Prevalence of Local Immune Response against Oral Infection in a Drosophila/Pseudomonas Infection Model

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Pathogens have developed multiple strategies that allow them to exploit host resources and resist the immune response. To study how Drosophila flies deal with infectious diseases in a natural context, we investigated the interactions between Drosophila and a newly identified entomopathogen, Pseudomonas entomophila. Flies orally infected with P. entomophila rapidly succumb despite the induction of both local and systemic immune responses, indicating that this bacterium has developed specific strategies to escape the fly immune response. Using a combined genetic approach on both host and pathogen, we showed that P. entomophila virulence is multi-factorial with a clear differentiation between factors that trigger the immune response and those that promote pathogenicity. We demonstrate that AprA, an abundant secreted metalloprotease produced by P. entomophila, is an important virulence factor. Inactivation of aprA attenuated both the capacity to persist in the host and pathogenicity. Interestingly, aprA mutants were able to survive to wild-type levels in immune-deficient Relish flies, indicating that the protease plays an important role in protection against the Drosophila immune response. Our study also reveals that the major contribution to the fly defense against P. entomophila is provided by the local, rather than the systemic immune response. More precisely, our data points to an important role for the antimicrobial peptide Diptericin against orally infectious Gram-negative bacteria, emphasizing the critical role of local antimicrobial peptide expression against foodborne pathogens.

Citation: Liehl P, Blight M, Vodovar N, Boccard F, Lemaitre B (2006) Prevalence of local immune response against oral infection in a Drosophila/Pseudomonas infection model. PLoS Pathog 2(6): e56. DOI: 10.1371/journal.ppat.0020056

Introduction

Host-pathogen interactions are complex relationships in which the success of each organism depends on its ability to overcome the other. Consequently, hosts have evolved surveillance and defense mechanisms to detect and eliminate invading microorganisms, whereas pathogens use sophisticated strategies to counteract such responses. In recent years, Drosophila has emerged as a powerful model for the study of host-pathogen interactions [1,2]. An attractive feature of the Drosophila system is the existence of multiple defense reactions that are shared with higher organisms [3–5]. These strategies include physical barriers, together with the local and systemic immune responses. Several tissues participate in a coordinated defense against microbial infection. Firstly, epithelia, such as alimentary tract and tracheae, are the first line of defense against pathogens and produce both antimicrobial peptides (AMPs) and reactive oxygen species. Secondly, specialized hemocytes participate in phagocytosis and encapsulation of foreign intruders. Finally, the fat body, a functional equivalent of the mammalian liver, is the site of the humoral response. Several genetic studies have demonstrated the regulation of AMP synthesis by the Toll and immune-deficient (Imd) pathways. The Toll pathway is mainly activated by Gram-positive bacteria and fungi, and controls, to a large extent, the expression of AMPs (e.g., Drosomycin) through the nuclear factor-kB transactors Dif and Dorsal. In contrast, the Imd pathway mainly responds to Gram-negative bacterial infections and controls different AMP genes (e.g., Diptericin) via the activation of the nuclear factor-kB transactivator Relish (Rel) [3,4,6]. In addition, the Imd pathway plays a predominant role in the regulation of AMPs in the alimentary tract and tracheal epithelia [7].

Our knowledge of the Drosophila immune response is mainly based on the analysis of host reactions following direct injection of non-pathogenic bacteria into the insect hemocoel. One limitation associated with this approach is that it bypasses the initial steps of naturally occurring infections, including bacterial colonization and persistence and host local immune responses. Septic injury is probably of little consequence in nature, unlike oral infection upon ingestion. In addition, the response to non-pathogenic microorganisms does not necessarily reflect a natural host reaction to a real pathogen. Recently we have developed the use of natural oral infection to dissect host responses of Drosophila after challenge with bacteria demonstrated to be infectious for this insect. We have isolated several Erwinia carotovora strains, such as Ecc13, for their capacity to persist in...
**Synopsis**

Normal feeding and digestion involves the ingestion of many microorganisms. Many are innocuous, some are commensal, and others may be pathogenic. Eukaryotes have thus evolved complex mechanisms to detect, control, and if necessary, eliminate intestinal microbes. Insects are no exception, and the fruit fly, *Drosophila*, employs a physical barrier within the intestinal lumen and the peritrophic matrix, and an innate immune response which exhibits similarities to the mammalian counterpart. *Pseudomonas entomophila* was identified as a novel entomopathogenic bacterium that can infect and colonize the gut of *Drosophila*. In this paper, Liehl et al. describe one specific secreted virulence factor of *P. entomophila*, the zinc metalloprotease, AprA, which they demonstrate to be required for defense against the host gut epithelial immune response. AprA defends *P. entomophila* against the *Drosophila* antimicrobial peptides, produced by the gut innate immune response. *P. entomophila* aprA mutants are attenuated for virulence in wild-type *Drosophila* but are equally infective as wild-type bacteria in immune-deficient mutant flies that do not express these antimicrobial peptides. Although secreted proteases have previously been described as a potentially important defense against host immune proteins, this is one of the rare examples of an in vivo demonstration of such a specific role against insect antimicrobial peptides.

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**Results**

*P. entomophila* Secretes an Abundant Protease

A common strategy used by bacterial pathogens is to secrete toxins and other virulence factors that damage host tissues. To test whether *P. entomophila* could secrete such toxic factors, a supernatant filtrate from a bacterial culture was tested for its ability to kill *Drosophila*. Figure 1A shows that a concentrated *P. entomophila* culture filtrate had a moderate but significant effect on *Drosophila* larval survival following ingestion. Although the filtrate did not kill adult flies after feeding (unpublished data), it was highly toxic by direct injection into the hemocoel (Figure 1B). No killing of either larvae or adults was observed with a culture filtrate derived from the avirulent *P. entomophila* strain carrying a Tn5 transposon in the gacA gene [9]. These data suggested that *P. entomophila* secreted one or more factors with toxic activity under the control of the GacS/GacA two-component system.

In an attempt to identify the factor(s) responsible for toxicity, we analyzed the proteins present in the supernatant. The protein profile for wild-type *P. entomophila* supernatant (Figure 1C, lane 2) shows a major protein band at 51 kDa and several minor bands. MALDI-TOF analysis of tryptic fragments of the 51-kDa band identified this protein as a homolog of the previously characterized Apr proteases from *Pseudomonas* spp., Prt proteases from *Erwinia* spp. and *Photorhabdus* spp., and serralysins from *Serratia* spp. [12–14]. All of these proteases are members of the zinc metzincin family of Type I-secreted RTX proteins [15]. We subsequently purified this protease, termed AprA, to homogeneity from the *P. entomophila* supernatant by anion exchange chromatography followed by size exclusion chromatography (Figure 1D). Injection of pure AprA into the hemocoel rapidly killed adult flies, identifying this protein as a bacterial toxin (Figure 1E). Feeding of larvae with high concentrations (1.5 mg/ml) of AprA led to modest lethality (unpublished data), recapitulating the properties of the *P. entomophila* supernatant.

Interestingly, in a gacA mutant the number of proteins in the supernatant was greatly reduced, including the complete absence of the 51-kDa band corresponding to AprA (Figure 1C, lane 3). In *P. fluorescens*, membrane-localized anti-sigma factor PrtR cleaves the extracytoplasmic sigma factor PrtI, resulting in increased expression of multiple genes, including one encoding for a metalloprotease, aprX [16]. Two independent Tn5 insertions in the prtR gene affecting virulence have recently been identified in a random insertion mutagenesis screen of *P. entomophila* [9]. To test a possible role of prtR in the regulation of aprX expression, we analyzed the proteins present in the supernatant of prtR mutants. Interestingly, both prtR mutants (CL25 and CU1) displayed a secreted protein profile identical to the wild-type strain except for a marked decrease of AprA (Figure 1C, lanes 4 and 5). Measurement of in vitro protease activity with azocasein revealed that the gacA mutant secretes no detectable protease activity and that prtR mutants retain only 30% of wild-type supernatant activity, thus indicating a role for PrtR in the...
regulation of *P. entomophila* protease secretion (Figure 1F). Furthermore, the *prtR* supernatant showed no toxicity toward flies after injection, suggesting a correlation between AprA levels and virulence (unpublished data). Altogether, this analysis indicates that *P. entomophila* *gacA* and *prtR* genes regulate the secretion of a protease with toxic activity when injected into flies or fed to larvae.

The *P. entomophila* *aprA* Mutant Displays Attenuated Pathogenicity

The experiments described above suggested that AprA plays an important in vivo role in *P. entomophila* virulence. To further test this hypothesis, we used a genetic approach by inactivating the *aprA* gene. Sequence analysis of the locus corresponding to *aprA* revealed a genetic organization characteristic of this class of Type I-secreted proteases [14]. The structural gene for the metalloprotease, *aprA*, is followed by a gene encoding its periplasmic inhibitor, *aprI*, and three genes encoding the Type I transporter, *aprD, E*, and *F* (Figure 2A). Studies in other bacteria have shown that AprD, E, and F participate in the elaboration of a Type I transporter required for AprA secretion to the external medium [17]. We inactivated the *aprA* gene by inserting a tetracycline resistance cassette by a double homologous recombination event. SDS-PAGE protein profiles of culture supernatants clearly demonstrated the absence of the 51-kDa band, and in vitro protease activity of the *aprA* mutant confirmed the concomitant absence of secreted protease activity (Figure 1C, lane 6, and 1F).

To analyze the in vivo contribution of AprA to *P. entomophila* virulence, we performed a survival analysis of *Drosophila* larvae and adults after oral infection with various *P. entomophila* derivatives. Only 40% of the *aprA*-infected larvae succumbed within 3 days, while 70% of the larvae died after infection with wild-type *P. entomophila*, demonstrating that the *aprA* mutant was attenuated for virulence (Figure 2B). Similarly, the *aprA* mutant showed reduced pathogenicity after oral infection of adult flies (Figure S1). Using the same assay, both *prtR* and *gacA* mutants were almost avirulent toward *Drosophila* indicating that both genes regulate other virulence factors in addition to AprA (Figure 2B). We next compared persistence of wild-type *P. entomophila* and the *aprA* mutant by quantifying the number of bacteria in larvae and adults at different time points after infection. Whereas the wild-type *P. entomophila* titer remained high, *aprA* bacterial levels decreased significantly with time (Figure 3A, not shown for larvae). However, *aprA* gut persistence remained higher than that of *gacA* mutants.

We could not exclude that the *aprA* mutant phenotype was due to polar effects of the insertion in the operon upon expression of *apr I, D, E, and F*. In support of this hypothesis, previous studies have demonstrated for both *Serratia marcescens* and *P. fluorescens* that Type I transporters can be polyvalent and secrete different types of proteins such as proteases and lipases [18,19]. Complementation of the *aprA* mutant with the wild-type *apr* operon restored protease secretion and activity (Figure 1C, lane 7, and 1F) and resulted in a complete regain of function with respect to all virulence phenotypes (Figures 2B and S1). In contrast, no rescue was observed with a plasmid containing the full *apr* operon but carrying a non-polar mutation in *aprA* (Figures 2B and S1).
and homogenized intestines was confirmed by measuring the absorbance of blue dye with larval extracts in a HEPES Buffer at [pH 8] (unpublished data).

DOI: 10.1371/journal.ppat.0020056.g002

These data confirmed that the protease AprA itself contributes to P. entomophila virulence.

The aprA Mutant Retains the Capacity to Trigger an Immune Response and to Induce Food-Uptake Cessation

In addition to its pathogenicity, P. entomophila infection provokes a food-uptake cessation in larvae and triggers an immune response in both larvae and adults [9]. Feeding of larvae with medium containing bromophenol blue, leads to a clearly visible food uptake, discernable by blue coloration throughout the gut. In contrast, P. entomophila-infected larvae show only a pale blue coloration indicating that infected larvae cease to ingest food. Whereas this food-uptake cessation was still observed in aprA and prtR mutants, it was not evident in gacA mutant-infected larvae (Figure 2D and data not shown for prtR).

The kinetics of the expression of the AMP, Dipterican, in larvae and adults after natural infection by P. entomophila and its derivative mutants, was analyzed by real time quantitative PCR (RT-qPCR). Dipterican was expressed at a wild-type level following oral infection with the aprA and prtR mutants, but no expression was detected in the case of the gacA mutant (Figures 2C and 5A). Observation of green fluorescent protein (GFP) fluorescence in infected larvae carrying a Dipterican-GFP reporter gene confirmed that prtR and aprA mutants elicited an immune response, whereas gacA mutants failed to do so (unpublished data).

The data presented above clearly indicate that AprA is a major virulence factor of P. entomophila that promotes bacterial persistence and killing of the host, but that other virulence factors controlled by gacA and prtR exist. It also reveals that P. entomophila virulence is multi-factorial with a clear distinction between factors that promote pathogenicity and those that trigger the immune response. The observation

Figure 2. The aprA Mutant Exhibits Attenuated Virulence

(A) Genetic organization of the P. entomophila apr locus and its associated Type I transporter. The locus contains the structural gene for the protease (aprA), followed by the genes encoding its putative inhibitor (aprI) and those coding for the associated Type I transporter (ABC Transporter, aprD; Membrane Fusion Protein, aprE; Outer Membrane Protein, aprF). The apr operon organization was deduced from [10].

(B) Survival analysis of Drosophila larvae (n = 60) after feeding with wild-type P. entomophila, aprA, aprI, aprD, aprE, aprF and apr operon carrying a non-polar mutation in the aprA gene (pUCP20-aprI). This experiment was repeated twice and yielded similar results. Log-rank analysis demonstrated a statistically significant difference in survival of flies fed with wild-type P. entomophila and flies fed with the aprA mutant (p < 0.0001).

(C) Dipterican expression measured by RT-qPCR in Drosophila larvae following natural infection with wild-type P. entomophila, aprA, and aprI mutants. Infection with wild-type P. entomophila and the aprA mutant induced sustained Dipterican expression unlike the gacA mutant. Larvae were collected at different time intervals after oral infection. Dipterican expression was normalized to rp49 mRNA. For each time point the values represented are the mean of three independent experiments (± standard deviation). 100% value corresponds to the level of Dpt mRNA obtained 24 h after infection with wild-type P. entomophila. rp49: ribosomal protein 49.

(D) Ingestion of aprI or prrR mutant bacteria induces a food-uptake cessation in larvae in contrast to animals fed with the gacA mutant. Larvae fed with medium containing 0.5% (w/v) bromophenol blue displayed a clearly discernable blue coloration throughout the gut whereas larvae fed with both P. entomophila wild-type, aprA, or prrR mutants and bromophenol blue showed only a pale blue coloration. Images were taken 6 h after infection. This visual effect was not due to a change of gut pH (acidification) that would result in yellow rather than blue coloration since the overall level of bromophenol blue in dissected larvae with medium containing bromophenol blue, leads to a clearly visible food uptake, discernable by blue coloration throughout the gut. In contrast, P. entomophila-infected larvae show only a pale blue coloration indicating that infected larvae cease to ingest food. Whereas this food-uptake cessation was still observed in aprA and prtR mutants, it was not evident in gacA mutant-infected larvae (Figure 2D and data not shown for prtR).

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Figure 3. Bacterial Persistence in Wild-Type and Rel Flies

(A) Bacterial persistence was measured in live wild-type flies by plating appropriate dilutions of homogenates of five surface-sterilized adults on LB medium containing rifampicin. The flies had been previously orally infected with rif’ strains of wild-type P. entomophila and its aprA and gacA derivatives. AprA mutants persisted less than wild-type P. entomophila but better than the gacA mutant.

(B) Persistence of wild-type P. entomophila aprA and gacA mutants in live Rel flies. AprA mutant bacteria persisted at a level similar to wild-type bacteria in Rel mutant flies whereas gacA bacterial levels decreased with time.

The number of cfus per fly represented in each histogram corresponds to the average of six independent experiments (± standard deviation). cfu, colony-forming unit.

DOI: 10.1371/journal.ppat.0020056.g003
that both factors are under the control of the GacS/GacA virulence. Our previous study had already revealed that AprA was required to promote persistence in wild-type flies and larvae, but was dispensable in a Rel background, and indicates that AprA plays an important role in the protection against the Imd-dependent immune response.

To investigate the connection between activation of the Imd pathway and the outcome of P. entomophila infection in more detail, we focused our analysis on the adult stage when it is easier to monitor survival. P. entomophila infection triggers Imd pathway activation within hours. To determine at which time Imd was required to impede P. entomophila infection, we monitored survival to P. entomophila in flies in which the Imd pathway was artificially activated in a time-dependent manner. We utilized an engineered fly line (UAS-imd, hs-Gal4) over-expressing imd under the control of a heat-shock promoter via the UAS-Gal4 system, which consequently leads to strong expression of Imd-dependent AMP genes. Figure 4A shows that over-expressing imd 12 h prior to infection protected flies from a P. entomophila infection, whereas over-expressing imd 6 h before contributed only modestly to survival of the flies. This experiment indicates that P. entomophila can be eliminated when the Imd pathway is activated at a high level prior to infection. It further suggests that P. entomophila is sensitive to immune defenses during a short time frame and subsequently becomes insensitive. Since P. entomophila is pathogenic under normal wild-type conditions, it may overcome the immune response by establishing a niche in the gut before the Imd pathway is activated. It is interesting to note that in agreement with the results described above, persistence of both the aprA mutant and wild-type bacteria was significantly altered in fly lines over-expressing the Imd pathway, and that aprA mutants survived less than wild-type bacteria (Figure 4B). Altogether, these data reveal that the Imd pathway is crucial for host survival after oral infection with P. entomophila and that AprA plays a key role in counteracting this effect.

Local, but Not Systemic Immunity, Contributes to Resistance against Oral Infection with P. entomophila

P. entomophila, but not the gacA derivative, rapidly activates both the local and systemic Imd-dependent immune responses in Drosophila adults, which can be demonstrated using RT-qPCR with fat body and gut extracts (Figure 5A). Use of Dipterica-GFP or lacZ reporter genes reveals strong Dipterica expression in the proventriculus, an organ that acts as a valve between the oesophagus and the anterior midgut (Figure 5B). This suggests a critical role of this organ in the elimination of bacteria. We next examined the respective contribution of the local gut and the systemic immune responses to controlling P. entomophila infection. We thus compared resistance to P. entomophila infection of flies that were previously either orally infected with Ecc15 (to activate a local immune response) or pricked with Ecc15 (to activate a systemic immune response). The rationale behind this experiment is based on previous observations that an oral infection with Ecc15 triggers a local, but not a systemic immune response at the adult stage (our unpublished data) [8]. Figure 5C shows that a prior infection with Ecc15 protected flies against a subsequent P. entomophila infection only when Ecc15 was administrated orally, indicating that the local, but not the systemic immune response, plays an important role in P. entomophila clearance. To ascertain that
Figures 5A-E. The Local Immune Response Plays a Critical Role against *P. entomophila*.

(A) Time course analysis of *Diptericin* expression measured by RT-qPCR in *Drosophila* fat body and gut extracted from females following natural infection with wild-type *P. entomophila*, *aprA*, and *gacA* mutants. Infection with wild-type *P. entomophila* and the *aprA* mutant induced a rapid and sustained *Diptericin* expression unlike the *gacA* mutant. Fat bodies (carcasses) and digestive tracts were dissected from adults collected at different time intervals after oral infection. *Diptericin* expression was normalized to *rp49* mRNA. 100% value corresponds to the level of *Dpt* mRNA obtained 4 h after infection with wild-type *P. entomophila*. *rp49*: ribosomal protein 49; *Dpt*: *Diptericin*.

(B) *P. entomophila* induces *Diptericin* expression in the cardia of adult *Drosophila*. Histochemical staining of β-galactosidase activity shows that *Dpt-lacZ* is expressed in the anterior midgut at the level of the proventriculus of infected flies carrying the *Dpt-lacZ* reporter gene (left panel). Similar results were obtained with a *Dpt-GFP* transgene (right panel). Adults were collected 24 h after infection. The control pictures display the cardia of uninfected flies. A constitutive expression was observed in the anterior part of the cardia, *Dpt*, *Diptericin*.

(C) Survival of wild-type flies (*n* = 60) to *P. entomophila* after previous infection with *Ecc15*. Flies were first infected with *Ecc15* either orally (OD$_{600}$ = 100) or by septic injury (OD$_{600}$ = 200). 20 h after this *Ecc15* infection, flies were fed with *P. entomophila*. Survival curves demonstrate that *Drosophila* flies primed with *Ecc15* were protected from a subsequent *P. entomophila* infection only when *Ecc15* was orally administrated. Log-rank analysis demonstrated a statistically significant difference in survival to *P. entomophila* infection of flies primed with *Ecc15* by septic injury (SI) or natural infection (NI) (*p* < 0.0001).

(D) Survival of wild-type and *Rel* flies (*n* = 60) to *P. entomophila* after previous infection with *Ecc15* evf- *Rel* flies were first infected with *Ecc15* evf- orally (OD$_{600}$ = 100). 20 h after this *Ecc15* infection, flies were fed with *P. entomophila*. Survival curves demonstrate that wild-type, but not *Rel* *Drosophila* flies primed with *Ecc15* evf-, were protected from a subsequent *P. entomophila* infection. This experiment was repeated three times and gave similar results. Log-rank analysis demonstrated a statistically significant difference in survival to *P. entomophila* infection of flies primed with *Ecc15* evf- by natural infection (NI) and flies without previous priming (*p* < 0.0001).

(E) *Rel* flies expressing a UAS-Rel transgene in the midgut under the control of the gut-specific *cad-Gal4* driver survive better to *P. entomophila* infection than *Rel* mutant flies. Survival experiments were performed on 30 flies orally infected with *P. entomophila* (OD$_{600}$ = 50). This experiment was repeated three times and yielded similar results. Log-rank analysis demonstrated a statistically significant difference in survival of UAS-Rel/*cad-Gal4*; *Rel* flies, and UAS-Rel/Rel; *Rel* flies to *P. entomophila* infection (*p* < 0.01).

DOI: 10.1371/journal.ppat.0020056.g005

The protection was due to *Imd* pathway activation and not to a competition between *P. entomophila* and *Ecc15*, we next monitored resistance to *P. entomophila* of wild-type and *Rel* flies orally infected by *Ecc15* evf-. We used the evf-deficient derivative because this bacterium triggers a local immune response without persisting in the gut. This experiment shows that wild-type, but not *Rel* flies previously infected with *Ecc15* evf-, resisted a second challenge by *P. entomophila* (Figure 5D). This demonstrates that the protection was indeed due to *Imd* pathway activation in the gut.

The results described above were corroborated by the over-expression of *Imd* in the gut or in the fat body using specific *Gal4* drivers. Over-expression of *Imd* in the gut protected against *P. entomophila*, whereas its activation in the fat body did not (Figure S2). However, the previous results did not address whether the *Imd* pathway induced during the course of a natural *P. entomophila* infection contributes to fly resistance to *P. entomophila*. Thus, we next compared survival of *Rel* flies specifically expressing a wild-type copy of *Rel* in the intestine using *caudal (cad)-Gal4*. These flies lack a functional *Imd* pathway except in the gut where *cad-Gal4* is expressed. In these flies, the *Imd* pathway was not constitutively active in the gut but could be induced upon oral bacterial infection similarly to the wild-type situation (Figure S3A). Figure 5E shows that *Rel* flies expressing *Rel* in the gut survive better than *Rel* mutant flies. These data demonstrate that the *P. entomophila*-induced local immune response in the intestine plays an important role in the defense against this Gram-negative bacterium.

**AprA Confers Protection against Diptericin**

We have shown that AprA protects *P. entomophila* against the *Imd*-regulated immune response. This immune pathway regulates the expression of several AMP genes, as well as many other immune genes. Previous studies indicated that *Attacin A* and *Diptericin* are the AMP genes most strongly induced in the gut following ingestion of infectious bacteria [7]. It has also been suggested that proteases homologous to AprA in other bacterial species degrade AMPs in vitro, thereby enabling...
pathogens to withstand the attack of the host immune system [20,21]. We confirmed that *P. entomophila* AprA rapidly degrades Cecropin A in vitro (unpublished data). To investigate a possible in vivo role of AprA in the protection against AMPs synthesized in the gut, we compared survival to *P. entomophila* in *imd*-deficient flies over-expressing Attacin A or Diptericin under the control of the da-Gal4 driver. aprA mutants persist less than wild-type *P. entomophila* in lines over-expressing Diptericin in contrast to flies mutated in the *imd* locus. The number of cfu per fly represented in each histogram corresponds to the average of six independent experiments (± standard deviation). cfu, colony-forming unit.

**Discussion**

Pathogens have developed a plethora of strategies that allow them to exploit host resources and resist the attacks of the host immune response. To study how flies deal with infections in a natural context, we investigated the interactions between *Drosophila* and a newly identified entomopathogen, *P. entomophila*. Using a combined genetic approach for both the host and pathogen we reveal the importance of the local immune response in host defense against gastrointestinal infections and provide an in vivo demonstration for the role of a bacterial metalloprotease in protection against AMPs. Our analysis reveals that *P. entomophila* virulence is multi-factorial, as one might expect, but can be decrypted through genetic analysis of the two interacting partners.

**Local Expression of AMP Genes Plays a Major Role against Food-Borne Infections**

The use of GFP reporter transgenes in *Drosophila* has revealed that, in addition to the fat body, AMP genes can be expressed in several barrier epithelia that are in direct contact with microorganisms from the environment [7,23]. The precise relevance of this local immune response in *Drosophila* has not been established to date. The present study demonstrates a key role for the local Imd immune response in the gut against oral infection by bacterial pathogens. The Imd pathway regulates a large number of immune genes including those encoding AMPs; and two peptides, Diptericin and Attacin, are preferentially synthesized following bacterial infection in the digestive tract of *Drosophila* [7]. To explore the potential implication of AMPs in the Imd immune defense against oral infection by *P. entomophila*, we compared survival of flies expressing only a single AMP. Our study reveals that over-expression of either Diptericin or Attacin confers protection against *P. entomophila*. This observation, combined with our data demonstrating that *P. entomophila* expresses specific virulence factors in order to resist AMPs, underlines the critical role of local AMP expression against food-borne pathogens.

Previous studies revealed only a modest contribution of Diptericin to resistance against Gram-negative bacteria during systemic infection [22]. In contrast, our data reveal that Diptericin plays an essential role in the defense against Gram-negative bacteria when they are ingested. The antibacterial activity of Diptericin may be enhanced in the gut by pH and other factors such as lysozymes [24]. It is also possible that Diptericin could reach high concentration levels in restricted areas of the *Drosophila* gut such as the proventriculus and thus be more effective against pathogens than in other body compartments. Our study suggests that Diptericin expression in the anterior gut provides an efficient early barrier allowing *Drosophila* to rapidly eliminate most ingested bacteria.

Finally, it remains surprising that *P. entomophila* infection triggers a systemic immune response that has no overt function against the bacteria that remain in the gut lumen. This response could be interpreted as anticipation of possible breaching of the gut barrier. Alternatively, our results suggest the possibility that bacteria such as *P. entomophila* may subvert insect host defenses by triggering this systemic response. This can be compared with many human pathogens for which activation of an inflammatory response represents a part of their invasive strategy [25].
AprA Protects *P. entomophila* from Antibacterial Peptides

In bacteria, nitrogen and carbon sources are frequently provided by enzymatic degradation of extracellular biopolymers by proteases and glycosidases. Proteases, especially metalloproteases, are also known to contribute to virulence in some pathogenic bacteria including *Pseudomonas aeruginosa*, *S. marcescens*, and *Bacillus thuringiensis* [20,21,26]. Molecular mechanisms for pathogenesis attributed to these proteases include degradation of structural matrices and destruction of proteins involved in host protective functions such as AMPs or complement factors. However, in most cases, attempts to evaluate the role of metalloproteases in virulence have failed to obtain conclusive results with respect to a specific function [20,21].

Thus, our approach focusing on both host and pathogen is the first to clearly demonstrate a key role of the AprA metalloprotease in the protection against AMPs in vivo. This conclusion is based on our observations that (i) aprA mutants show attenuated virulence, (ii) aprA mutants survive less well than wild-type *P. entomophila* in *Drosophila* and are more sensitive to Imd-mediated defense, and (iii) AprA provides specific protection in vivo against Diptereticin. Altogether, our study reveals that local AMP expression plays an important role in defense against oral pathogens and that entomopathogens such as *P. entomophila* can counteract this effect by expressing aprA. It is interesting to note that *S. marcescens*, another potent oral pathogen of *Drosophila*, also expresses a protease that can degrade AMPs in vitro [12]. However, in the absence of a *Serratia* protease mutant, the in vivo relevance of the protease is not yet established. Taken together, this observation and our findings suggest that proteases may represent a common strategy used by *Drosophila* pathogens to circumvent the potent antimicrobial host defense.

We cannot exclude that AprA degrades other immune effectors or participates directly as a toxic factor by degrading the gut epithelium or the peritrophic matrix. In *B. thuringiensis*, it has been proposed that the protease InhA2 participates in the degradation of the gut, thus facilitating the action of the Cry toxins [27,28]. Alternatively, in *Photorhabdus luminescens*, an AprA homologue, PrtA, has been proposed not to be a virulence factor, but rather to be involved in degrading host tissues following host death in order to promote efficient nutrition and development of its symbiotic nematode host [14,29]. The observation that injection of AprA into the hemocoel is lethal to adult flies supports the hypothesis of additional roles for the protease in bacterial pathogenesis. However, oral ingestion of purified AprA induces only low levels of lethality, indicating that AprA alone is not sufficient to kill the host via this route of entry, and other factors participate in oral toxicity.

**P. entomophila** Virulence Is Multi-Factorial

In agreement with previous studies, we show that aprA expression in *P. entomophila* depends on both PrtR and the GacS/GacA system. The GacS/GacA two-component regulatory system is conserved in numerous Gram-negative bacteria and has been shown to regulate a wide variety of cellular functions and virulence factors [30,31]. Our study indicates that it is the master regulator of *P. entomophila* virulence. In a gacA mutant background, AprA synthesis is not restored when a plasmid with the aprA locus is expressed in trans (unpublished data), which is in agreement with post-transcriptional regulation of aprA by gacA via the two small non-coding RNAs RsmY and RsmZ [11]. As opposed to the pleiotropic effects of GacS/GacA, PrtR appears to be a more specific regulator of aprA expression in *P. entomophila*, reminiscent of aprX regulation in *P. fluorescens* [32].

Many pseudomonads and other bacteria express proteases similar to AprA but are not able to infect *Drosophila* by oral ingestion. This indicates that AprA is not the sole virulence factor required for persistence in the *Drosophila* gastrointestinal tract. The difference in pathogenicity exhibited by gacA, prtR, and aprA mutants underlines the complexity of *P. entomophila* virulence factors. The observation that both aprA and prtR, but not gacA, mutants retained the capacity to trigger a systemic immune response indicates a clear distinction between pathogenicity and immune activation. Our current hypothesis is that systemic immune activation is linked to bacterial persistence in the gut and release of peptidoglycan fragments small enough to cross the gut barrier [33]. Our results indicate that the GacS/GacA two-component system regulates one or several genes that promote bacterial survival in the gut. This hypothesized persistence-promoting factor may have a function similar to the *Erwinia virulence factor* (evf) gene of *E. carotovora* Ecc15 that promotes persistence of Gram-negative bacteria in the larval gut [34]. Since gacA mutants did not persist in imd-deficient mutant hosts, we speculate that this persistence-promoting factor provides general protection against gut intestinal conditions rather than a specific protection against the fly immune response.

Another interesting feature of *P. entomophila* virulence is the food-uptake cessation which is observed in *prtR* and aprA but not in gacA mutants. Food-uptake cessation or blockage in insects is induced by several other entomopathogenic bacteria including *S. entomophila* and *Yersinia pestis*, enabling persistence in the digestive tract of their insect hosts [35,36]. This observation suggests that peristaltic movements of the gut may also play an important role in the elimination of bacteria, and that entomopathogens have developed strategies to abrogate these movements. In *S. entomophila*, it has been shown that genes encoded by a prophage were responsible for the anti-feeding reaction in its natural insect host, the grass grub *Costelytra zealandica* larvae [35]. *Y. pestis* is able to multiply in the flea midgut and forms cohesive aggregates. The absence of homologous genes to these factors in *P. entomophila* indicates that other factors are probably implicated in this bacterium [10]. Determining the cause of this food-uptake cessation and its possible link to the persistence-promoting factor will be essential for the elucidation of the initial events involved in gut colonization. Finally, our study shows that aprA, but not gacA, mutants of *P. entomophila* retain a moderate capacity to kill both adult flies and larvae. This confirms the existence of other bacterial virulence factors. The genome of *P. entomophila* contains several genes encoding putative insecticidal toxins (e.g., Tc toxins, hemolysins, and lipopeptides). Therefore, it remains to be determined whether the strategies developed by *P. entomophila* to persist in the larval gut and to kill its host involves genes related to those identified in other entomopathogenic bacteria and how the different factors contribute to pathogenesis.
Materials and Methods

Insect stocks. OregonK (OrK) flies were used as a wild-type strain. The RelE [30] and the Dpteriens-lacZ (Dpt-lacZ), Dpteriens- GFP (Dpt-GFP), UAS-md heat shock (hsp)-Gal4, caudal (caud-Gal4), and daughters (daGal4) fly lines were previously described [30,9]. C. carotovora Ecc15 was expressed in the posterior region of the midgut and in the Malpighian tubules [38]. By standard genetic crosses, we generated smd flies carrying two copies of an UAS-AMP and one copy of da-Gal4. Additional information on these fly lines is provided in [22]. The fly lines UAS-Rel, Rel, and caudal (caud-Gal4); Rel (kindly provided by Won-Jae Lee) were used to produce Rel flies expressing Rel only in the gut (UAS-Rel-Gal4; RelE [30]). F1 progeny carrying the caud-Gal4 driver were transferred to 29ºC at late larval-early pupal stages for optimal GALA efficiency. Dro sophila stocks were maintained at 25ºC. Infections were performed by feeding as described before [20,].

Bacterial strains, plasmids, and culture media. P. entomophila and E. coli aprA and carotovora Ecc15 have been previously described [8,9]. P. entomophila was grown at 37ºC in LB medium with appropriate antibiotics when required (rifampicin 100 µg/ml and carbenicillin 600 µg/ml) at 30ºC in Luria-Bertani (LB) medium. Escherichia coli BW25142 was used for cloning experiments and was grown at 37ºC in LB medium. E.coli SM10;pir was utilized for in vivo allelic replacements as described before, and the pMTL22 vector was applied for general cloning manipulations [40].

Construction of the aprA knockout mutant and complementation study. An aprA knockout mutant was constructed by a double crossover approach using vector pKT1001 containing a 3.4 kb fragment inactivated with a tetracycline resistance cassette. A 2.085-bp aprA fragment was cloned with Xhol into the multi-cloning site of the ampr resistant pMTL22 vector. The aprA fragment was inactivated by inserting the 1.402-bp tetracycline resistance cassette in the XhoI site. The 3.4-kb Xhol fragment was then cloned into Sall digested pKN101 suicide vector. The construct was named pKN101-A, and transformed into E. coli SM10;pir. This E. coli strain was subsequently conjugated with P. entomophila. Detection of double recombination events was performed as previously described [49]. AprA knockout mutants were confirmed by PCR. To complement the aprA mutants, the entire aprA operon was subcloned from a pBeloBAC derivative containing the region surrounding the aprA operon with EcoRV/EcoRI into pUCP20 [41] cleaved with EcoRI. The plasmid with the incorporated apr operon was called pUCP20-apr. To specifically inactivate aprA gene, a 120-bp fragment containing a part of the promoter region and the initiation codon of the aprA gene was cloned in frame with aprr resistant pMTL22 vector. This plasmid construct, 730 bp of the apr operon were deleted. This strategy produces an in-frame deletion that should not have polar effects on downstream genes expression. The proteolytic activity was assessed on 5% skim milk agar plates.

Infection experiments. Natural bacterial infection of larvae: Approximately 200 third instar larvae were placed in a 2-ml tube containing 200 µl of a concentrated bacterial pellet (optical density at 600 nm [OD600] = 290) from an overnight culture and 400 µl of crushed banana. The larvae, bacteria, and banana were thoroughly mixed in the microfuge tube, which was closed with a foam plug and incubated at room temperature for 30 min. The mixture was then transferred to a standard corn meal fly medium and incubated at 29ºC. Larvae were collected at different time points for RT-QPCR analysis and bacterial counts. For bacterial counting experiments, larvae were first rinsed in water, dried in 70% ethanol (three times, 5 s) for external sterilization, and then homogenized and spread onto LB plates containing rifampicin (100 µg/ml). Larval feeding experiments with concentrated supernatant or pure protease: Approximately 200 third instar larvae were placed in a 2-ml tube containing 200 µl of 60-fold concentrated filtered bacterial culture supernatant and incubated at room temperature for 30 min. The mixture was then transferred to an apple juice medium plate which was spayed with paraformaldehyde and incubated at 29ºC. Dead larvae were counted at indicated time points after infection.

Natural bacterial infection of adults: For oral infection, flies were incubated 2 h at 25ºC in an empty vial in order to starve them and then placed in a fly vial with a filter soaked in a food solution. This food solution was obtained by mixing a pellet of an overnight culture of bacteria with a 5% sucrose solution in equal parts. The final bacterial concentration was OD600 = 100 except when otherwise mentioned.

Injection of flies: Solution (buffer, culture supernatant, or protease solution) was injected into the thorax of female adults (aged 3–4d) with a Nanoject apparatus (Drummond, Broomall, Pennsylvania, United States).

Protein analysis and AprA purification. A concentrated super- natant was prepared by the centrifugation of an overnight bacterial culture. The supernatant was filtered through a 0.45-µm filter. The clarified solution was concentrated 60-fold by using 5-kDa cutoff Centricron membranes (Amicon©, Millipore, Billerica, Massachusetts, United States). Proteins were analyzed by SDS-PAGE. Culture supernatant fractions were prepared by centrifuging bacterial cultures at 14,000 g and 4 ºC for 5 min and precipitating supernatant proteins with 10% trichloroacetic acid (TCA) for 30 min on ice. Precipitated proteins were pelleted at 14,000 g and 4 ºC for 30 min and washed once with 500 µl cold (−20 ºC) 80% acetone, followed by resuspension in a 15 µl buffer and analysis by SDS-PAGE. For AprA purification, P. entomophila was grown in 1 L of LB medium at 29 ºC to the late stationary phase of growth (24 h). The culture supernatant was retained following centrifugation of the culture at 8,000 g (4 ºC) for 30 min. Solid ammonium sulphate was added to the supernatant to a final saturation of 80%, and proteins were precipitated at 4 ºC with gentle stirring. Precipitated material was collected by centrifugation at 10,000 g (4 ºC) for 30 min, and the pellets were combined and solubilized in a minimum volume of 50 mM Tris-HCl [pH 8.5], 1 mM EDTA. The resuspended pellet was dialyzed against 1 L of buffer with two buffer changes at 4 ºC. Chromatography procedures were performed on an AKTA FPLC (Amersham Biosciences, Little Chalfont, United Kingdom) system. Following a final clarification step of the solubilized supernatant by centrifugation at 10,000 g (4 ºC) for 30 min and filtering through a 0.45-µm filter, the solubilized proteins were loaded onto a MonoQ HR 5/5 column (Amersham- Pharmacia Biotech) that had been equilibrated with 50 mM Tris-HCl [pH 8.5], 1 mM EDTA. The protease activity of the fractions was determined using azocasein (see protease assay). Fractions containing protease activity were pooled in the flow-through which was subsequently concentrated with an Amicon Ultra 5000 MW cut-off filter and loaded onto a size exclusion chromatography column (Superdex 200 10/300 GL Tricorn, Amersham-Pharmacia Biotech) equilibrated in running buffer (20 mM Tris-HCl, 5 mM CaCl2, 150 mM NaCl [pH 8.0]). Fractions were collected at a flow rate of 1 ml/min and assayed for protease activity as described below.

Protease assay. Supernatant samples were assayed for proteolytic activity using azocasein (Sigma, St. Louis, Missouri, United States) as a substrate. Aliquots (50 µl) of samples were added to 200-µl azocasein (5 mg/ml) 50 mM Tris-HCl [pH 8.5]. 1 mM EDTA. Fractions were then incubated at 37ºC for 30 min and filtered through a 0.45-µm filter. The absorbance values of the resulting supernatants were measured at 440 nm. Increased absorbance indicates the presence of proteolytic activity. The blank was obtained by precipitating the substrate plus the sample in TCA without incubation.

RT-QPCR. For Dpteriens mRNA quantification from whole animals, RNA was extracted using RNA TRizol™. cDNAs were synthesized using SuperScript II (Invitrogen, Carlsbad, California, United States) and RT-QPCR was performed using dsDNA dye SYBR Green I (Roche Diagnostics, Basel, Switzerland). Primer pairs for Dpteriens (sence, 5'-GCT GCC CAA TCG CAT CTA CT-3' and antisense 5'-TGG TGG AGT GGG CAT CAT G-3'), and control primers for aprA (sense 5'-GAC GCT TCA AGG AGC AGT ATC TG-3', and antisense 5'-AAA CGC GGT TCT GCA TGA G-3') were utilized. SYBR Green analysis was performed on a Lightcycler (Roche). The amount of mRNA detected was normalized to control rp49 mRNA values. We used normalized data to quantify the relative levels of a given mRNA according to cycling threshold analysis (ΔCt). For the Y-axis, we used the value ΔCt Dpt-Dpt/ΔCt apr normalized to control ΔCt DptΔCt apr (100%).

Supporting Information

Figure S1. The aprA Mutant Exhibits Attenuated Virulence towards Adults
Survival analysis of Dro sophila adult flies (n = 30) after feeding with wild-type P. entomophila, gac, prR, and aprA mutants; the aprA mutant complemented with the wild-type apr operon (pUCP20-apr); and an aprA mutant complemented with the apr operon carrying a non-polar
mutation in the aprA gene (PUCP20-aprA). This experiment was repeated twice and yielded similar results. Log-rank analysis demonstrated a statistically significant difference in survival of flies fed with wild-type P. entomophila and flies fed with the aprA mutant (p < 0.01).

Found at DOI: 10.1371/journal.ppat.0020056.sg001 (1.0 MB TIF).

Figure S2. Over-Expression of Imd in the Gut Protected against P. entomophila whereas Its Activation in the Fat Body Did Not
(A) Over-expression of an UAS-imd construct with the gut-specific driver caudal-Gal4 protects flies from an oral infection with P. entomophila. No protection was observed when the fat body driver pumpless-Gal4 was used. Log-rank analysis demonstrated a statistically significant difference in survival of wild-type flies and flies over-expressing an UAS-imd construct in the gut after oral infection with P. entomophila (p < 0.0001).

(B) Diptericin expression measured by RT-qPCR in Drosophila gut extracts from flies used in (A). A high Diptericin expression was observed in the gut of flies carrying both the UAS-imd and the caudal-Gal4 constructs.

Found at DOI: 10.1371/journal.ppat.0020056.sg002 (1.2 MB TIF).

Figure S3. Expression of AMP Genes
(A) Diptericin expression measured by RT-qPCR in Drosophila gut extracted from wild-type and Rel1 males following natural infection with P. entomophila. Over-expression of an UAS-Relish with the caudal-Gal4 driver restores the immune inducibility of the Diptericin gene. Diptericin expression was normalized to rp49 mRNA. 100% value corresponds to the level of Diptericin mRNA obtained after infection of wild-type flies with P. entomophila, rp49, ribosomal protein 49.

(B) The quantification of AMP gene expression shows that IMD overexpression in the gut protects against infection with P. entomophila. Diptericin and fringe expression were normalized by the corresponding values of the rp49 signal. AttA, Attacin A, Dpt, Diptericin.

Found at DOI: 10.1371/journal.ppat.0020056.sg003 (1.8 MB TIF).

Accession Numbers
The FlyBase (http://flybase.bio.indiana.edu) accession numbers for the Drosophila strains produced include Attacin A (CG10146), Caudal (CG1750), Diptericin (CG12763), IMD (CG5576), Pumpless (CG7778), and Relish (CG1992).

Accession numbers for the bacterial genes are Erwinia carotovora esp (AY167732), P. entomophila aprA, aprD, aprE, aprF, aprI, gacA, and prlR (CT573326).

Acknowledgments
We thank Won-Jae Lee for providing fly stocks; Marisa Vinals, Christoph Scherfer, and Anna Zaidman-Remy for helpful comments; and Brigitte Maroni, Michæl Poidevin, and Michèle Valens for technical help.

Author contributions. PL, FB, and BL conceived and designed the experiments. PL and MB performed the experiments. PL, FB, and BL analyzed the data. NV contributed reagents/materials/analysis tools. PL, MB, and BL wrote the paper.

Funding. The laboratory of BL was funded by the Agence Nationale de la Recherche, the Schlumberger and Bettencourt Foundations, and the association “Vaincre La Mucoviscidose.”

Competing interests. The authors have declared that no competing interests exist.
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