more efficiently than the parental strain. Next, we tested this hypothesis by conducting an assay for the phagocytosis of *E. coli in vitro* using hemocytes isolated from *Drosophila* larvae. The extent of phagocytosis was assessed by determining the ratio of hemocytes that had accomplished phagocytosis as well as the number of *E. coli* engulfed by a given number of hemocytes, but we observed no significant difference between Δ*hfq* and the parental strain in either index (Fig. 5A). Then, we examined a possible difference in the rate of killing of *E. coli* in hemocytes after engulfment. For this examination, l(2)mbn cells, a cell line derived from

larval hemocytes, were used as phagocytes because larval hemocytes spontaneously lysed and could not be maintained in culture for a period long enough to analyze the killing of engulfed bacteria. l(2)mbn cells and *E. coli* were co-incubated, unengulfed *E. coli* were washed out, and phagocytes, after engulfment, were maintained in culture further for 1.5 h. Whole-cell lysates of l(2)mbn cells at 0 and 1.5 h of incubation were inoculated onto agar-solidified medium to determine the number of bacteria capable of forming colonies that existed in the phagocytes. We found that Δ*hfq* decreased more rapidly than its parental strain (Fig. 5B), showing that the Hfq-lacking strain is more effectively killed in phagocytes after engulfment. These results collectively indicated that Hfq helps *E. coli* evade killing by *Drosophila* phagocytes after engulfment.

The observation that flies deficient in the Imd pathway (Figs. 3B and 3C) or hemocyte phagocytosis (Figs. 4A and 4B) succumbed more severely to infection with *E. coli* compared to control flies indicates the roles of both humoral and cellular immunity in the protection of *Drosophila* from bacterial infection. Thus, we examined whether these two immune responses are related. To do so, flies were abdominally injected with latex beads or left untreated, infected with the parental *E. coli* strain, and analyzed for the level of antimicrobial peptide mRNA. Also, hemocytes isolated from larvae of Imd-lacking and control flies were tested for the phagocytosis of the parental *E. coli* strain *in vitro*. We found that impaired phagocytosis did not influence the production of antimicrobial peptide mRNA in flies after infection, and the compromised humoral response did not alter the hemocyte phagocytosis of *E. coli* (Supplemental Fig. 1). These results suggest that humoral and cellular responses are independently involved in the antibacterial immunity of *Drosophila*. A possible explanation for the observation that the inhibition of either immune response causes earlier fly deaths after bacterial infection could be that two responses are induced at different times: phagocytosis is an immediate reaction for the direct elimination of invading pathogens followed by the production of antimicrobial peptides to kill the remaining pathogens.

σ^{38}-mediated Hfq function in controlling E. coli *persistence*

E. coli genes whose expression is controlled by Hfq include *rpoS*, which codes for the stress-responsive sigma factor σ^{38} (13, 49, 50), and the phenotype of Δ*hfq* described above resembles what is seen in the *rpoS* mutant, as we previously reported (41). To test the possible involvement of σ^{38} in the Hfq-mediated control of the persistence and killing effect of *E. coli* in *Drosophila*, we examined whether the forced expression of *rpoS* restores the phenotypes of *E. coli* observed after a loss of Hfq. When the lysates of *E. coli* were analyzed by Western blotting using Ab specific to σ^{38}, the signal was less intense in Δ*hfq* than in the parental strain (left panel in Fig. 6A), confirming the Hfq control of *rpoS* expression. The forced expression of *rpoS* in Δ*hfq*, which was confirmed by Western blotting (right panel in Fig. 6A), partly recovered a host-killing effect (Fig. 6B). Also, *rpoS*-expressing Δ*hfq* a higher level of persistent infection in adult flies compared to Δ*hfq* that harbored vector alone (Fig. 6C). Furthermore, Δ*hfq* became more resistant to killing in l(2)mbn cells, after engulfment, by the expression of *rpoS* (Fig. 6D). These results indicated that *rpoS* partly accounts for the Hfq-dependent persistent infection of *E. coli* in *Drosophila*, and suggested the presence of other Hfq-controlled genes besides *rpoS* that explain the entire actions of Hfq. Target genes of σ^{38} include *katE* and *katG* that code for an enzyme detoxifying reactive oxygen species. In our previous study (41), forced expression of *katE* or *katG* recovered lowered persistence and host-killing effect seen in σ^{38}-lacking *E. coli*. We thus examined if the defective phenotypes of Hfq-lacking *E. coli* were rescued by the expression of *katG* (Fig. 6E). The data indicated that overexpression of *katG* (left panel) restored, though partly, host-killing effect (middle panel) and persistence of Δ*hfq* (right panel). It is thus likely that the Hfq-mediated inhibition of phagocytic killing is achieved through augmented expression of *katG*.

Post-transcriptional increase of σ^{38} mRNA by Hfq during infection

Finally, we examined the mechanism of the control of *rpoS* expression by Hfq. We first

determined the level of σ^{38} in the whole-cell lysates of Δ*hfq* by Western blotting and found that σ^{38} increased after the expression of His-tagged Hfq (Fig. 7A), confirming a positive control of *rpoS* expression by Hfq. Hfq as an RNA chaperone is thought to influence the level of mRNA independent of transcription. The strength of the *rpoS* promoter was then analyzed using GFP as a reporter, and the level of GFP determined in Western blotting was almost the same between the parental and Hfq-lacking *E. coli* strains (Fig. 7B), suggesting no influence of Hfq on the promoter activity of *rpoS*. We found that σ^{38} mRNA increased in adult flies infected with the parental but not Hfq-lacking *E. coli* strains (Fig. 7C), indicating the Hfq-mediated expression of *rpoS* after infection. The expression of *rpoS* is post-transcriptionally enhanced by RprA (71–73), a small non-coding RNA controlled by Hfq, and Hfq remains abundant in *E. coli* regardless of the phase of its growth (11). We thus determined a possible change in the level of this RNA by RT-qPCR after infection. The level of RprA was higher in the parental, but not RpoS-lacking, strains in adult flies (Fig. 7D), suggesting the presence of a positive feedback loop in the expression of *rpoS* and RprA-encoding genes. Collectively, an elevated level of RprA in the parental *E. coli* most likely accounts for the Hfq-dependent increase of σ^{38} after infection.

Discussion

There are a number of reports describing the role of Hfq in the virulence and drug resistance of bacteria (14–17, 74, 75). However, the mechanism by which Hfq is involved in the control of bacterial behavior under infectious conditions has yet to be clarified. In the present study, we showed that Hfq is required for the maintenance of the persistent infection of *E. coli* in *Drosophila*. Hfq seemed to inhibit the killing of bacteria in hemocytes, a major phagocytic cell type in *Drosophila* (76, 77), after engulfment, which should be a mechanistic explanation for the Hfq-mediated persistent infection of *E. coli*. There was no difference in the efficacy of phagocytosis of *E. coli* between the parental and Hfq-lacking strains, while the Hfq-lacking strain was more rapidly killed in phagocytes after engulfment than the parent strain. Therefore, it is possible that the Hfq-lacking strain is more efficiently engulfed by *Drosophila* phagocytes than the parental strain.

Hfq is known as an RNA chaperone that binds small non-coding RNAs to facilitate their actions in post-transcriptionally controlling the stability and translational efficacy of mRNAs (3, 12, 13). One of the target RNAs of Hfq is RprA, which positively controls the expression of *rpoS* coding for the stress-inducing sigma factor σ^{38} (71–73). The levels of RprA and σ^{38} mRNA rose in *E. coli* after infection of adult flies. Furthermore, we showed that the forced expression of *rpoS* or *katG* restored, though partly, the *E. coli* phenotype of lowered persistence caused by the lack of Hfq. These findings allowed us to propose the following mechanism for the Hfq- and σ^{38}-mediated persistent infection of *E. coli* in *Drosophila*: the expression of RprA is augmented after infection; Hfq forms a complex with RprA; Hfq-bound RprA stabilizes σ^{38} mRNA; the production of σ^{38} is enhanced; σ^{38} induces the transcription of genes including *katG*; killing of *E. coli* engulfed by hemocytes is inhibited; and the persistent infection of *E. coli* is achieved. As a result, an increase in the abundance of bacteria in *Drosophila* brings about early death of the host organism. Rescue of the defective phenotype in Hfq-lacking *E. coli* by the forced expression of

rpoS and *katG* was partial. This suggests that other genes besides *rpoS* are induced by Hfq and involved in the control of *E. coli* persistence in *Drosophila*, and that other genes in addition to *katG* are induced by σ^{38} and responsible for the inhibition of the phagocytic killing of *E. coli* in *Drosophila* hemocytes. Further study is required for identifying and characterizing such genes.

The expression of *hfq* seems to remain unchanged regardless of the phase of *E. coli* cell growth (11). We showed an increase of RprA, a small non-coding RNA that stabilizes σ^{38} mRNA, in adult flies. It is therefore likely that the post-transcriptional control of *rpoS* expression depends on increased RprA levels after infection. Thus, another important issue to be resolved is the mechanism behind the augmented expression of the RprA-encoding gene in *Drosophila*. The two-component signal transduction system (TCS) senses environmental changes and causes the alteration of gene expression so that bacteria adapt to the new environment and survive (78–80). The TCS consists of two distinct proteins, a membrane protein called the sensor kinase that serves as a receptor, and a DNA-binding transcription factor called the response regulator that is phosphorylated by the sensor kinase for activation (79). It is probable that the TCS control of gene expression is related to the levels of persistence and pathogenicity of bacteria in host organisms. Indeed, we previously found that EnvZ-OmpR, a TCS of *E. coli*, is required for bacteria to maintain low pathogenicity in *Drosophila* (81). More specifically, positive control of the expression of RprA-encoding gene by RcsC/RcsD-RcsB and CpxA-CpxR was reported previously (82). To examine the possible involvement of such TCSs in the control of *rprA* expression under infectious conditions, further investigation is required.

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Footnotes

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[3]Abbreviations used in this paper: Hfq, host factor required for phage Qβ RNA replication; PGRP, peptidoglycan recognition protein(s); Rp49, ribosomal protein 49; RpoA, α subunit of *E. coli* RNA polymerase; RpoS, σ^{38} subunit of *E. coli* RNA polymerase; RprA, RpoS regulator RNA A; TCS, two-component regulatory system.

Figure Legends

FIGURE 1. Decreases in the persistent infection and host killing effect of *E. coli* with the loss of Hfq. (**A**) Growth rates of Hfq-lacking (Δ*hfq*) and parental strains of *E. coli* were determined in a Luria-Bertani liquid medium. Representative results from three independent experiments with similar results are shown. (**B**) The ratio of live *Oregon R* flies was determined at the indicated time points after abdominal infection with Δ*hfq* and parental strains. Data were analyzed using the log-rank test. Results from one of three independent experiments that gave similar results are shown. (**C**) The level of colony-forming bacteria was determined in lysates of *Oregon R* flies at the indicated time points after abdominal infection with Δ*hfq* and parental strains. Data from five (parent) and three (Δ*hfq*) independent experiments are shown after analysis by Student's *t* test.

FIGURE 2. Recovery of the persistence and host killing effect of Δ*hfq* by the forced expression of His-tagged Hfq. The mutant *E. coli* strain Δ*hfq*, which had been transfected with a plasmid for *hfq* expression (*hfq*) or a vector alone (vector), was analyzed. (**A**) The levels of Hfq, endogenous Hfq and ectopically expressed His-tagged Hfq, in lysates of the indicated bacteria were determined by Western blotting with anti-Hfq Ab. Representative data from one of two independent experiments with similar results are shown. (**B**) The growth rate of the indicated bacteria was determined in a Luria-Bertani liquid medium. Data from one of three independent experiments with similar results are shown. (**C**) The survival of *Oregon R* flies was analyzed after abdominal infection with bacteria. Data from one of three independent experiments are shown after analysis by the log-rank test. (**D**) The level of colony-forming bacteria was determined in lysates of *Oregon R* flies at the indicated time points after abdominal infection with bacteria. Results from three (parent) and four (Δ*hfq*) independent experiments are shown after analysis by Student's *t* test.

FIGURE 3. No relationship of the control of *E. coli* persistence by Hfq with host humoral

immunity. (**A**) mRNA levels of diptericin and attacin were determined by RT-qPCR with RNA prepared from *Oregon R* flies infected with the indicated *E. coli* strains for 3 h. Values for the mRNAs of attacin and diptericin are shown after normalization with those of Rp49 mRNA. Results from 13 (attacin) and 11 (diptericin) independent experiments are shown after analysis by Student's *t* test. NS, difference not significant. (**B**) Levels of the persistence and killing effect of *E. coli* were determined using the fly line imd^1 defective in the Imd pathway as the host. (left) The fly lines imd^1 and w^{1118}, as a control with the same genetic background, were abdominally infected with the parental *E. coli* strain, and the proportion of live flies was determined at the indicated time points. Data were analyzed by the log-rank test. Representative results from one of three independent experiments that gave similar results are shown. (middle) The ratio of viable imd^1 flies was determined at the indicated time points after infection with Δ*hfq* or the parental strain. Data were analyzed by the log-rank test. Representative results from one of three independent experiments with similar results are shown. (right) Level of colony-forming activity in lysates of imd^1 flies, which had been infected with Δ*hfq* or the parental strain, was determined at the indicated time points after infection. *Oregon R* flies infected with the parental strain were similarly analyzed as a control. Data from three (*Oregon R*) and four (imd^1) independent experiments are shown after analysis by Student's *t* test. (**C**) The killing effect of *E. coli* was determined using the fly line $PGRP\text{-}LE^{112};PGRP\text{-}LC^{7454}$ lacking both PGRP-LC and PGRP-LE as the host. (left) The fly lines $PGRP\text{-}LE^{112};PGRP\text{-}LC^{7454}$ and $y^1\ w^{1118}$, as a control with the same genetic background, were abdominally infected with the parental *E. coli* strain, and the proportion of live flies was determined at the indicated time points. Data were analyzed by the log-rank test. Representative results from one of three independent experiments that gave similar results are shown. (middle) The proportion of live $PGRP\text{-}LE^{112};PGRP\text{-}LC^{7454}$ flies was determined at the indicated time points after infection with Δ*hfq* or the parental strain. Data were analyzed by the log-rank test. Representative results from one of three independent experiments that gave similar results are shown. (right) The level of colony-forming activity in lysates of $PGRP\text{-}LE^{112};PGRP\text{-}LC^{7454}$ flies,

which had been infected with Δ*hfq* or the parental strain, was determined at the indicated time points after infection. *Oregon R* flies infected with the parental strain were similarly analyzed as a control. Data from three (*Oregon R*) and four (*PGRP-LE*^{112}*;PGRP-LC*^{7454}) independent experiments are shown after analysis by Student's *t* test.

FIGURE 4. Involvement of phagocytosis in Hfq-mediated persistence of *E. coli*. (**A**) (left) Cells contained in the hemolymph of adult flies that possessed the trans-gene *pxn-GAL4 UAS-GFP* or *pxn-GAL4 UAS-rpr UAS-GFP* were examined by fluorescence microscopy. Phase contrast and fluorescence views of the same microscopic fields are shown. Scale bar, 20 μm. (middle) Flies possessing the trans-gene *pxn-GAL4 UAS-rpr*, *pxn-GAL4* or *UAS-rpr* were abdominally infected with the parental *E. coli* strain, and the proportion of live flies was determined at the indicated time points. Data were analyzed by the log-rank test. Representative results from one of three independent experiments that gave similar results are shown. (right) The proportion of live flies possessing *pxn-GAL4 UAS-rpr* was determined after infection with the indicated *E. coli* strains. Data were analyzed using the log-rank test. NS, difference not significant. Representative results from one of three independent experiments that gave similar results are shown. (**B**) (left) *Oregon R* flies, which had been pre-injected with latex beads or PBS alone followed by the infection of the parental *E. coli* strain, were subjected to a survival assay. Data were analyzed by the log-rank test. Representative results from one of three independent experiments that gave similar results are shown. (right) The proportion of live *Oregon R* flies, which had been pre-injected with latex beads followed by the infection with the indicated *E. coli* strains, was determined at the indicated time points. Data were analyzed by the log-rank test. NS, difference not significant. Representative results from one of three independent experiments that gave similar results are shown. (**C**) The level of colony-formable bacteria was determined in the whole-fly lysates of *Oregon R*, which had been pre-injected with latex beads, at the indicated time points after abdominal infection with the parental or Δ*hfq E. coli* strain. Flies not treated with

latex beads and infected with the parental bacteria were similarly analyzed as a control. Results from three (no bead injection) and five (the others) independent experiments are shown after analysis by Student's *t* test. NS, difference not significant. (**D**) Levels of the killing effect (left) and persistence (right) of *E. coli* were determined using *imd*^{1} flies that had been pre-injected with latex beads as a host. Data were analyzed by the log-rank test (left, representative data from one of three independent experiments shown) or Student's *t* test (right, results from five independent experiments analyzed). NS, difference not significant.

FIGURE 5. Hfq inhibition of *E. coli* phagocytosis by *Drosophila* phagocytes. (**A**) Hemocytes obtained from third-instar larvae of *Oregon R* flies were incubated with the indicated *E. coli* strains, and the level of phagocytosis was determined using two indices. Data from four independent experiments are shown after analysis by Student's *t* test. NS, difference not significant. (**B**) The hemocyte cell line l(2)mbn that had been incubated with the indicated *E. coli* strains was maintained in culture further for 1.5 h, and their lysates were subjected to an assay of colony-forming bacteria. Data from three independent experiments are shown after analysis by Student's *t* test.

FIGURE 6. σ^{38}-mediated function of Hfq in maintaining the persistent infection of *E. coli*. (**A**) Lysates of parental *E. coli* and Δ*hfq*, which had been transfected with *rpoS*-expressing plasmid (*rpoS*) or an empty vector, were subjected to Western blotting with anti-σ^{38} Ab. Data from one of two independent experiments that gave similar results are shown. (**B**) *Oregon R* flies were infected with Δ*hfq* possessing *rpoS*-expressing plasmid (*rpoS*) or an empty vector, and the proportion of live flies was determined at the indicated time points. Flies infected with the parental bacteria were similarly analyzed as a control. The data from one of four independent experiments that yielded similar results are shown after analysis by the log-rank test. (**C**) Lysates of *Oregon R* flies that had been infected with the indicated bacterial strains were analyzed for the

level of colony-forming bacteria at the indicated time points after infection. Data from three independent experiments are shown after analysis by Student's *t* test. (**D**) l(2)mbn cells that had been incubated with the indicated *E. coli* strains were maintained in culture further for 1.5 h, and their lysates were subjected to an assay of colony-formable bacteria. Data from three (parent) and four (Δ*hfq*) independent experiments are shown after analysis by Student's *t* test. (**E**) The killing effect and persistence of Δ*hfq* in adult flies were determined after forced expression of *katG*. (left) Proteins in whole-cell lysates of the indicated bacteria were separated by SDS-PAGE followed by staining with Coomassie brilliant blue. The arrowhead points to the position of KatG. Representative data from one of three independent experiments with similar results are shown. (middle) Killing effect of the indicated bacteria was determined using *Oregon R* flies as a host. Data were analyzed by the log-rank test. Representative results from one of three independent experiments that gave similar results are shown. (right) Persistence of the indicated bacteria in *Oregon R* flies was determined. Data from three independent experiments are shown after analysis by Student's *t* test.

FIGURE 7. Increases of σ^{38} mRNA and RprA in *E. coli* after infection. (**A**) Lysates prepared from parental *E. coli* and Δ*hfq*, which had been transfected with a plasmid for the expression of His-tagged Hfq (*hfq*) or a vector alone, were analyzed for the level of σ^{38} by Western blotting with anti-σ^{38} Ab. Data from one of three independent experiments with similar results are shown. (**B**) Lysates from parental *E. coli* and Δ*hfq* harboring plasmid for GFP expression driven by *rpoS* promoter were analyzed by Western blotting with anti-GFP Ab. Data from one of three independent experiments with similar results are shown. (**C**) RNA prepared from *Oregon R* flies that had been infected with the parental or Δ*hfq E. coli* strain for 5 min together with the RNA of the same *E. coli* strains was subjected to RT-qPCR analysis for the level of the mRNA of σ^{38} and RpoA. Values for σ^{38} mRNA are shown after normalization with those of RpoA mRNA.

Results from 15 (parent), eight (Δ*hfq*, no infection), and seven (Δ*hfq*, infection) independent experiments are shown after analysis by Student's *t* test. NS, difference not significant. (**D**) RNA prepared from *Oregon R* flies, after the infection with parental or σ^{38}-lacking (Δ*rpoS*) *E. coli* strain for 5 min together with RNA of the same *E. coli* strains, was subjected to RT-qPCR for the level of RprA and RpoA mRNA. Values of RprA were normalized with those of RpoA mRNA, and results from nine (left panel: parent, no infection), six (left panel: parent, infection), three (right panel: parent, no infection), eight (Δ*rpoS*, no infection), and seven (Δ*rpoS*, infection) independent experiments analyzed by Student's *t* test are shown.

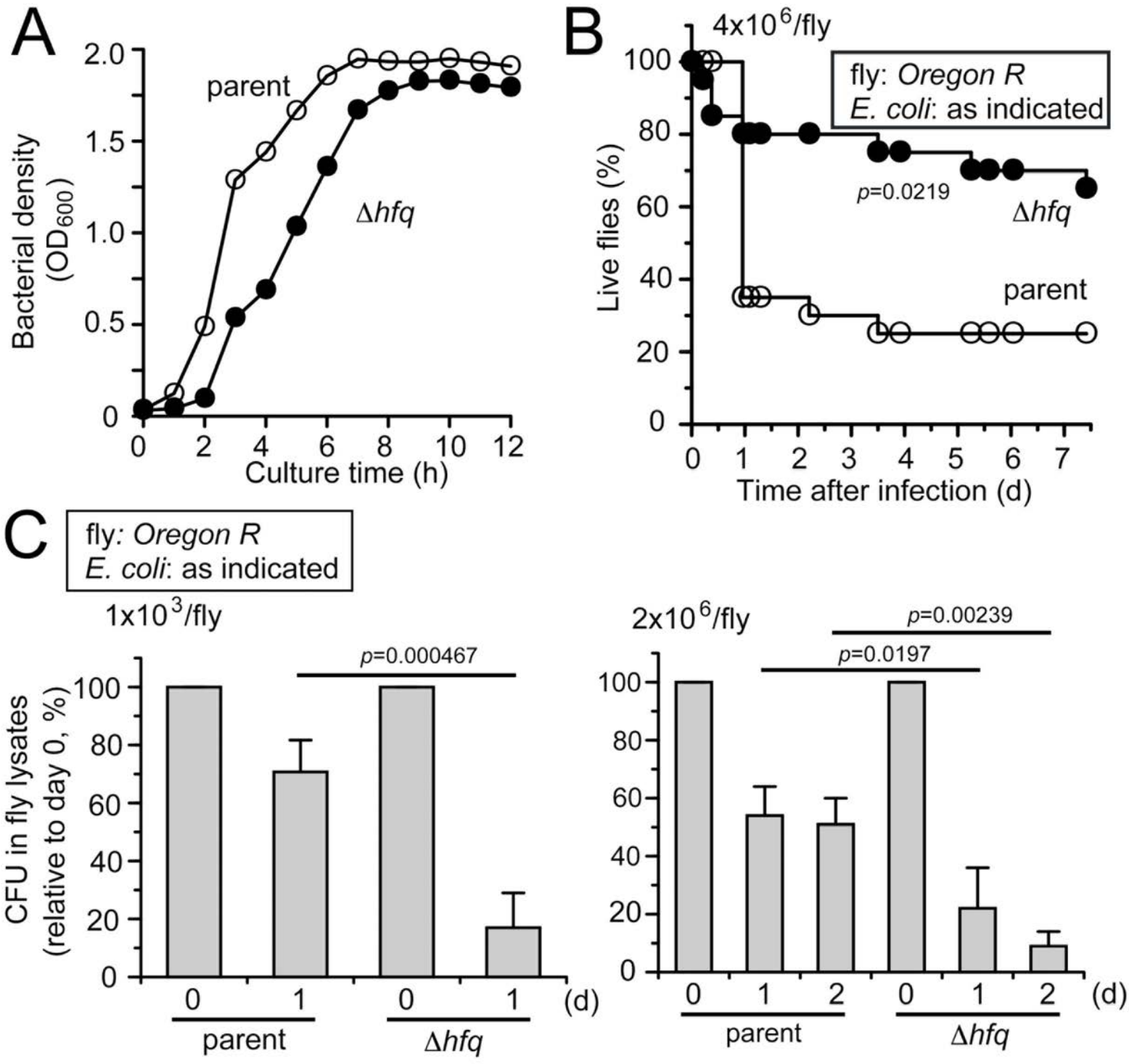

A
Bacterial density (OD600)
parent
Δhfq
Culture time (h)
B
4x10^6/fly
fly: Oregon R
E. coli: as indicated
Live flies (%)
p=0.0219
Δhfq
parent
Time after infection (d)
C
fly: Oregon R
E. coli: as indicated
1x10^3/fly
CFU in fly lysates (relative to day 0, %)
p=0.000467
parent
Δhfq
(d)
2x10^6/fly
p=0.00239
p=0.0197
parent
Δhfq
(d)

Fig. 1

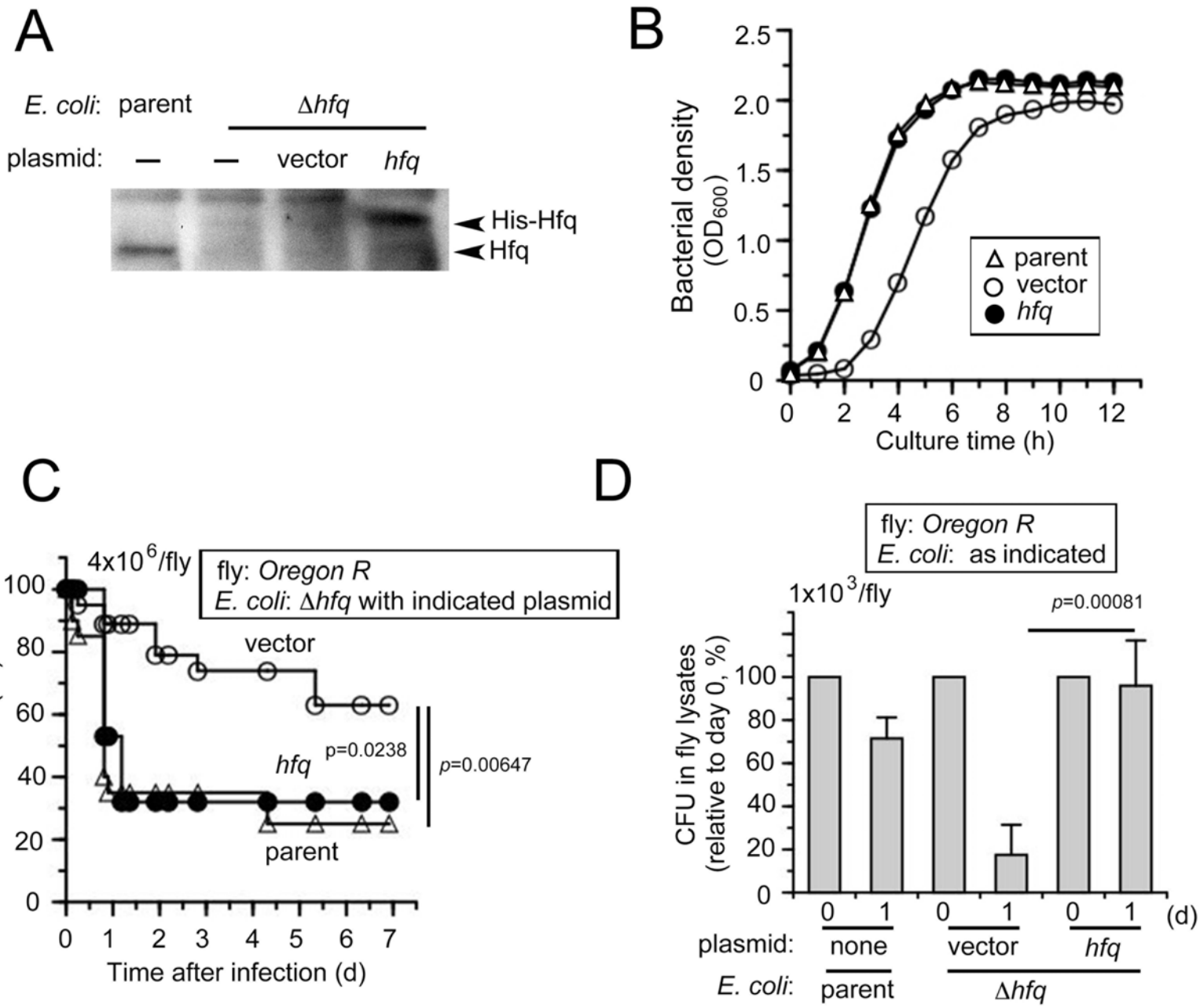

A
E. coli: parent
Δhfq
plasmid: —
—
vector
hfq
His-Hfq
Hfq
B
Bacterial density (OD600)
Culture time (h)
parent
vector
hfq
C
4x10^6/fly
fly: Oregon R
E. coli: Δhfq with indicated plasmid
vector
hfq
parent
p=0.0238
p=0.00647
Live flies (%)
Time after infection (d)
D
fly: Oregon R
E. coli: as indicated
1x10^3/fly
p=0.00081
CFU in fly lysates (relative to day 0, %)
plasmid: none
vector
hfq
E. coli: parent
Δhfq
(d)

Fig. 2

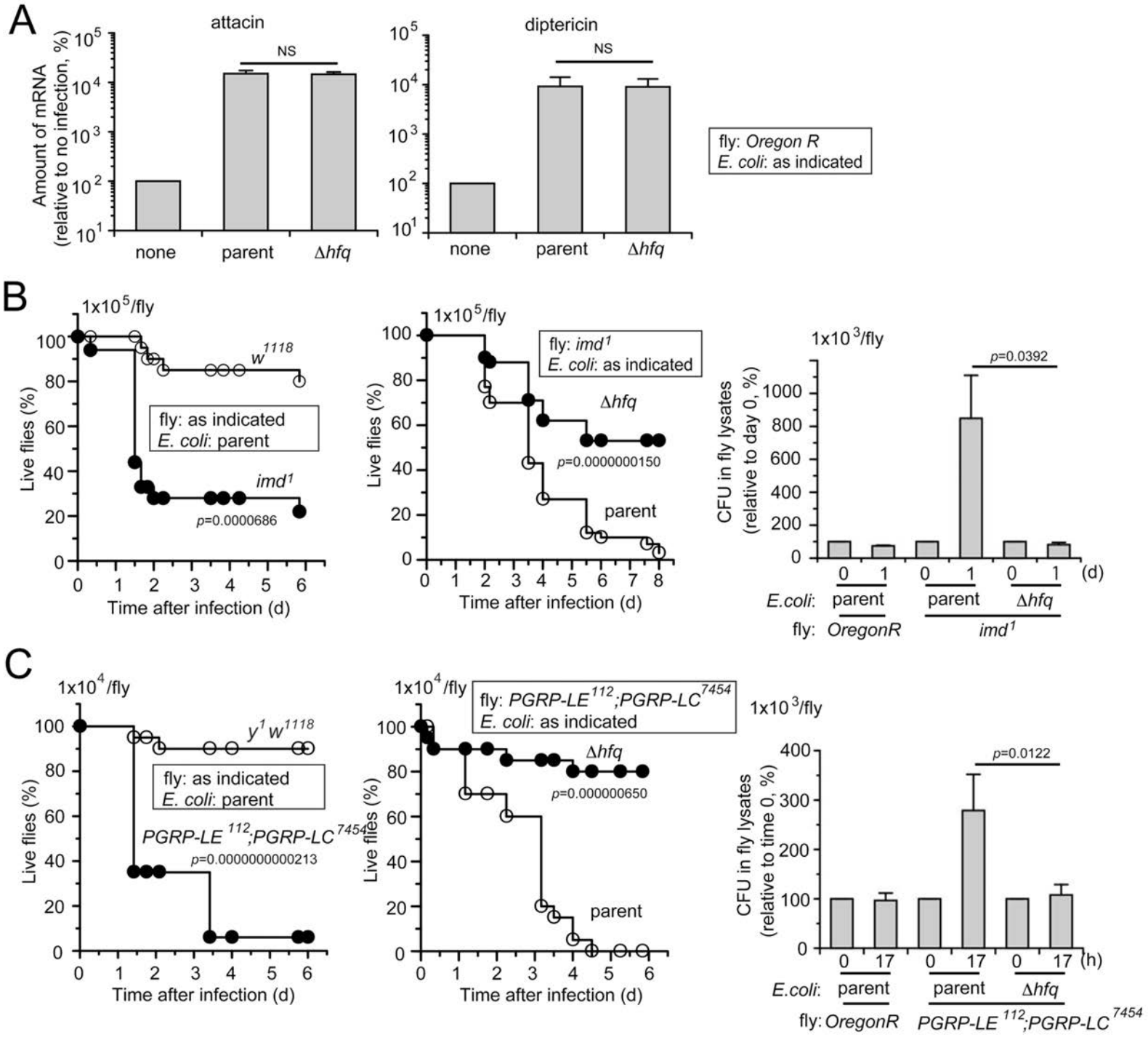

A
attacin
diptericin
Amount of mRNA (relative to no infection, %)
NS
none
parent
Δhfq
fly: Oregon R
E. coli: as indicated
B
1x10^5/fly
w^1118
fly: as indicated
E. coli: parent
imd^1
p=0.0000686
Live flies (%)
Time after infection (d)
fly: imd^1
E. coli: as indicated
Δhfq
p=0.0000000150
parent
1x10^3/fly
p=0.0392
CFU in fly lysates (relative to day 0, %)
E.coli:
fly: OregonR
imd^1
C
1x10^4/fly
y^1 w^1118
PGRP-LE^112;PGRP-LC^7454
p=0.0000000000213
fly: PGRP-LE^112;PGRP-LC^7454
p=0.000000650
p=0.0122
CFU in fly lysates (relative to time 0, %)

Fig. 3

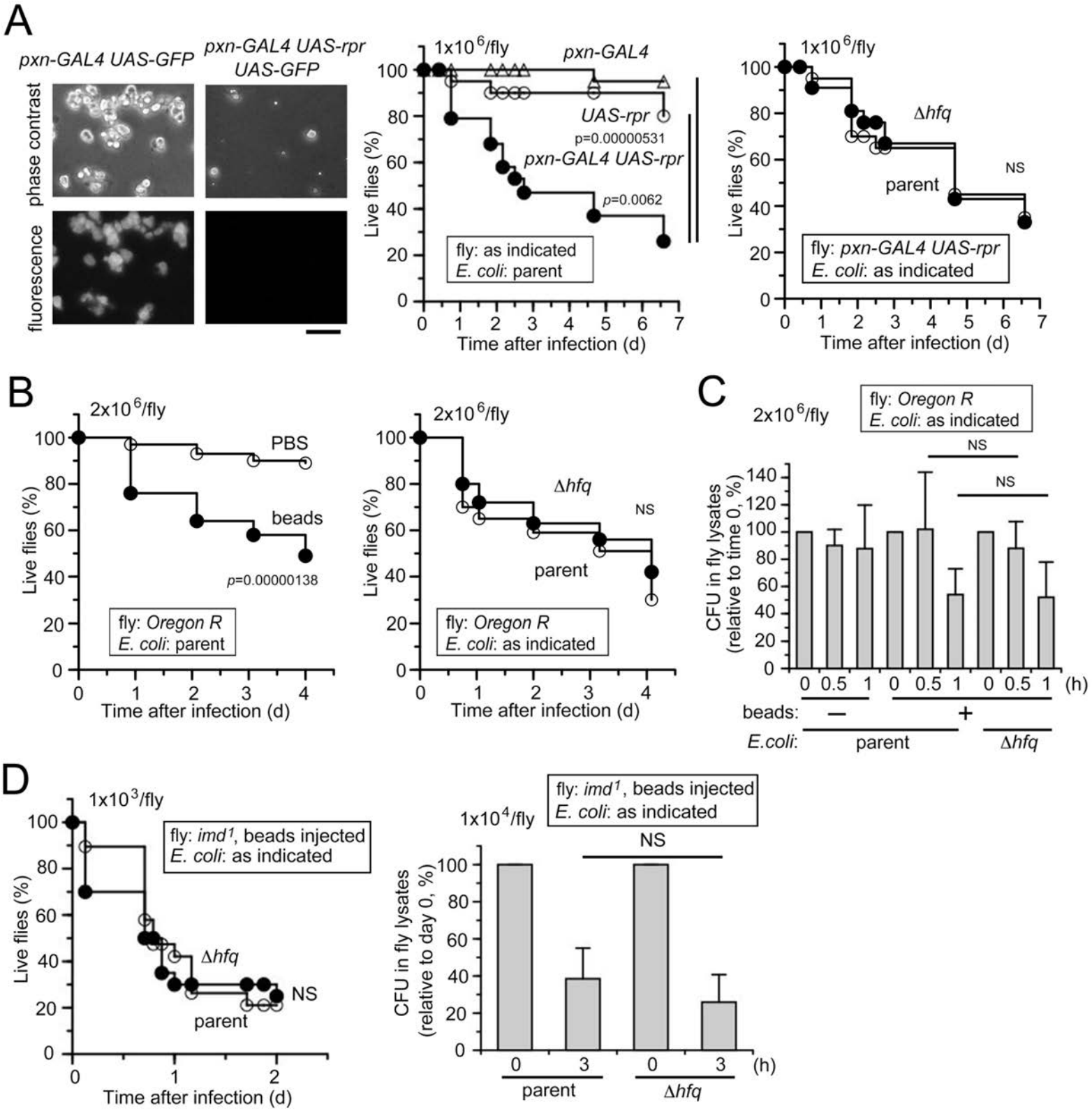

A
pxn-GAL4 UAS-GFP
pxn-GAL4 UAS-rpr UAS-GFP
phase contrast
fluorescence
1x10^6/fly
pxn-GAL4
UAS-rpr
p=0.00000531
pxn-GAL4 UAS-rpr
p=0.0062
fly: as indicated
E. coli: parent
Live flies (%)
Time after infection (d)
1x10^6/fly
Δhfq
parent
NS
fly: pxn-GAL4 UAS-rpr
E. coli: as indicated
B
2x10^6/fly
PBS
beads
p=0.00000138
fly: Oregon R
E. coli: parent
Δhfq
NS
parent
fly: Oregon R
E. coli: as indicated
C
2x10^6/fly
fly: Oregon R
E. coli: as indicated
CFU in fly lysates (relative to time 0, %)
NS
NS
0 0.5 1 0 0.5 1 0 0.5 1 (h)
beads: – +
E.coli: parent Δhfq
D
1x10^3/fly
fly: imd1, beads injected
E. coli: as indicated
Δhfq
NS
parent
1x10^4/fly
fly: imd1, beads injected
E. coli: as indicated
NS
CFU in fly lysates (relative to day 0, %)
0 3 0 3 (h)
parent
Δhfq

Fig. 4

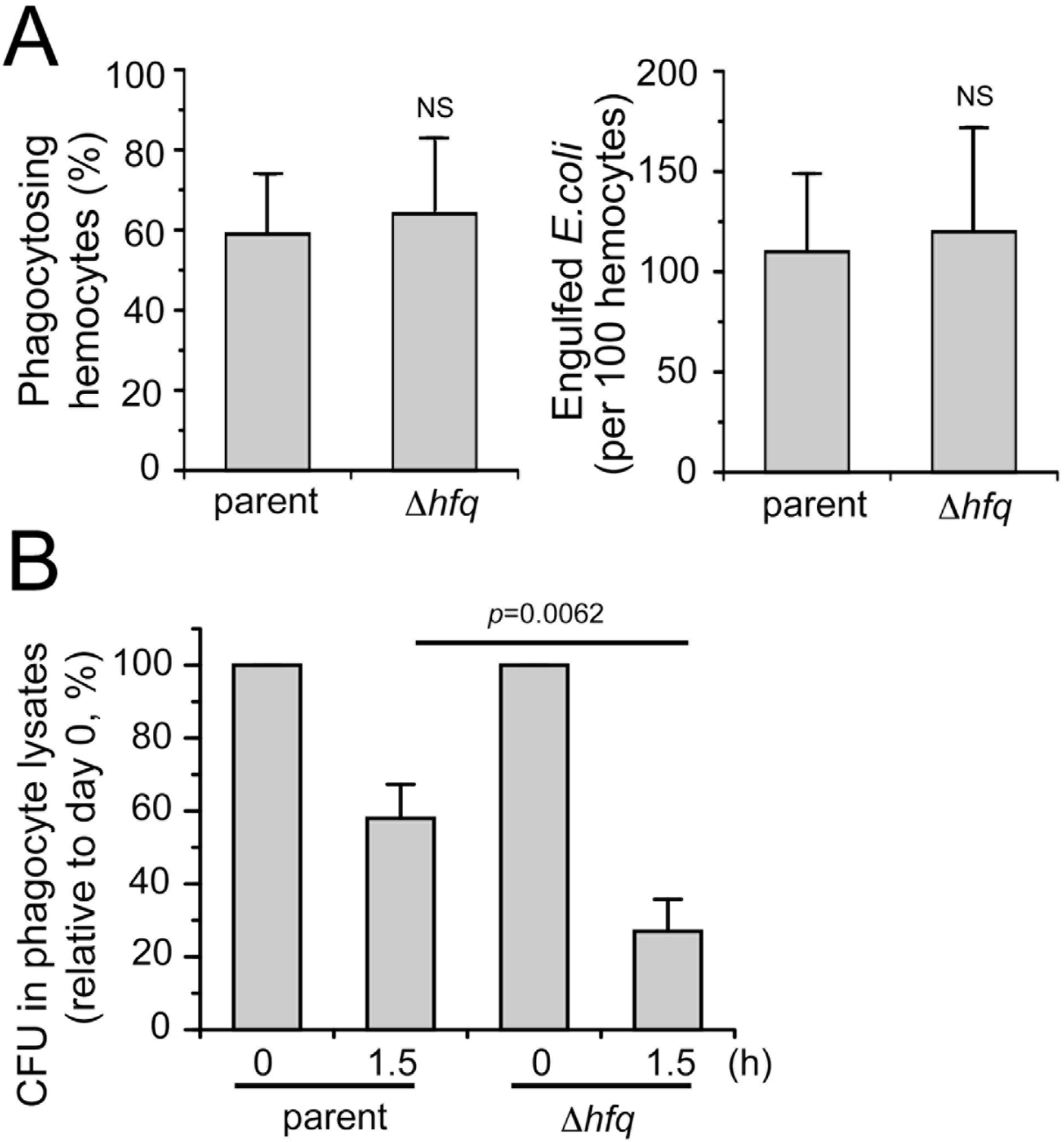

A
Phagocytosing hemocytes (%)
100
80
60
40
20
0
NS
parent
Δhfq
Engulfed E.coli (per 100 hemocytes)
200
150
100
50
0
NS
parent
Δhfq
B
CFU in phagocyte lysates (relative to day 0, %)
p=0.0062
100
80
60
40
20
0
0
1.5
0
1.5
(h)
parent
Δhfq

Fig. 5

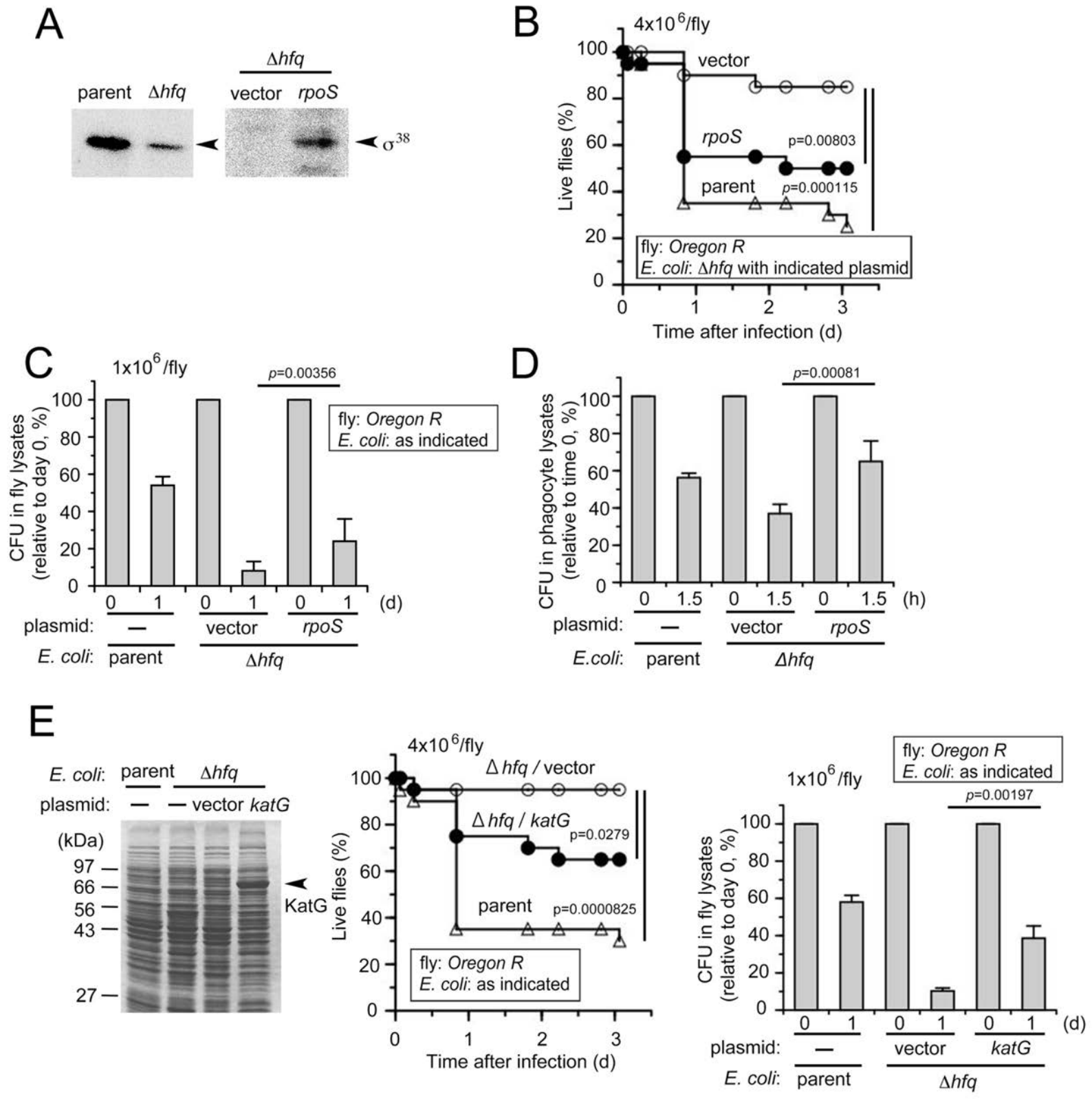

A
Δhfq
parent
Δhfq
vector
rpoS
σ38
B
4x10^6/fly
vector
rpoS
p=0.00803
parent
p=0.000115
Live flies (%)
fly: Oregon R
E. coli: Δhfq with indicated plasmid
Time after infection (d)
C
1x10^6/fly
p=0.00356
CFU in fly lysates (relative to day 0, %)
fly: Oregon R
E. coli: as indicated
(d)
plasmid:
vector
rpoS
E. coli:
parent
Δhfq
D
p=0.00081
CFU in phagocyte lysates (relative to time 0, %)
(h)
plasmid:
vector
rpoS
E.coli:
parent
Δhfq
E
E. coli:
parent
Δhfq
plasmid:
vector
katG
(kDa)
97
66
56
43
27
KatG
4x10^6/fly
Δ hfq / vector
Δ hfq / katG
p=0.0279
parent
p=0.0000825
Live flies (%)
fly: Oregon R
E. coli: as indicated
Time after infection (d)
1x10^6/fly
fly: Oregon R
E. coli: as indicated
p=0.00197
CFU in fly lysates (relative to day 0, %)
(d)
plasmid:
vector
katG
E. coli:
parent
Δhfq

Fig. 6

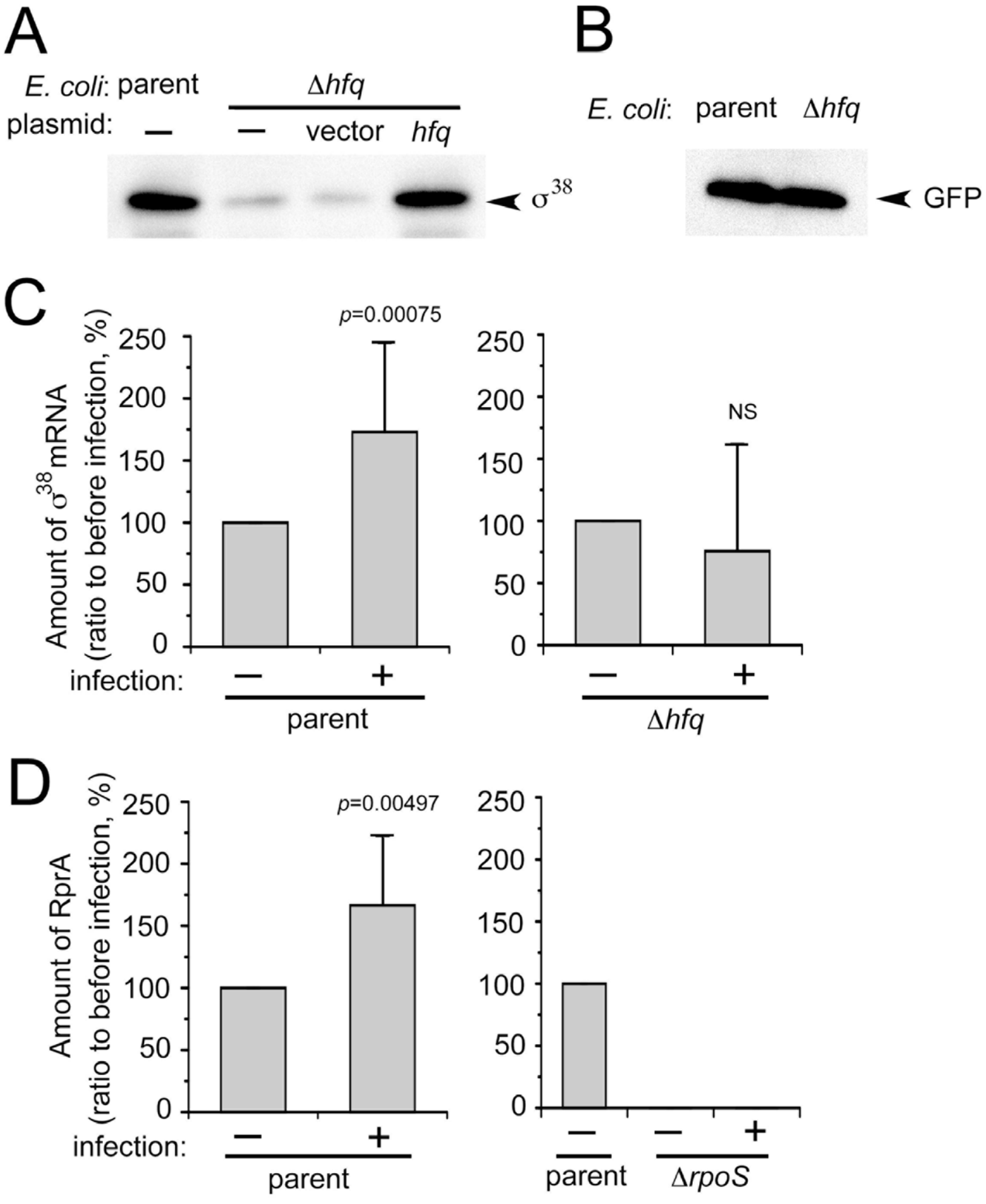
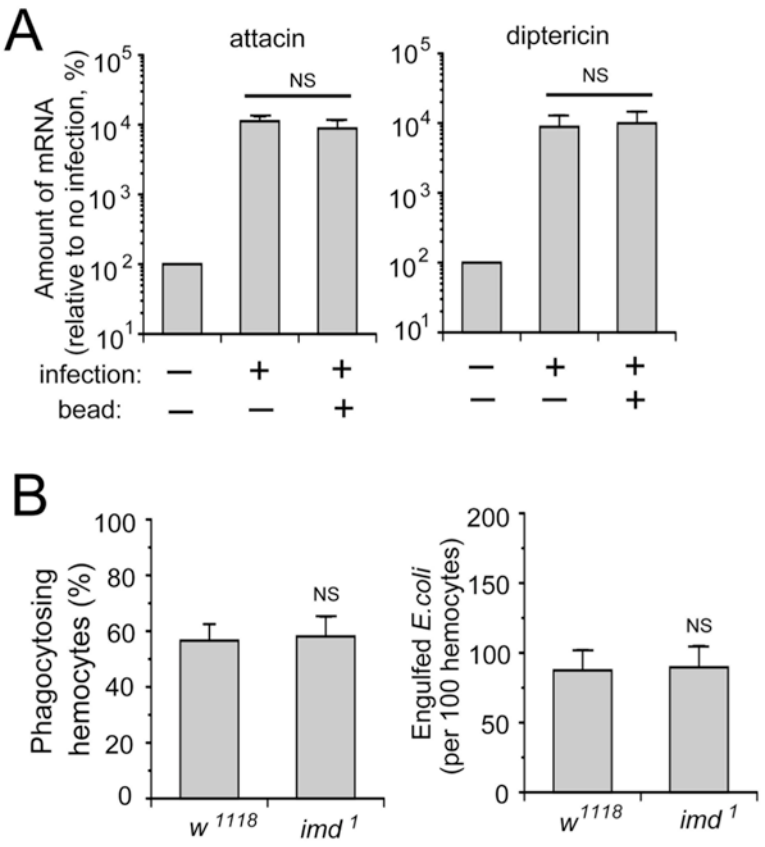

A
E. coli: parent
Δhfq
plasmid:
—
—
vector
hfq
σ38
B
E. coli: parent Δhfq
GFP
C
p=0.00075
NS
Amount of σ38 mRNA (ratio to before infection, %)
250
200
150
100
50
0
infection:
—
+
parent
Δhfq
D
p=0.00497
Amount of RprA (ratio to before infection, %)
parent
ΔrpoS

Fig. 7



Supplemental Figure 1

No relation between the production of antimicrobial peptide mRNA and the phagocytosis of *E. coli* by hemocytes. (**A**) Adult flies of *Oregon R* were injected with latex beads or PBS prior to the abdominal infection with the parental *E. coli* strain. After 3 h, RNA was prepared from those flies as well as the *E. coli* strain before infection, and subjected to RT-qPCR for the mRNAs of attacin, diptericin, and Rp49. The values for the mRNAs of attacin and diptericin were normalized with those of Rp49 mRNA, and the data from independent experiments are shown as the mean ± SD after analysis by Student's *t* test. NS, difference not significant. The numbers of replicated experiments are: (in the analysis of attacin mRNA, from left to right) four, seven, and eight; (in the analysis of diptericin mRNA, from left to right) five, three, and eight. (**B**) Hemocytes were isolated from third-instar larvae of the indicated fly lines and used as phagocytes in the phagocytosis reaction *in vitro* with the parental *E. coli* strain as the target. Data from three independent experiments are shown as the mean ± SD after analysis by Student's *t* test. NS, difference not significant.