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A Toll pathway effector protects *Drosophila* specifically from distinct toxins secreted by a fungus or a bacterium

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The *Drosophila* systemic immune response against many Gram-positive bacteria and fungi is mediated by the Toll pathway. How Toll-regulated effectors actually fulfill this role remains poorly understood as the known Toll-regulated antimicrobial peptide (AMP) genes are active only against filamentous fungi and not against Gram-positive bacteria or yeasts. Besides AMPs, two families of peptides secreted in response to infectious stimuli that activate the Toll pathway have been identified, namely Bomanins and peptides derived from a polyprotein precursor known as Baramicin A (BaraA). Unexpectedly, the deletion of a cluster of 10 Bomanins phenocopies the Toll mutant phenotype of susceptibility to infections. Here, we demonstrate that *BaraA* is required specifically in the host defense against *Enterococcus faecalis* and against the entomopathogenic fungus *Metarhizium robertsii*, albeit the fungal burden is not altered in *BaraA* mutants. BaraA protects the fly from the action of distinct toxins secreted by these Gram-positive and fungal pathogens, respectively, Enterocin V and Destruxin A. The injection of Destruxin A leads to the rapid paralysis of flies, whether wild type (WT) or mutant. However, a larger fraction of wild-type than *BaraA* flies recovers from paralysis within 5 to 10 h. BaraAs' function in protecting the host from the deleterious action of Destruxin is required in glial cells, highlighting a resilience role for the Toll pathway in the nervous system against microbial virulence factors. Thus, in complement to the current paradigm, innate immunity can cope effectively with the effects of toxins secreted by pathogens through the secretion of dedicated peptides, independently of xenobiotics detoxification pathways.

Baramicin A | microbial toxins | Destruxin A | enterocin O16 | resilience/disease tolerance

Significance

Major immune response pathways control the expression of hundreds of genes that represent potential effectors of the immune response. The *Drosophila* Toll pathway is required in the host defenses against several Gram-positive bacterial infections as well as against fungal infections. The current paradigm is that peptides secreted in the hemolymph during the systemic immune response are either bona fide antimicrobial peptides or likely ones. The finding of a dual role for one Toll pathway effector in the resilience to both *Enterococcus faecalis* and *Metarhizium robertsii* infections underscores an original concept in insect innate immunity. Evolution can select effectors tailored to protect the host from the action of microbial toxins of prokaryotic or eukaryotic origin, independently of antibodies or detoxification pathways.

The study of host defense against infections has essentially focused on the immune response and the mechanisms used by the organism to directly attack, kill, or neutralize invading pathogens. This dimension of host defense is known as resistance and in insects is mediated primarily by antimicrobial peptides (AMPs) and also by the cellular immune response and melanization (1–4). However, there is a second complementary dimension known as disease tolerance or resilience whereby the organism is able to withstand and, in some cases, repair damages inflicted by the virulence factors of pathogens or the host's own immune response (5–7). Some instances of resilience have been reported in *Drosophila*, e.g., the removal of oxidized lipids by Malpighian tubules through the lipid-binding protein Materazzi, the requirement for *CrebA* in regulating secretion during the immune response or the enterocyte cytoplasmic purge against pore-forming toxins (8–10). One way to discriminate between resistance and resilience is to monitor the microbial burden of infected hosts. It will be increased during infection of immunodeficient as compared to immunocompetent hosts. In contrast, it will not change much in organisms with defective resilience, which will tend to succumb to a lower load of pathogens, as monitored by measuring the pathogen load upon death (PLUD) (11, 12).

In *Drosophila*, the Toll pathway is one of the two NF-κB (nuclear factor kappa light chain enhancer of activated B cells) pathways that regulate the systemic immune response to microbial infections and through the MyD88 adapter complex is required in the host defense against many Gram-positive and fungal infections. It regulates the expression of more than 250 genes (9, 13–16). A few AMPs active against filamentous fungi have been identified (Drosomycin, Metchnikowin, and Daisho) (17–19). However, effectors solely regulated by the Toll pathway able to attack pathogenic yeasts or Gram-positive bacteria in vitro have not been described so far. Mass spectrometry analysis performed on the hemolymph of single immune-challenged flies has led to the identification of more than 30 peaks corresponding to Drosophila immune-induced molecules (DIMs) (20, 21). Some of them correspond to known AMPs, whereas others belong to a family of 12 proteins that contain a domain known as the Bomanin domain (21, 22). Ten such *Bomanin* genes are located at the 55C locus, including DIMs 1 to 3, now referred to BomS1 to S3.

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The authors declare no competing interest.

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The deletion of this locus strikingly phenocopies the Toll mutant phenotype, being sensitive to filamentous fungi, pathogenic yeasts, and Gram-positive bacteria such as *Enterococcus faecalis* (22). Some short Bomanins that essentially contain only the Bomanin domain may be active against *Candida glabrata* in vivo (23).

Several DIMs (5, 6, 8, 10, 12, 13, 22, 24) are actually derived from a polyprotein precursor known as IMPPP and until recently their function has not been understood (20, 21). A recent study renamed this protein as Baramicin A (BaraA) and proposed that some of the derived peptides function as antifungal AMPs (24). Here, we report our analysis of *BaraA* mutants. While we confirm a sensitivity to entomopathogenic fungi, our data clearly establish a susceptibility also to *E. faecalis*, but not to other pathogens we have tested. Interestingly, the fungal burden does not appear to be altered in the mutants, from the beginning to the end of the infections. Our data indicate that a major function of *BaraA* is in the resilience against distinct toxins, Destruxin A (DtxA), a pore-forming toxin, and Enterocin V (EntV), a bacteriocin, respectively, secreted by *Metarhizium robertsii* and *E. faecalis*. BaraA helps the host recover from DtxA-induced paralysis and appears to be required in glial cells but not in neurons.

Results

The *BaraA* locus encodes a polyprotein precursor that is likely processed by a furin-like enzymatic activity, which leads to the release in the hemolymph of multiple DIM peptides. These peptides share extensive sequence similarity, except for the N-terminal DIM24 protein that defines an evolutionarily conserved independent domain (25) (Fig. 1 *A* and *B*). For convenience, we shall refer to specific BaraA-derived peptides by their DIM names. Of note, *BaraA* lies next to the *CG18278* gene and the two genes are found as a perfect duplication in some wild and laboratory lines (25) (*SI Appendix*, Fig. S1 *A* and *B*).

BaraA gene expression is induced by a challenge with the Gram-positive bacteria *Micrococcus luteus* (used as a reference) and *E. faecalis* and by injected *M. robertsii* spores in a *MyD88*-dependent manner (Fig. 1*C*), in keeping with previous data at the transcriptional and peptide levels (20, 24). In contrast, the *CG18278* gene does not appear to be induced by any of these challenges (*SI Appendix*, Fig. S2*A*) and also not upon natural infection (*SI Appendix*, Fig. S2*B*).

***BaraA* Contributes to the Host Defense against *E. faecalis*.** In this work, we have generated a CRISPR–Cas9-mediated knock-out (KO) line, a *mCherry* knock-in (KI) line, and also used an RNAi line for knockdown (KD) experiments (*SI Appendix*, Fig. S1 *A*, *C* and *D*). In these lines, the induction of *BaraA* expression by an immune challenge is hardly detected, both at the transcriptional (*SI Appendix*, Fig. S1*E*) and protein levels (*SI Appendix*, Fig. S3 and Table S1). We have also generated two *CG18278* KO lines and tested them along a KD line with an *E. faecalis* challenge: No consistent susceptibility phenotype was detected (*SI Appendix*, Fig. S2 *C–F*). In contrast, we observed a significant susceptibility of *BaraA* mutant lines isogenized in the wild-type (WT) w^{A5001} background after the injection of this Gram-positive bacterial strain (Fig. 2*A*). Contrary to the *MyD88* line, measurements of the bacterial burden did not reveal any difference between the KO/KI lines and the isogenic w^{A5001} control line except for the 24 h time point, also within 30 min after death (bacterial load upon death: BLUD (11); Fig. 2 *B–D*). Note that at the 24 h and 48 h time points, the distribution of bacterial loads is already bimodal in the *BaraA* mutant, reflecting that the fate of infected flies is likely settled earlier in the mutant than in the wild-type flies (11). These observations at 24 to 48 h opened the possibility that BaraA may be partially involved in resistance to *E. faecalis*. We therefore monitored the melanization arm of the immune response by visualizing the proteolytic cleavage that activates pro-phenoloxidase (PPO) into a catalytically active enzyme after a microbial challenge; we observed a lessened maturation of PPO1 into PO1 in the *BaraA* KO mutant in three out of four experiments (*SI Appendix*, Fig. S4 *A* and *A'*). As regards the cellular immune response, we noted that the m*Cherry* gene that replaces the *BaraA–CG18278* locus was more strongly expressed in adult hemocytes after an *E. faecalis* challenge (*SI Appendix*, Fig. S4 *B* and *B'*). As expected, the *BaraA* KD line led to an enhanced sensitivity to *E. faecalis* and to *M. robertsii* when the short RNA hairpin was ubiquitously expressed (*SI Appendix*, Fig. S4 *C* and *D*). When we silenced *BaraA* by RNAi in hemocytes, we did observe that

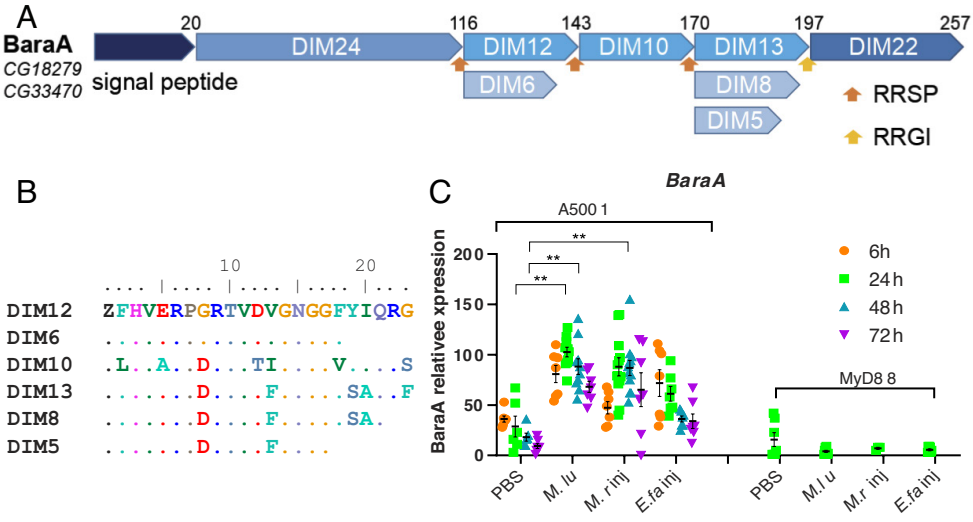

Fig. 1. Structure of the BaraA precursor protein and induction of *BaraA* expression by an immune challenge. (*A*) Schematic structure of the BaraA polyprotein. The name of the peptides derived from the processing of the precursor upon furin cleavage is shown as Drosophila-induced immune molecules (DIM), their original name. The type of internal furin-like cleavage sites is indicated by orange and yellow arrows (RRSP, RRGI). (*B*) Alignment of the short DIM peptides derived from BaraA, referred to by their DIM numbers. (*C*) Expression of the *BaraA* gene monitored by qRT-PCR at various time points after the injection of the indicated microbes; *M. luM. luteus*; *M. rM. robertsii*; *E. fa*: *E. faecalis*. The measured expression of *BaraA* 24 h after a *M. luteus* challenge is taken as reference for all other data points and given a 100% value. The means ± SEM are shown in black. Pooled data from three independent experiments, $^{**}P < 0.01$.

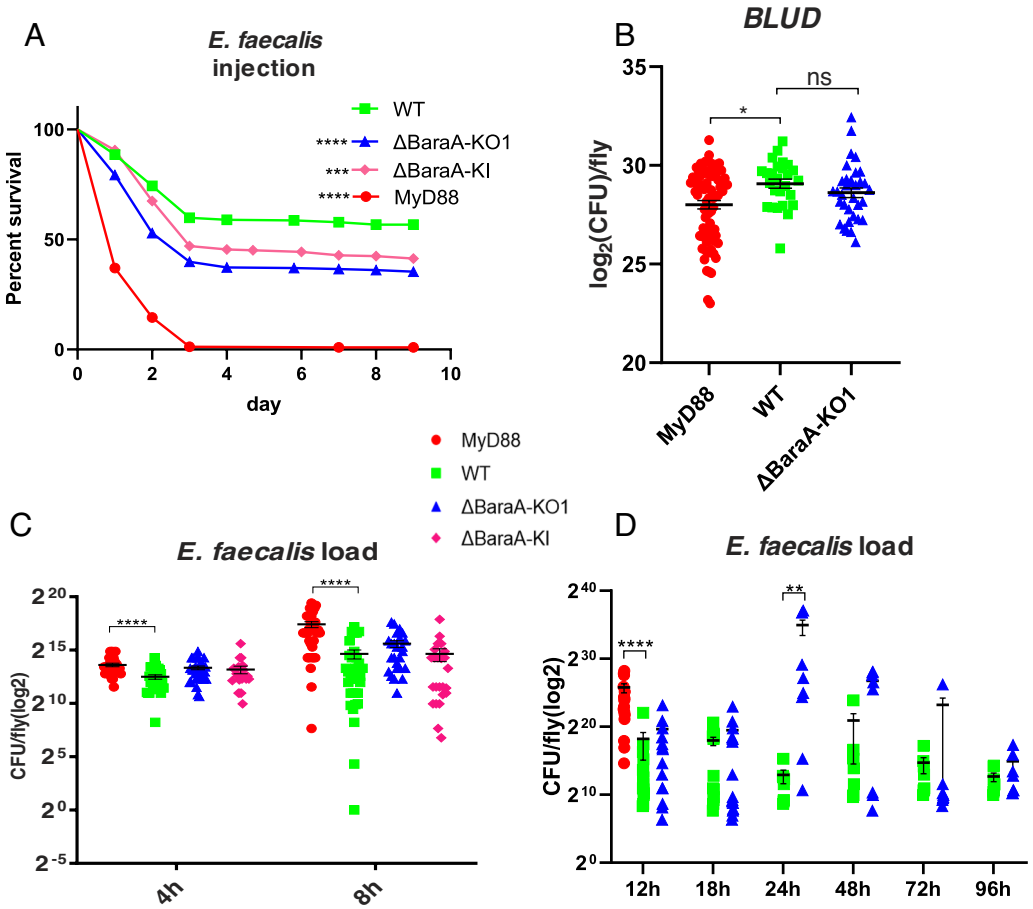

Fig. 2. Susceptibility of *BaraA* mutant flies to *E. faecalis* infection. (*A*) Survival curves of the isogenic *BaraA* KO and KI flies infected with *E. faecalis* NCTC 775. The WT corresponds to a wild-type w^{A5001} line isogenized in parallel to the KO and KI lines, which behaves like the w^{A5001} line used for isogenization. Pooled data from six independent experiments, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. (*B*) Bacterial load upon death (BLUD) of *E. faecalis* OG1RF in WT and *BaraA* KO and KI lines. Pooled data from three independent experiments. (*C* and *D*) Bacterial load of *E. faecalis* NCTC 775 (*C*) and OG1RF (*D*) in WT and *BaraA* KO and KI lines from early time points to 6 d after infection. No significant differences were detected between WT and *BaraA* mutants at each time point, except for the 24 h one, $^{**}P < 0.01$. *MyD88* was significantly different from WT, $^{****}P < 0.0001$. The caption applies to panels *B*–*D*. Pooled data from three independent experiments. Data are expressed as means ± SEM.

these flies were significantly more susceptible to injected *E. faecalis* bacteria (*SI Appendix*, Fig. S4*E*).

We conclude that the *BaraA* mutant lines display an intermediate sensitivity to *E. faecalis* infection. It is however not fully clear whether the altered bacterial burden measured at 24 h results from a lessened activation of melanization, or a hemocyte-mediated response, albeit an impaired antimicrobial activity can also not be excluded.

The *BaraA* Mutant Is Susceptible to *M. robertsii* Infection Only in the Septic Injury Model. The *BaraA* KO and KI lines as well as the KD line consistently exhibited a moderately enhanced sensitivity to the injection of 50 *M. robertsii* conidia (Fig. 3*A* and *SI Appendix*, Fig. S4*C*). We did not detect an increased microbial titer in these mutants compared to wild-type controls during the infection, in contrast to *MyD88*; the fungal loads upon death (FLUD) were also similar (Fig. 3 *B* and *C*). Interestingly, no susceptibility to *M. robertsii* in the natural infection model was observed, even though the *BaraA* gene is induced by this challenge (*SI Appendix*, Fig. S5 *A* and *A'*). We have also tested a panel of other bacterial and fungal strains and did not observe any sensitivity to those infections (*SI Appendix*, Fig. S5 *B*–*F*).

In conclusion, we have found that *BaraA* appears to be required rather specifically in the host defense against a bacterial opportunistic pathogen, *E. faecalis*, and an entomopathogenic fungus, *M. robertsii*. Interestingly, the fungal burden did not appear to be altered in the *BaraA* mutants, which suggests that *BaraA* is not required in the resistance against *M. robertsii*.

The Transgenic Overexpression of BaraA Rescues the Sensitivity of *MyD88* Flies to *E. faecalis* and to *M. robertsii* to a Limited Degree. A complementary strategy to the loss-of-function analysis reported above consists in overexpressing the *BaraA* gene in a wild-type context, thus determining whether it might constitute a limiting factor in host defense against infections. The overexpression of *BaraA* at the adult stage using transgenic lines failed to enhance the protection of wild-type hosts against *M. robertsii* and to *E. faecalis* (*SI Appendix*, Fig. S6 *A* and *B*) yet rescued the *BaraA* sensitivity phenotype to these pathogens (*SI Appendix*, Fig. S6 *C* and *D*).

We next tested the overexpression of *BaraA* in a sensitized *MyD88* background. The *BaraA* transgene partially rescued the sensitivity of *MyD88* flies to *M. robertsii* and *E. faecalis* (*SI Appendix*, Fig. S6 *A* and *B*), suggesting that BaraA can function in the absence of Toll-induced Bomanins. We also checked by Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) spectrometry that the transgenic polyprotein was correctly processed, in the *MyD88* background, so that the endogenous signal would not mask that of the transgene-derived protein. As shown in *SI Appendix*, Fig. S6*E* and Table S2, *MyD88* is not required for the processing of the precursor into the DIM10, DIM12, DIM13, or DIM24 proteins by a putative furin. We also infer that the Toll

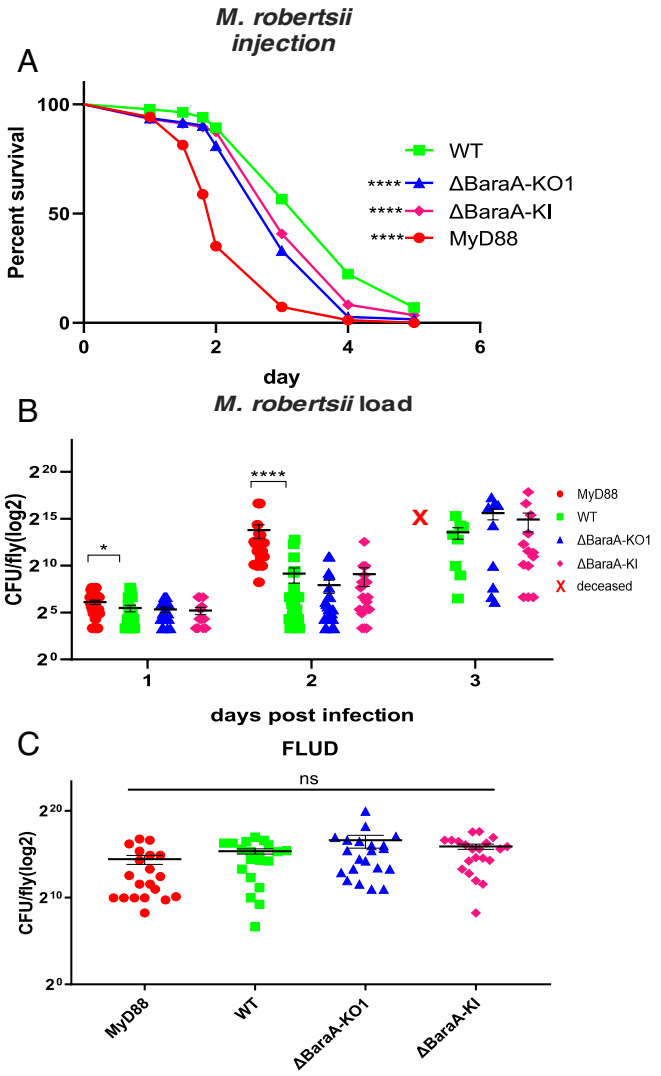

Fig. 3. Susceptibility of *BaraA* mutants to *M. robertsii* infection. (*A*) Survival of isogenic *BaraA* mutants injected with 50 *M. robertsii* conidia. Pooled data from 10 independent experiments. Statistical significance between WT and the KO or KI mutants: ****$P < 0.0001$. (*B*) Kinetics over 3 d of the fungal load of the *BaraA* KO and KI mutants injected with 50 *M. robertsii* conidia. No significant difference was detected between WT and *BaraA* mutants at each time point. (*C*) Fungal load upon death (FLUD) of single isogenic *BaraA* KO and KI flies injected with 50 *M. robertsii* conidia. No significant differences between WT and the isogenic mutant flies were detected. Three independent experiments have been performed and pooled (*B* and *C*). Data are expressed as means ± SEM.

pathway-dependent Bombardier activity needed to stabilize the expression of short Bomanins is not required for the stability of DIM10, DIM12, and DIM13 (26).

We conclude that the transgenic overexpression of BaraA is not sufficient to confer additional protection against *E. faecalis* or *M. robertsii* in the context of a wild type but can partially compensate for the Toll-deficient host defense against these two pathogens.

BaraA Does Not Modulate the Induction of the Toll Pathway. Besides a potential role of effectors, proteins that are induced by immune signaling pathways may play a role in their feedback regulation. We therefore monitored Toll pathway activation using the steady-state mRNA levels of AMP genes known to be regulated by the Toll pathway such as *Drosomycin*, *Metchnikowin*, and *IM1* (27–29). As shown in *SI Appendix*, Fig. S7, we did not observe any influence of the isogenized *BaraA* KO or KI null mutations over 48 h on their expressions.

***BaraA* Protects *Drosophila* from the Action of Secreted Microbial Toxins.** Our data thus far are not in keeping with a function for *BaraA* in resistance against *M. robertsii* and are compatible with this possibility as regards *E. faecalis*. The PLUD data are not indicative of a function in resilience.

The concept of pathogen load and PLUD relies on the assumption that the virulence of the pathogen correlates with the microbial burden. We have recently established that the function of the Toll pathway in the host defense against *Aspergillus fumigatus* is not to directly fight off this pathogen, as immunodeficient flies are killed by a limited number of pathogens that are trapped at the injection site. Rather, we have discovered that Toll function in the host defense against *A. fumigatus* is to limit or counteract some of its secreted mycotoxins (30). As mycotoxins, namely Destruxins (Dtx), have been described as important virulence factors from generalist *Metarhizium* entomopathogenic fungi (31, 32), we therefore injected DtxA into wild-type and *BaraA* flies. Interestingly, *BaraA* KO and KI mutants as well as *MyD88* flies reproducibly succumbed to a larger extent than wild-type flies to the injection of DtxA (Fig. 4*A*), a result confirmed in axenic flies (*SI Appendix*, Fig. S8*A*). We next determined that *BaraA* mutants are not more susceptible than wild-type flies to a challenge with a *Dtx* mutant *M. robertsii* strain (32) (Fig. 4*B*). Taken together, these results suggest that a major function of *BaraA* in the host defense against *M. robertsii* is to alleviate or counteract the effects of Destruxins secreted by the fungus in the septic injury model.

We then wondered whether *BaraA* might function in a similar manner in the host defense against *E. faecalis*. We therefore injected the *E. faecalis* culture supernatant into flies. Strikingly, whereas wild-type flies survived this challenge well, about 50% of *BaraA* KO flies and 20% of *BaraA* KI and *MyD88* flies succumbed to the injected supernatant (Fig. 4*C*). Filtration experiments allowed us to determine that the toxic component of the supernatant can be recovered in a 3 to 10 kD fraction (Fig. 4*D*). Even though the noxious activity in the *E. faecalis* supernatant was heat resistant, it was nevertheless susceptible to proteinase K treatment, suggesting a protein component (*SI Appendix*, Fig. S8 *B* and *C*). Interestingly, it has been reported that the bacteriocin enterocin O16 is an *E. faecalis* virulence factor in *Drosophila* (33). Enterocin O16 is also known as EntV, which is heat resistant and able to kill some lactobacilli strain as well as to inhibit the hyphal growth, the virulence, and the biofilm formation of *Candida albicans* (34–36).

We therefore asked whether the toxic activity in the supernatant is still present when using a bacterial *entV*$^-$ strain. We observed that the supernatant from the complemented *E. faecalis* strain *entV*$^+$/*entV*$^-$ behaved as that from the wild-type bacterial strain, that is, it killed *MyD88* and *BaraA* KO and KI mutants more than wild-type flies. Strikingly, the supernatant from an *entV*$^-$ *E. faecalis* mutant strain killed wild-type and *BaraA* mutants at a similar rate, whereas *MyD88* flies succumbed to the same extent to the mutant or complemented wild-type supernatants (Fig. 4*E*). Similarly, the direct infection-complemented *E. faecalis* strain *entV*$^+$/*entV* killed the immunodeficient *MyD88* and *BaraA* flies more than the wild-type control flies and thus behaved like the wild-type bacterial strain. In contrast, the *entV*$^-$ mutant *E. faecalis* strain did not kill *BaraA* KO flies to the same extent than the complemented bacterial strain (Fig. 4*F*). The EntV peptide derives from the open reading frame found in the *ef1097/entV* gene, which encodes a preproprotein. This precursor protein gets cleaved by the GelE protease into a 7.2 kDa active peptide (35, 37). As expected, a *gelE E. faecalis* mutant strain did not kill *BaraA* flies faster than wild-type flies (*SI Appendix*, Fig. S8*D*). We conclude that *BaraA* protects the flies from the action of the EntV bacteriocin.

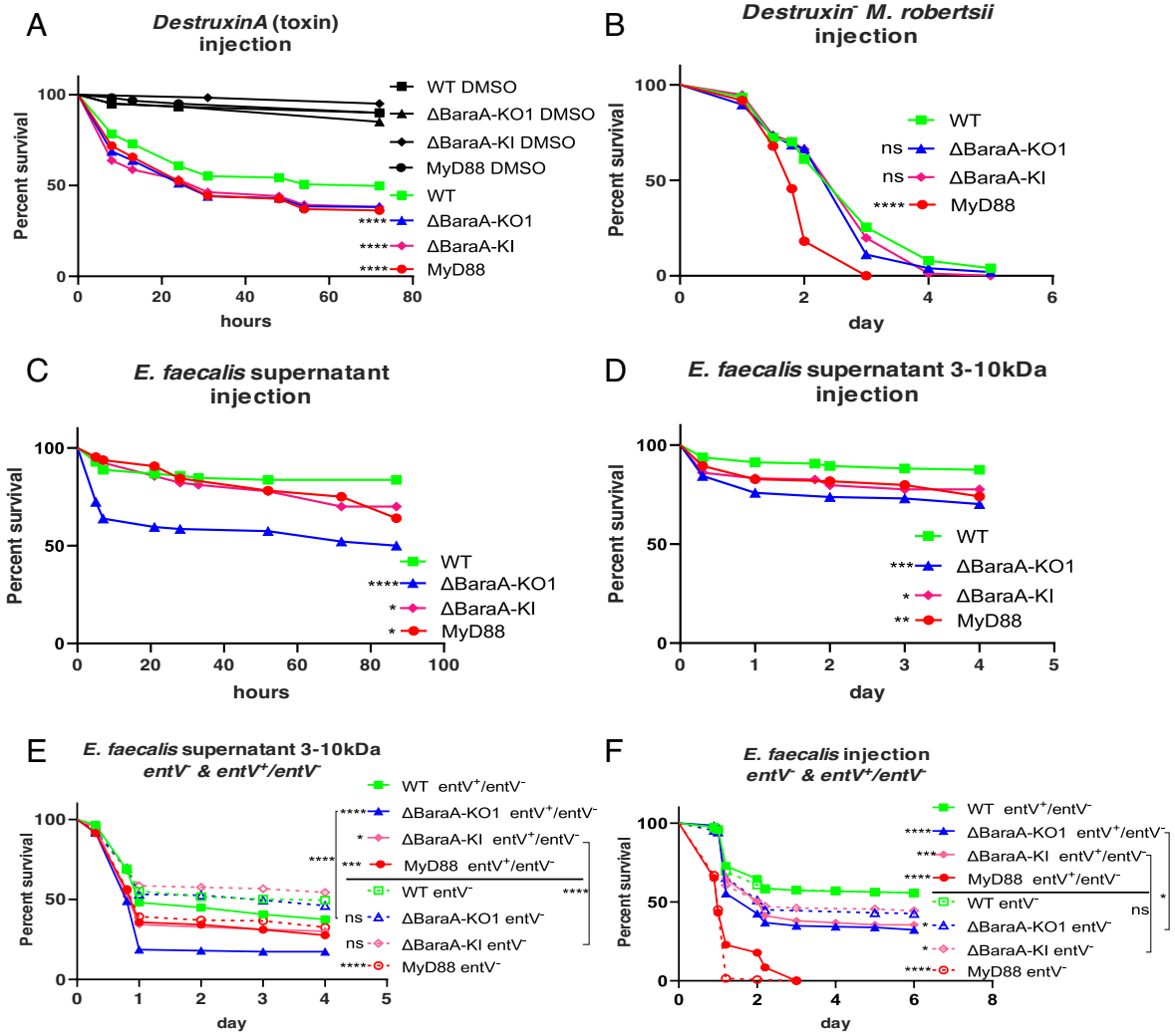

Fig. 4. *BaraA*-dependent protection of *Drosophila* flies from the noxious effects of microbial toxins. (*A*) Mutant flies were injected with 4.6 nl, 8 mM Destruxin A toxin. 80% DMSO was injected as vehicle control. Pooled data from eight independent experiments. Statistically significant differences between wild-type and *BaraA* mutants, ****$P < 0.0001$. (*B*) *BaraA* mutants were injected with 50 spores of *DestruxinS1⁻ M. robertsii* mutant strain in which the biosynthesis of Destruxins is blocked. No significant difference was observed between wild-type and *BaraA* flies; pooled data from five independent experiments. (*C*) Wild-type, *MyD88*, and *BaraA* KO1 and KI mutant flies were injected with the concentrated supernatant from overnight *E. faecalis* OG1RF cultures. About 50% of *BaraA* KO1 mutant but not wild-type flies succumbed to this challenge, while 30% of the KI and MyD88 flies succumbed to this challenge. Pooled data from four independent experiments, *$P < 0.05$, ****$P < 0.0001$. (*D*) Same as (*C*), except that the supernatant was size filtered to retain molecules ranging from 3 to 10 kDa. Pooled data from four independent experiments, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (*E*) The 3 to 10 kDa fraction supernatant from *E. faecalis* OG1RF was collected from *entV⁻* and *entV⁺/entV⁻* strains. The supernatant from the *entV⁻* strain killed *BaraA* mutants at the same rate as wild-type flies, whereas *BaraA* KO1 mutants were killed by the complemented *entV⁺/entV⁻* supernatant significantly faster than wild-type flies. Pooled data from eight independent experiments. (*F*) 0.5 OD, 4.6 nl of the mutant and rescued *E. faecalis* OG1RF strains were injected. Rescued strain *entV⁺/entV⁻* killed *BaraA* KO1 faster than wild-type flies. Significant differences between wild-type and *BaraA* mutant flies were detected upon *entV⁻* infection. *BaraA* KO1 infected by *entV⁺/entV⁻* strain was killed faster than *BaraA* KO1 infected by *entV⁻* strain, while no such significant difference was observed in the case of the *BaraA* KI line. Pooled data from four independent experiments. (*E* and *F*) For each condition (above or below the line in the caption), mutant flies are compared to wild-type flies submitted to the same challenge for statistical analysis, *$P < 0.05$; ***$P < 0.001$; ****$P < 0.0001$.

Taken together, our data suggest that a major function of *BaraA* in *Drosophila* host defense is to protect the fly from specific secreted microbial toxins, whether of prokaryotic or eukaryotic origin.

BaraA Helps *Drosophila* Recover from DtxA-Induced Paralysis and Is Required in Glial Cells. A striking phenotype observed upon the injection of DtxA is the immediate paralysis it induces as flies do not recover from anesthesia as untreated flies do. A careful scrutiny revealed that some 60% of wild-type flies progressively recovered their activity within 5 to 10 h of DtxA injection in contrast to less than 40% for *BaraA* KO flies (Fig. 5 *A* and *B* and Movies S1–S3). In contrast, the injection of the *E. faecalis* supernatant only temporarily slowed down the flies, a phenomenon difficult to quantify accurately.

This set of data suggested that the toxins may somehow interfere with the nervous system. We therefore silenced *BaraA* gene expression either in neurons or in glia and monitored the survival of flies to injected DtxA or *E. faecalis* supernatant. Silencing *BaraA* expression in glial cells but not in neurons enhanced the sensitivity of flies to these challenges (Fig. 5 *C*–*F*). In the case of DtxA, the effect was as strong as that observed upon using a ubiquitous driver (Fig. 5 *C* and *G*). In contrast, the degree of enhanced susceptibility to the injection of the *E. faecalis* supernatant was modest and unexpectedly none was detected upon the ubiquitous silencing of *BaraA* (Fig. 5*H*).

We conclude that BaraA function is required in glial cells where it may mediate the protection against the effects of DtxA on the nervous system.

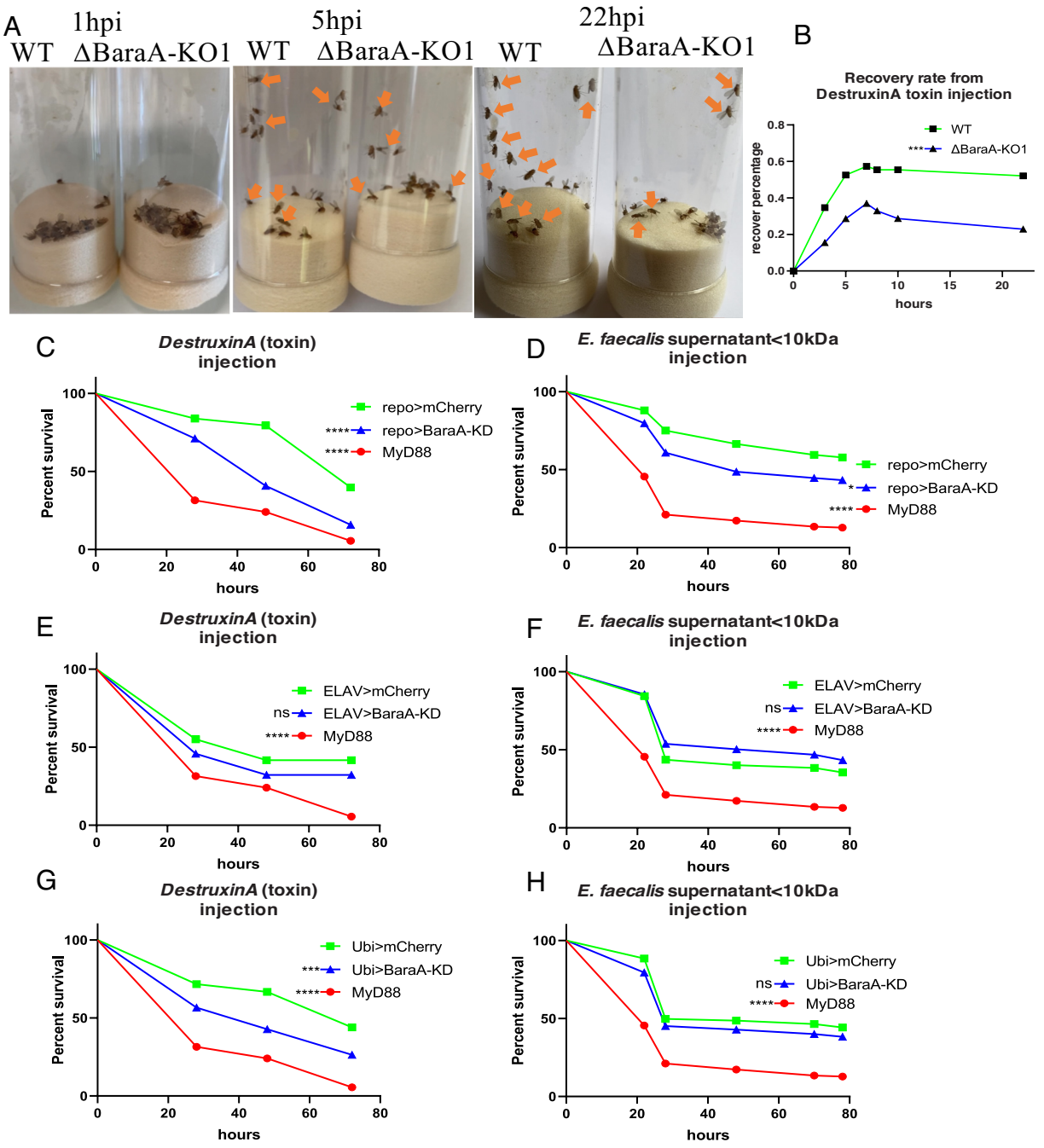

Fig. 5. BaraA counteracts the paralysis induced by exposure to Destruxin A and is required in glial cells. (*A*) Wild-type flies or *BaraA*-KO1 mutant flies were injected with 4.6 nl, 8 mM Destruxin A toxin. Pictures were taken at 1, 5, and 22 h postinfection (see also the corresponding Movies S1–S3). After 1 h postinfection, all flies were paralyzed. At 5 h postinfection, 11 wild-type flies woke up, whereas only six *BaraA*-KO1 mutant flies woke up from toxin injection. At 22 h postinfection, 12 wild-type flies woke up, whereas four *BaraA*-KO1 mutant flies woke up. (*B*) Quantification of (*A*). Pooled data from two independent experiments, ***$P < 0.0001$. (*C* and *D*) *BaraA*-KD flies were silenced in glial cells (*repo-Gal4*) and injected with 4.6 nl, 8 mM Destruxin A toxin (*C*) or 23 nl of *E. faecalis* supernatant <10 kDa (*D*). *BaraA*-KD flies displayed significant difference from wild-type control flies. Pooled data from four independent experiments, *$P < 0.05$, ****$P < 0.0001$. (*E* and *F*) *BaraA*-KD flies were silenced in neurons (*elav-Gal4*) and injected with 4.6 nl, 8 mM Destruxin A toxin (*E*) or 23 nl of *E. faecalis* supernatant <10 kDa (*F*). *BaraA*-KD flies showed no significant (ns) difference from wild-type control flies. Pooled data from four independent experiments. (*G* and *H*) *BaraA*-KD flies were silenced ubiquitously (*ubi-Gal4*) and were injected with 4.6 nl, 8 mM Destruxin A toxin (*G*) or 23 nl of *E. faecalis* supernatant <10 kDa (*H*). *Ubi-Gal4>BaraA* KD flies showed significant difference from wild-type control flies after Destruxin A injection (*G*). No significant difference between *BaraA* KD and wild-type control upon *E. faecalis* OG1RF supernatant injection. Pooled data from four independent experiments.

Discussion

Our analysis of the *BaraA* infection mutant phenotype revealed a sensitivity to specific pathogens and not to broad categories of microorganisms as is the case for Toll pathway mutants. Interestingly, we observed a susceptibility to *E. faecalis* and to *M. robertsii*, respectively, a Gram-positive bacterium and an entomopathogenic fungus. For both pathogens, specific secreted virulence factors killed a significant fraction of *BaraA* mutants, whereas the *BaraA* phenotype of enhanced sensitivity to infection was lost when the corresponding virulence factor genes were mutated in the pathogen. Taken together, these results indicate that a major function of *BaraA* in *Drosophila* host defense is to protect it from the action of specific secreted toxins. Indeed, whereas in a concurrent study we showed that Toll pathway mutant flies are sensitive to *A. fumigatus* restrictocin (30), *BaraA* mutants did not exhibit any enhanced sensitivity to

restrictocin, nor to Beauvericin, a toxin made by *Beauveria bassiana*, another entomopathogenic fungus that has been reported to kill *BaraA* mutants faster than wild-type flies (*SI Appendix*, Fig. S9) (24).

A recently published study proposed that BaraA is involved in resistance to infection to entomopathogenic fungi as an AMP since, besides being sensitive to *B. bassiana* and *Metarhizium rileyi*, *BaraA* mutants exhibit an increased *B. bassiana* load 48 h after infection (24). In addition, BaraA-derived IM10-like peptides synergize with a membrane-active antifungal compound to kill *C. albicans* in vitro (24). The fact that BaraA is a polyprotein that produces multiple DIM10-like peptides and that the *BaraA* locus is found to be duplicated in about 14% of wild-type *Drosophila* strains caught at one location is in keeping with this possibility, as these strains would be expected to produce twice as much BaraA-derived peptides (25). Because BaraA encodes a polyprotein precursor, we cannot formally exclude such an AMP function for one or several of these BaraA-derived peptides, possibly acting locally to achieve an effective antimicrobial concentration, for instance in the brain. Indeed, the Bomanin family presents a similar situation: Whereas we have shown a function for some specific 55C *Bomanins* in the resilience to *A. fumigatus* mycotoxins (30), it is known that at least some *Bomanin* genes are required for resistance to *E. faecalis* (22), a finding we have directly confirmed for at least one 55C *Bomanin* gene (38). We note that if DIM10-like peptides indeed act as AMPs, they would need to act rather specifically against *E. faecalis* and *M. robertsii* since we did not detect an enhanced sensitivity to the other pathogens tested in this study. Very specific antibacterial functions for some *Drosophila* AMPs have been documented (39–41); however, we are not aware of AMPs having dual specificities against both particular bacterial and fungal species. We observed an increased *E. faecalis* burden at one specific time point, which supports a function in resistance that may be mediated through antimicrobial activity as discussed above, or through a function in mediating melanization or the cellular immune response (Fig. 2 and *SI Appendix*, Fig. S4). Nevertheless, the findings that bacterial and fungal toxin mutants kill *BaraA* mutant as efficiently as wild-type mutant flies and that ubiquitous *BaraA* overexpression does not enhance the protection against *E. faecalis* or *M. robertsii* support the concept that an important function of BaraA is to neutralize or counteract the action of specific secreted microbial toxins in the case of *E. faecalis* or *M. robertsii* infections.

Interestingly, several studies have shown that besides their direct antimicrobial functions, mammalian α-defensins have the remarkable property to neutralize some microbial pore-forming toxins or enzymes that need to cross the host cell plasma membrane to act on their intracellular targets (42 and references therein). The proposed mechanism of action of these AMPs relies on a common property of these microbial virulence factors: a relative thermodynamic instability that is required for the necessary flexibility to insert into or cross the plasma membrane. α-defensins are constituted by amphipatic α-helices that through hydrophobic interactions with the targeted enzymes are able to destabilize them (42). The unfolded proteins can then be degraded. We know little about the biochemistry of BaraA-derived peptides and no antimicrobial activity at physiological concentrations has been yet found in vitro in the absence of a cofactor. While a similar mechanism may be at play with the EntV protein, it is less likely to function with a hexadepsipeptide that is circular (31) and thus likely difficult to destabilize and degrade because of its circular conformation, even though it is rather hydrophobic.

A study on the evolution of BaraA as well as two related paralogs generated by independent duplication events suggests that the core domain of these three proteins is the N-terminal DIM24 domain, which is associated with only two DIM10-like domains in BaraB and none in BaraC (25). The expression of *BaraB* and *BaraC* has been reported not to be induced by an immune challenge (25), a finding we have independently confirmed for *BaraB*. We did not find a susceptibility of *BaraB* KO mutant to *E. faecalis* infection (43), in keeping with a reported lack of detectable immune function (25). BaraB function is essential in neurons, whereas BaraC appears to be expressed in glial cells (25). Interestingly, a *BaraA* expression fluorescent reporter is detected in brain tissues (24). Thus, it is likely that the DIM24 domain may have a function distinct from the DIM10-like peptides that are thought to act more like AMPs, although definitive evidence is presently lacking. These observations taken together with our finding of a requirement for *BaraA* expression in glial cells to protect the host against the noxious effects of DtxA therefore open the possibility that the DIM24 peptide might mediate this function, a proposition that requires experimental validation.

The exact mode of action of BaraA-derived peptides in the resilience to microbial toxins remains to be characterized, in as much as they act against distinct types of toxins. Destruxins have been isolated 60 y ago and they appear to act as ionophores that deplete cellular ions such as H^+, Na^+, and K^+ through the formation of pores in the membranes in a reversible process (44), although many other functions have been proposed (31). ClassII bacteriocins also form pores on the membrane of targeted bacteria, but the specific molecular mechanism of action of EntV on eukaryotic cells remains unknown. It presents an activity against the formation of biofilms by the dimorphic yeast *C. albicans* or the monomorphic yeast *C. glabrata*. Furthermore, it prevents filamentation of the former in vitro and in vivo (34). Thus, it is unclear whether BaraA would act directly on both toxins, through the same or distinct BaraA-derived peptides, would counteract a common process triggered by Destruxins and EntV such as intracellular ion depletion, or would indirectly alter the physiology of cells exposed to the action of these toxins. It will be important to determine how the toxins act on the host and whether they target preferentially some tissues. For instance, it will be interesting to determine whether the function of BaraA in glial cells is linked to the blood–brain barrier. An emerging theme is that some of the Toll pathway effectors act in the brain and counteract the noxious effects of toxins that also act on the nervous system as exemplified here with the requirement for BaraA expression in glial cells. Interestingly, we have recently found that *BomS6* overexpression in the brain protects the flies from the effects of the injected *A. fumigatus* toxin verruculogen (30).

A specificity of the Toll pathway is that it is required in the host defense against both prokaryotic and eukaryotic pathogens. As compared to the Immune deficiency (IMD) pathway, one interesting feature is that the Toll pathway can be activated by proteases secreted by invading pathogens (45–47). It is interesting to note here that the function of BaraA against two distinct secreted virulence factors, likely pore-forming toxins, provides another point of convergence for the dual role of the Toll pathway, this time at the effector level. It is thus an open possibility that one of the selective pressures that shaped the function of the Toll pathway would be the need to cope with pathogens secreting virulence factors in the extracellular compartment.

Taken together with a concurrent study (30), our work underscores that the Toll pathway mediates resilience against the action of multiple toxin types such as pore-forming toxins, ribotoxins, or tremorgenic toxins, which are mediated by specific Bomanins or BaraA-derived proteins. It is likely that other uncharacterized effectors are able to counteract other toxins to which *Drosophila* flies are exposed to in the wild. In contrast to the current paradigm

according to which secreted peptides act as AMPs, our discoveries illustrate an original concept in insect innate immunity, the ability of the host to counteract secreted microbial virulence factors by dedicated effectors of the immune response.

Materials and Methods

Fly Strains. Fly lines were raised on media at 25° with 65% humidity. For 25 L fly food medium, 1.2 kg cornmeal (Priméal), 1.2 kg glucose (Tereos Syral), 1.5 kg yeast (Bio Springer), and 90 g nipagin (VWR Chemicals) were diluted into 350 mL ethanol (Sigma-Aldrich), 120 g agar-agar (Sobigel), and water qsp.

w^{A5001}(48) and occasionally *yw* flies were used as wild-type controls as needed. The positive controls for infection assays for Gram-positive/fungal infections and Gram-negative infections were, respectively, *MyD88* and *key* in the w^{A5001} background. Where stated, mutant flies were isogenized in the w^{A5001} background. For RNAi experiments, virgin females carrying the *Ubi-Gal4, ptub1-Gal80^{ts}* (*Ubi-Gal4, Gal80^{ts}*), *repo*-Gal4, and *elav*-Gal4 transposon were crossed to Trip lines males carrying an UAS-RNAi (Upstream Activation Sequence-RNA interference) transgene (TRiP) from the Tsinghua RNAi Center: THU0393 (*BaraA* KD), THU02336.N (*CG18278* KD). The control flies were the offspring of the cross of the driver to UAS-mCherry RNAi VALIUM20 (Bloomington Stock Center #BL35785). Crosses with the *Ubi-Gal4, Gal80^{ts}* driver were performed at 25 °C for 3 d, then the progeny was left to develop at the nonpermissive 18 °C temperature. The hatched flies were kept at 29 °C for 5 d prior to the experiment to allow Gal4-mediated transcription. All crosses involving flies without RNAi expression were performed at 25 °C. Unless stated otherwise, female flies were 5 to 7 d old at the beginning of each experiment.

To generate axenic flies, standard fly media was autoclaved. Antibiotics were added (ampicillin 50 µg/mL, kanamycin 50 µg/mL, tetracyclin 50 µg/mL, erythromycin 15 µg/mL) when it cooled down to 50 to 60°. The embryos were bleached and then cultured on the sterilized media. The sterility of axenic flies (20 d old) was checked on Lysogeny Broth (LB), Bushnell-Haas medium (BHB), Yeast Extract–Peptone–Dextrose (YPD), and deMan, Rogosa, Sharpe (MRS) plates.

Pathogen Infections. The bacterial strains used in this study include the Gram-negative bacterium *Pectobacterium carotovorum carotovorum* 15 (strain *Ecc15,* Optical Density $(OD)_{600} = 50$) and the Gram-positive strains *E. faecalis* National Collection of Type Cultures (NCTC) 775 (American Type Tissue Culture Collection (ATCC) 19433) or OG1RF (ATCC 47077) (OD = 0.1), *Micrococcus luteus* (OD = 200), and *Staphylococcus albus* (OD = 10), as well as *gelE*, *entV*$^{-}$, and complemented *entV*$^{+}$/*entV*$^{-}$ and, which are derivatives of the wild-type *E. faecalis* OG1RF strain (OD = 0.5) (kind gifts of Profs. Garsin and Lorenz, Houston, USA) (34). The fungal strains we used include filamentous fungi, *A. fumigatus* (5×10^7 spores/mL, 250 spores in 4.6 nL), *M. robertsii* (ARSEF2575, 1×10^7 spore/mL, natural infection 5×10^4/mL), and *DestruxinS1* mutant strain (1×10^7 spore/mL), a kind gift from Prof. Wang, Shanghai, China (32). Besides, we used yeast as well, *C. albicans* (pricked) and *C. glabrata* (1×10^9 yeasts/mL). The following media were used to grow the strains: Yeast extract–Peptone–Glucose Broth Agar (YPDA, *C. albicans* and *C. glabrata*) or LB–all others at 29 °C (*Ecc15*, *M. luteus*, *C. albicans*, *C. glabrata*) or 37 °C, *entV*$^{-}$, *entV*$^{+}$/*entV*$^{-}$ *E. faecalis*, Brain Heart Infusion (BHI) medium, 37 °C overnight, rifampicin 100 µg/mL. Spores of *M. robertsii* and *A. fumigatus* were grown on Potato Dextrose Agar (PDA) plates at 25 °C or 29 °C (*A. fumigatus*) for approximately 1 wk or 3 wk (*A. fumigatus*) until sporulation. We injected 4.6 nL of the suspension into each fly thorax using a Nanoject III (Drummond). Natural infections were initiated by shaking anesthetized flies in 5 mL 0.01% tween-20 solution containing *M. robertsii* conidia at a concentration of 5×10^4/mL. Infected flies were subsequently maintained at 29 °C (*C. albicans*, *C. glabrata*, *A. fumigatus, M. robertsii*) or at 25 °C (for all other pathogens, except for experiments with RNAi KD flies performed at 29 °C). The flies were anesthetized with light CO2 for about 3 min during the injection procedure and were observed 3 h after injection to confirm recovery from manipulations. Survival experiments were usually performed on three batches of 20 flies tested in parallel and independent experiments pooled for statistical analysis using the log-rank test.

Quantification of the pathogen burden in infected flies. To characterize the dynamics of within-host microbial loads or BLUDs or FLUDs, live flies were taken at each time point postinjection for pathogen load or flies were infected with *E. faecalis* or *M. robertsii* and vials were monitored every 30 min for newly dead flies (PLUD). These flies were then individually homogenized with a bead in 100 µL Phosphate Buffered Saline (PBS) with 0.01% tween-20 (PBST: Phosphate Buffered Saline tween) or PBS. Homogenates were diluted serially (10-fold dilutions, checked by fivefold dilutions for some BLUD experiments) in PBST (or PBS) and spread on LB (*E. faecalis*) or PDA (*M. robertsii*) plates for incubation at 37 °C (*E. faecalis*) or 25 °C (*M. robertsii*) overnight. Colonies were counted manually. Data were obtained from at least three independent experiments and pooled.

Western Blots. For western blots, hemolymph samples were collected from 50 flies in a protease inhibitor solution (PBS+:phenylmethylsulfonyl fluoride (PMSF)). Protein concentration of the samples was determined by Bradford assay. 30 µg protein was separated on an 8% gel by SDS-PAGE and transferred to a PVDF membrane. After blocking in 5% bovine serum albumin in PBST for 1 h at room temperature, samples were incubated at 4 °C overnight with rabbit antibodies against *Drosophila* PPO1 at a 1:10,000 dilution (a kind gift from Prof. Erjun Ling). After washes, a goat anti-rabbit-horseradish peroxidase (HRP) secondary antibody at a 1:20,000 dilution was incubated for 1 h at room temperature. Enhanced chemiluminescence substrate (ECL, General Electric Healthcare) was used according to the manufacturer's instructions to reveal the blot.

Gene Expression Quantitation. We followed the protocol as described (49) using primer pairs displayed in *SI Appendix*, Table S4.

Survival Tests. Survival tests were performed using 20 to 25 flies per vial in biological triplicates. Female adult flies used for survival tests were 5 to 7-d old. For survival tests using RNAi-silencing genes, flies were crossed at 25 °C for 3 d for laying eggs and then transferred to 18 °C; after hatching, flies were kept for at least 5 d at 29 °C prior to infections. Flies were counted every day. Each experiment shown is representative of at least two independent experiments.

Toxin Injection. Destruxin A (MedChemExpress) was resuspended in high-quality-grade dimethyl sulfoxide (DMSO) and was diluted in PBS to a 8-mM concentration. 4.6 nL of the solution or of control DMSO diluted in PBS at the same concentration was injected into flies using the Nanoject III microinjector (Drummond). Restrictocin (Sigma) was resuspended in PBS to the concentration of 1 mg/mL, 4.6 nL was injected. Beauvericin (Sigma) was resuspended in high-quality-grade DMSO. 20 mM, 9.2 nL was injected.

Collection and Preparation of *E. faecalis* Supernatants. Filter-sterilized supernatant phases were obtained from 10 mL overnight cultures grown in LB medium that were collected by centrifugation at 4,000 rpm for 10 min. The supernatants were sterilized by passing through a 0.2-µm-pore-size sterile syringe filter. The sterilized supernatants were centrifuged through a 15-mL Amicon Centricon filter (Millipore) to separately collect the molecules larger or lower than 10 kDa. 1.5 mL Eppendorf tubes were used to collect the supernatant lower than 10 kDa, which were vacuum freeze-dried for 24 h. The powder was resuspended with H_2O and thus concentrated 10 to 20-fold. The solution was filtered on 3 kDa Amicon Centricon filter columns (Millipore) by centrifugation at 10,000 rpm for 30 min. The nonfiltered fraction was then injected into flies with a volume optimized according to the batch (16 to 69 nL) and the same volume of buffer was used for the controls. All experiments were performed at least three times.

Statistics. Statistical analyses were performed using GraphPad software Prism 6 and R. Data were expressed as means ± SEM. RT-qPCR data were analyzed by ANOVA (one-way) with Dunnett's multiple comparisons test, with a significance threshold of $P = 0.05$. Log-rank tests were used to determine whether survival curves of female flies were significantly different from each other. Experiments measuring microbial loads (log2 values) were analyzed using linear models (lm) or linear mixed-effect models (lmer, package lme4) (50) in order to include the different factors of the experiment, such as the fly line or the treatment, and to include random factors, such as the experimental replicates. Significance of interactions between factors was tested by comparing models fitting the data with and without the interactions, using ANOVA. Models were simplified when interactions were not significant. Pairwise comparisons of the estimates from fitted models were analyzed using lmerTest, lsmean, and multcomp packages. Details are included in the legend of each figure. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

Data, Materials, and Software Availability. Raw data have been deposited in Figshare (51).

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