Monalysin, a Novel β-Pore-Forming Toxin from the Drosophila Pathogen Pseudomonas entomophila, Contributes to Host Intestinal Damage and Lethality

Onya Opota1,*, Isabelle Vallet-Gély2, Renaud Vincentelli3, Christine Kellenberger3, Ioan lacovache1, Manuel Rodrigo Gonzalez1, Alain Roussel3, Françoise-Gisou van der Goot1, Bruno Lemaitre1

1 Global Health Institute, Ecole Polytechnique Fédérale Lausanne (EPFL), Lausanne, Switzerland, 2 Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette, France, 3 Structural Immunology, AFMB UMR 6098 CNRS/UI/U1I, Case 932, Marseille, France

Abstract

Pseudomonas entomophila is an entomopathogenic bacterium that infects and kills Drosophila. P. entomophila pathogenicity is linked to its ability to cause irreversible damages to the Drosophila gut, preventing epithelium renewal and repair. Here we report the identification of a novel pore-forming toxin (PFT), Monalysin, which contributes to the virulence of P. entomophila against Drosophila. Our data show that Monalysin requires N-terminal cleavage to become fully active, forms oligomers in vitro, and induces pore-formation in artificial lipid membranes. The prediction of the secondary structure of the membrane-spanning domain indicates that Monalysin is a PFT of the β-type. The expression of Monalysin is regulated by both the GacS/GacA two-component system and the Pvf regulator, two signaling systems that control P. entomophila pathogenicity. In addition, AprA, a metallo-protease secreted by P. entomophila, can induce the rapid cleavage of pro-Monalysin into its active form. Reduced cell death is observed upon infection with a mutant deficient in Monalysin production, showing that Monalysin plays a role in P. entomophila ability to induce intestinal cell damages, which is consistent with its activity as a PFT. Our study together with the well-established action of Bacillus thuringiensis Cry toxins suggests that production of PFTs is a common strategy of entomopathogens to disrupt insect gut homeostasis.

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* E-mail: onya.opota@epfl.ch (OO); bruno.lemaitre@epfl.ch (BL)

Introduction

The intestinal epithelium has a role in defining the barrier between the host and the external environment [1]. This barrier protects the host against invasion and systemic dissemination of both pathogenic and commensal microorganisms. Both resistance and tolerance mechanisms contribute to maintain the gut integrity from the assault of infectious bacteria [2]. Resistance mechanisms involve the activation of various local immune responses that directly target pathogens. In contrast, tolerance mechanisms involve the activation of repair and stress pathways that quickly seal damages caused by infectious agents. Pathogenic bacteria have the capacity to overcome gut defenses and impede the return to homeostasis [3]. To study how pathogenic bacteria disrupt gut homeostasis, we chose to investigate the interactions between Drosophila and a newly identified entomopathogen, Pseudomonas entomophila. P. entomophila is closely related to the saprophytic soil bacterium Pseudomonas putida [4,5]. It was originally isolated from a fly sampled in Guadeloupe and subsequently shown to be lethal to Drosophila larvae and adults after ingestion. P. entomophila can also effectively kill members of other insect orders (e.g. Bombyx mori, Anopheles gambiae, Galleria mellonella). After ingestion, P. entomophila is able to persist in the Drosophila gut. It induces the expression of antimicrobial peptide genes via the Imd pathway, both locally in the intestinal epithelium and systemically in the fat body, an organ analogous to the mammalian liver [4]. It was shown that P. entomophila virulence is under the control of two global regulatory systems: the well known GacS/GacA two component system, and a second system involving a secreted secondary metabolite synthesized by the pvf gene products [4,6]. The Gac system also controls the production of a secreted protease, AprA, which is important for P. entomophila to counteract the local immune response of Drosophila [7].

Recent studies revealed that upon bacterial infection, homeostasis in the gut is restored only when bacterial clearance is coordinated with the repair of infection-induced damage through epithelium renewal [8–10]. Epithelium renewal of the Drosophila gut is stimulated by the release of the secreted ligand Upd3 from damaged enterocytes, which then activates the JAK/STAT pathway in intestinal stem cells to promote both their division and differentiation, establishing a homeostatic regulatory loop [8,9]. In contrast to infection with non-lethal bacteria, P. entomophila infection inflicts strong damage to its host without triggering an epithelial renewal [8,11]. This suggests that the damages inflicted by P. entomophila are too severe to be repaired. How damages are inflicted however remains unknown. One hypothesis was that P. entomophila produces cytoxic factors that damage the intestinal epithelium.
Author Summary

Insects are potential reservoirs for microbes and ideal vectors for their transmission due to their motility and capacity to live in bacteria-rich environments. This is exemplified by fruit flies that live in rotting fruits and are capable of transmitting phytopathogenic bacteria. Insects are notably resistant to microbial infection allowing them to colonize these microbe-rich environments. To study how pathogenic bacteria disrupt gut homeostasis, we investigated the interactions between Drosophila and a newly identified entomopathogen, Pseudomonas entomophila. Ingestion of P. entomophila infects severe damage to the Drosophila intestine. How damages are inflicted, however, remains unknown. In this study, we identified a secreted protein that plays an important role in the damage inflicted by P. entomophila to the Drosophila gut. We showed that this protein is a pore-forming toxin (PFT) that we named Monalysin. Our study reveals that Monalysin oligomerizes into ring-like structures that form pores into the plasma membrane of target cells leading to the disruption of membrane permeability and cell death. Our work together with studies on the insecticidal Cry toxins produced by Bacillus thuringiensis suggests that production of PFTs is a common strategy of entomopathogenic bacteria to interfere with insect gut homeostasis.

Results

Identification of a secreted protein involved in P. entomophila pathogenicity

We previously showed that P. entomophila secretes large amount of the metalloprotease, AprA, which can degrade antimicrobial peptides [7]. The production of this protease is regulated by the GacS-GacA system, known to control secondary metabolite production, protein secretion, and virulence determinants in γ-proteobacteria [12]. To identify additional factors responsible for P. entomophila virulence, we analyzed the culture supernatant of the wild-type bacterium and a gacA mutant by SDS-PAGE (Figure 1A). Bands corresponding to major secreted proteins in the wild type strain, but not in the gacA mutant were submitted to analysis by mass spectrometry. This allowed us to confirm that one of the major bands corresponds to the 51 kDa AprA. Three bands contained Hcp, Vgr and Rhs, proteins known to be secreted by the Type VI Secretion System (T6SS). T6SS are bacterial needle-like structures involved in the infection of effectors into the cytoplasm of eukaryotic but also prokaryotic cells [13]. We also identified a band with an apparent molecular weight of 30 kDa, containing a protein encoded by the uncharacterized gene pseen0535 that we named Monalysin.

In order to investigate the role of these secreted proteins, we made a T6SS mutant (affecting the ORF pseen0535) and a monalysin (mnl) mutant (Annal), and tested their virulence in Drosophila. No difference could be observed between the wild type strain and the T6SS mutant. Interestingly, the mnl mutant presented a decreased pathogenicity. Indeed, survival analysis of Drosophila adults after oral infection with the wild-type strain, the gacA mutant, and the mnl mutant showed that only 40% of the flies infected with the mnl mutant succumbed within 3 days, while 70% of the flies died after infection with wild-type P. entomophila (Figure 1B and Figure S6). As previously shown [4], a gacA deficient mutant did not show any pathogenicity using this assay. The attenuated virulence of the mnl mutant was fully rescued by complementation with a wild-type copy of the monalysin gene.

A mutant deficient in Monalysin production is affected in its abilities to induce cell damage in the Drosophila gut

It was previously shown that P. entomophila virulence towards Drosophila is associated to its ability to persist in the gut and the transcription of antibacterial peptide genes both locally and systematically [4]. In order to better characterize the role of the monalysin gene in the infectious process, we next compared the ability of the mnl mutant (Annal) to persist to that of the wild type strain or a gacA mutant. Flies were infected by feeding and bacterial loads were quantified at two time points (Figure 1C). While bacterial loads were indistinguishable after 3 hrs, persistence of the mnl mutant and the gacA mutant were significantly decreased when compared to wild type bacteria [4]. We then compared the activation of the Imd pathway after infection by the wild type, the gacA, and the mnl mutant. We used reverse transcriptase quantitative PCR (RT-qPCR) to measure the expression of the Diptericin gene, a target of the Imd pathway, specifically in the gut (local response) or in whole flies (reflecting mostly the systemic expression of Diptericin by the fat body) (Figure 1D and E). As previously shown [7], Diptericin expression increased already 4 h after infection by P. entomophila and even more after 16 h, both in the gut and the fat body, an increase that was not observed for the gacA mutant [4]. The mnl mutant leads to an increase in Diptericin expression in the gut similar to that observed for the wild-type bacterium (Figure 1D). However, while Diptericin expression increased to wild-type levels in the fat body 4 h after infection, no further increase was observed (16 h) in flies infected with the mnl mutant (Figure 1E).

We next investigated the contribution of Monalysin in the damage caused by P. entomophila to the Drosophila gut. We first monitor the induction of cell death upon bacterial ingestion using an acridine orange staining. A high number of dead cells were detected in guts from flies infected by wild type P. entomophila, but not in guts from flies infected by a gacA mutant as previously reported [6]. Interestingly, a reduced level of cell death was observed in the mnl mutant (Figure 1F and S7). Oral infection with P. entomophila resulted in a decrease of the adherens junction marker Cadherin-GFP (Figure 2A) and to morphologically altered guts, with regions devoid of enterocytes indicative of a disruption of tissue integrity (see the lack of nuclear DAPI staining due to the loss of cell in Figure 2A3). Interestingly, gut collected 16 hrs after oral infection with gacA and mnl mutants did not show any Cadherin-GFP signal decreases or a rupture of the gut integrity (Figure 2A4 to 2A6). Previous studies showed that ingestion of P. entomophila activates both JAK-STAT and the Jun N-terminal kinase (JNK) pathway in the Drosophila gut [8] that participate in the repair and stress responses, respectively [8,14–16]. The activation of both pathways can be monitored by measuring by RT-qPCR the expression of puckered (puc) (a direct downstream target of JNK signaling) or upd3 (a target of JAK-STAT signaling) and Socs36E (a target of JAK-STAT signaling that encodes a negative regulator of this pathway). Figure 2B, 2C and 2D shows that the mnl mutant was less efficient than wild type P. entomophila to activate the JNK and JAK-STAT pathways, yet more efficient than a gacA mutant. Consistent with the RT-qPCR analysis, expression of the upd3-GFP reporter gene (upd3-Gal4, P. entomophila Pore-Forming Toxin Monalysin)}
Monalysin is a secreted cytotoxic protein

In order to characterize the activity of the secreted protein encoded by monalysin, we produced and purified a his-tag version of it in E. coli. Ingestion of the recombinant protein at high dose had no impact on fly survival. However, the Monalysin protein was highly toxic when directly injected in the body cavity (Figure 2E). Altogether, these data show that even though the null mutant retains some ability to cause intestinal damage, this ability is strongly diminished compared to wild type P. entomophila. This suggested a specific role of the Monalysin protein in P. entomophila cytotoxicity towards Drosophila.

Monalysin is a novel β-type pore-forming toxin

The Monalysin amino acid sequence does not show any homology to other sequences using P Blast, except for two uncharacterized orthologs found in Pseudomonas putida F1 strain (Figure S1). Neither the P. entomophila nor the P. putida gene products displayed any obvious protein domains. Nevertheless, the use of the HHpred software (Homology detection & structure prediction by HMM-HMM comparison) revealed the presence of an internal region with alternating polar and hydrophobic residues flanked by stretch of serine- and threonine residues, a hallmark of the membrane-spanning region of β-barrel pore-forming toxins (Figure 6A). PFTs can be classified according to the secondary structure of their membrane-spanning region or β- and β-PFTs. Far-UV circular dichroism analysis of Monalysin revealed a spectrum typical of structured proteins (Figure S2). The content of α-helices and β-sheets was estimated to be 13% and 40%, respectively in agreement with the secondary structure prediction obtained with the program JPRED giving 17% of α-helices and 35% of β-sheets as indicated in Figure 6A. This program also indicated that the putative membrane-spanning region of Monalysin was formed of a β-sheet. This sequence analysis suggests that Monalysin is related to PFT of the β-type.

β-PFTs are synthesized as soluble proteins and have the ability to multimerize into circular polymers at high concentration, a step that for certain toxins, such as Aerolysin, requires proteolytic activation [18]. We next investigated whether Monalysin shared these properties with PFTs. SDS-PAGE analysis of a fresh recombinant Monalysin solution revealed a major band at the expected size (30 kDa) as well as several high molecular weight bands corresponding to oligomers that were resistant to SDS (see below). Interestingly, a shorter form of the protein was observed upon storage of samples at 4°C (Figure S3A). This together with the observation that Monalysin is matured by AprA indicates the existence of a protease sensitive site in the N-terminus part of Monalysin (Figure 3B). The cleavage of the recombinant pro-Monalysin into its shorter form could also be induced by a limited trypsinolysis (Figure S3B). This processed form has a molecular
Figure 2. Monalysin contributes to *P. entomophila*-inflicted damage to the *Drosophila* gut. (A) Expression of the marker of adherens junction Cadherin-GFP 16 hours after infection with lethal doses of the indicated bacteria. Ingestion of wild type *P. entomophila* disrupts the pattern of Cadherin-GFP. A3 and A6 show DAPI of A2 and A5 respectively. The symbol (*) marks regions where DAPI staining is absent. (B–D) Analysis of puckered (*puc*), unpaired3 (*upd3*), and socs36E expression measured by RT-qPCR in guts of infected flies. Statistical analysis was performed using a Wilcoxon test and letters indicate significantly different values (*P* < 0.05). (E) Expression of the *upd3-Gal4, UAS-GFP* reporter in (E1) unchallenged flies or (E2–E4) 4 hours after infections with a sublethal dose of bacteria (OD600 = 10). In contrast to the wild type *P. entomophila* strain (E2), the Δmnl (E3) and the ΔgacA (E4) strains were unable to elicit upd3-GFP expression. Scale bars represent 50 μm.

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weight of 26.5 kDa as determined by MALDI-TOF analysis, as opposed to 30.2 kDa for the full-length pro-toxin. Interestingly, Monalysin had stronger hemolytic activity than pro-monalysin (Figure 4A), indicating that the removal of the N-terminal fragment constitutes a maturation step that enhances the cytotoxic activity. Processing of pro-monalysin to its mature form was accompanied by an increase and change in the higher order SDS-resistant complexes (Figure S3B).

While multiple size oligomers were observed by SDS-PAGE, a single species was observed by native PAGE analysis of Monalysin (Figure S3C). Multi-Angle Light Scattering analysis (MALS/UV/RI) confirmed the presence of a single species with a molecular mass of 546 kDa and hydrodynamic radius of 7.5 nm hence a diameter of 15 nm, which would correspond to about 18 monomers (Figure S4). This was further confirmed by electron microscopy of negatively stained recombinant Monalysin which

Figure 3. Monalysin encodes a cytotoxic protein secreted by P. entomophila. (A) Survival analysis of wild-type Oregon adult flies after injection of various quantities of Monalysin or heat-inactivated (denaturated) Monalysin. (B) Drosophila S2 cells and Spodoptera frugiperda Sf9 cells were treated with Monalysin (Final concentration = 100nM) and stained with a live-dead viability reagent. Living cells are stained in green with Calcein while dead cells are stained in red with Ethidium homodimer 1 (EthD1, red). (C) The loss of viability was quantified by measuring the release of lactate dehydrogenase (LDH) from S2 cells. (D) DNA fragmentation in S2 cells was monitored by ISNT (in situ nick translation). (E) Chromatin condensation on untreated and Monalysin treated S2 cells was examined by DAPI staining. Phase-contrast and fluorescence views of the same microscopic fields are shown. (−) untreated cells, (+) = cells treated with Monalysin 100nM. doi:10.1371/journal.ppat.1002259.g003
showed circular like (top view) and barrel like structures (side view) similar to that observed with other β-PFT [Figure 6B].

Sequence analysis of Monalysin, its ability to form ring like high order structures combined with its hemolytic activity strongly indicate that the toxin is a PFT. To address this issue directly, we analyzed its ability to form channels in planar lipid bilayers, an extremely sensitive electrophysiological method that enables the study of single-channel events. Addition of Monalysin led to a stepwise increase in membrane current, reflecting the formation of pores [Figure 6C]. Collectively, our data show that P. entomophila Monalysin is a bona fide pore-forming toxin of the β type.

Discussion

Many bacterial pathogens, both Gram-positive and Gram-negative, produce PFTs that contribute to their virulence [17]. Here we report the identification of a novel PFT that contributes to the virulence of P. entomophila against Drosophila. Our data show that Monalysin requires N-terminal cleavage to become fully active, forms oligomers in vitro, and induces pore formation in artificial lipid membranes. The prediction of the secondary structure of the membrane-spanning domain indicates that Monalysin is a PFT of the β-type. Outside of this domain, Monalysin does not show any homology to any other PFT and appears rather different from previously identified insecticidal PFTs such as B. thuringiensis Cry toxins. Nevertheless, Monalysin has two homologs in the closely related P. putida F1 strain. These proteins could participate to the interaction of some Pseudomonas species with eukaryotic cells, defining a new family of PFTs.

We previously showed that P. entomophila virulence is multifactorial and regulated by multiple signaling modules. Taking advantage of the genetic amenability of both the host and the pathogen, we aimed to identify P. entomophila and Drosophila pathways and effectors involved in the infectious process. Using this integrated approach, we previously proposed a role for the AprA metalloprotease in protection against antimicrobial peptides [7]. We now identify a second virulence factor, the β-PFT Monalysin. Like AprA, a mnl mutant is affected in several, but not all, aspects of P. entomophila virulence. This attenuated virulence of the mnl mutant is clearly shown by survival analysis, which

Figure 4. Monalysin hemolytic activity and cytotoxicity towards mammalian cells. (A) Hemolytic activity was measured with pro and matured Monalysin incubated with red blood cells. The mature Monalysin was obtained by limited trypsinolysis of a fresh extract of recombinant Pro-Monalysin. (B) Phase contrast microscopy of Hela cells untreated or treated with Monalysin 100 nM for 24h. Cells were shrinking and displayed irreversible loss of adherence.
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monitors the global outcome of infection. Our study indicates that Monalysin significantly contributes to the damage inflicted to intestinal cells by the bacterium, which is fully consistent with its activity as a pore-forming toxin. Supporting this notion, we observe that a mnl-deficient mutant induced less cell damage and a lower level of stress and repair pathway activity. The mnl mutant still induced a local immune response but the systemic immune response is drastically attenuated. This is also consistent with a role of Monalysin as a cytotoxin since activation of a systemic immune response is probably linked to damage of intestinal tract rendering possible the translocation of peptidoglycan, the bacteria elicitor activating the Imd pathway, from the lumen to the hemolymph compartment.

We also show that Monalysin production is regulated both by the GacS/GacA two component-system and the pef genes. However, a mnl-deficient mutant still causes higher levels of stress and damage to the intestinal epithelium than gacA or pef mutants. This indicates that these signaling modules regulate additional virulence factors contributing to P. entomophila cytotoxicity. Alternatively, it is possible that the overall cytotoxicity is caused by a synergy between the metalloprotease AprA and the PFT Monalysin, both of them being regulated by GacA. Along this line, we observed that AprA promotes the rapid cleavage of the pro-Monalysin into its active form. Since Monalysin can also be processed by trypsin, it is likely that AprA is not essential for PFT function of Monalysin in the Drosophila gut, as this toxin could also be processed by host enzymes. Both Monalysin and AprA are expressed in the algR mutant that affects a transcriptional regulator involved in alginate production as well as genes that are often associated to virulence (ie: pili biosynthesis, cyanide production…) [19]. The observation that an algR mutant is still avirulent (Vodovar 2005), although expressing both Monalysin and AprA, indicates the existence of additional virulence factors. Future studies should investigate at which level Pvf and GacS/GacA affect Monalysin production as well as identify other potential virulence factors regulated by the Pvf, Gac or AlgR.

Recent studies have shown that cells respond to PFTs by inducing repair and stress signal-transduction pathways to repair damage. Studies in C. elegans and mammalian cells have revealed a role for the P38 pathway, the unfolded protein response, and hypoxia in cellular resistance to the action of PFT [20–22]. The reduced expression of JAK-STAT and JNK pathway activities in guts infected with the mnl mutant indicate that Drosophila epibolic cells respond to PFT by activating stress and repair pathways. Thus, the P. entomophila/Drosophila interaction provides an interesting model to dissect the host response to PFTs in a natural infectious context.

Insects are potential reservoirs for microbes and are ideal vectors for their transmission due to their motility and their capacity to live in bacteria-rich environments [23]. This is exemplified by fruit flies that live in rotting fruits and are capable of transmitting phytopathogenic bacteria [24]. Insects are notably resistant to microbial infection allowing them to colonize these microbe-rich environments. This is largely due to the existence of very efficient physical barriers that block entry of microbes in the body cavity. As an illustration, injection of less than 10 cells of P. aeruginosa or Serratia marcescens in the body cavity rapidly kills flies, while high doses of these bacteria have only modest effects on survival when ingested [25]. In contrast to mammals, the gut of insects is lined with a chitinous matrix, the peritrophic matrix [26], that blocks the direct interaction between bacteria and epithelia cells and prevents the use of virulence devices such as type III and VI secretion systems that allow the injection of virulence factors directly into target cells. Rare bacterial species such as Photorhabdus luminescens can bypass this physical barrier since there are transported by symbiotic nematodes that can pierce the insect cuticle [27]. Other entomopathogens that enter through the oral route have to escape the local immune response and breach the gut barrier [23]. Despite the characterization of several virulence factors in few species, the mechanisms by which enteric pathogens kill insects remain poorly understood. This paper together with the well-characterized action of Bacillus thuringensis cytotoxin Cry suggests that PFTs efficiently promote bacterial colonization of the insect gut [28–30]. This heavy artillery strategy does not require a direct contact between bacteria and host cells since PFTs can cross the pores of the peritrophic matrix and reach intestinal cells. PFTs

Figure 5. Regulation and processing of Monalysin. (A) Western-blot analysis of proteins from crude cell extracts or filtrate supernatants shows that Monalysin was not produced in gacA and pef mutants, but was produced in the algR mutant. (B) Western-blot analysis of bacterial crude cell extracts and filtrated supernatants of Pe wt and _AprA_ shows that pro-Monalysin is not processed in the supernatant of an _AprA_ mutant. The stronger signal in the _AprA_ mutant lane is due to the fact that the serum recognized better the pro-Monalysin than the monalysin (see Figure S5).

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Figure 6. Monalysin is a β pore-forming toxin. (A) Protein sequence analysis of Monalysin reveals an internal domain with amphipathic patches flanked by serine- and threonine-rich sequences that shares similarities with the membrane-spanning domain of β-PFT (β-toxin from Clostridium perfringiens, aerolysin from Aeromonas hydrophila and MTX-3 from Bacillus sphaericus). The multiple sequence alignment reveals the presence in P. entomophila Monalysin of putative membrane exposed residues (Yellow stars), solvent-exposed residues (green stars), and serine and threonine residues (red stars). A black star (▲) shows the first amino acid detected by MALDI-TOF analysis of tryptic fragment of the recombinant Monalysin. The N-terminal residues of the mature Monalysin, identified by Edman sequencing, present in P. entomophila supernatant are underlined in red; a triangle (▼) indicates the potential cleavage site of pro-monalysin deduced from the N-terminal Edman sequencing. Purple cylinders indicate predicted α-helices and yellow arrows indicate predicted β-sheets. (B) Scanning Electron micrographs show that Monalysin forms circular-like structures (top view) and barrel-like aggregates (side view). Scale bar represents 100 nm. (C) Monalysin (5 µg.ml) is able to form pores in a planar lipid bilayer. doi:10.1371/journal.ppat.1002259.g006
can induce gut damage and rupture of intestinal homeostasis that in fine will lead to a weakening of the gut barrier and an inhibition of gut peristalsis promoting bacteria persistence. Gut damage and food uptake blockage are two symptoms of insect pathogenesis and could reflect the action of PFTs [23]. It would be interesting to know if other entomopathogens such as Serratia marcescens and Serratia entomophila also use PFT to colonize their insect host. In conclusion, this and other studies using different bacteria species contribute to uncovering strategies used by entomopathogens to breach insect barriers. A better knowledge of these strategies could also open the route to new methods of insect pests control.

Materials and Methods

Bacterial strains, media and antibiotics

P. entomophila L48 [4] was grown in LB for all experiments. P. entomophila mutated for the gacA, aprA, algR, and the pof gene are described elsewhere [4,5,6,7,11]. The mnl deletion construct was generated by amplifying flanking regions of the monalysin gene (pseen3174 or mnl) by PCR. The resulting PCR product was cloned into the plasmid pEXG2. This plasmid was then used to create the strain A3174 (alternatively A0mnl), containing a deletion of the gene pseen3174. Complementation construct were made by cloning into the plasmid pPSV35 of PCR-amplified DNA fragments from P. entomophila containing the mutated genes. Pseudomonas Isolation agar (PIA, Difco) was used for selection after conjugations and persistence experiments. When E. coli was grown, antibiotics were used when necessary at the following concentrations: G418, 25 μg/ml and tetracycline, 5 μg/ml. When P. entomophila was grown, antibiotics were used when necessary at the following concentrations: gentamicin, 50 μg/ml for liquid cultures and 150 μg/ml for solid media, tetracycline 40 μg/ml and rifampicin, 30 μg/ml. The bacterial strains used in this study and the culture conditions are presented in Table S1. All primer sequences are available upon request. Insertion constructs were generated as previously described [6,11].

Sequence analysis

DNA sequence searches and analysis were performed using the Pseudomonas genome database (www.pseudomonas.com). The monalysin gene (ORF PSEEEN3174) corresponds to the accession number YP_608728.1. Monalysin putative orthologs in Pseudomonas putida Pput_1063 and Pput_1064 correspond to the accessions numbers YP_001266408.1, YP_001266409.1 respectively. The ORF PSEEEN0535 involved in the production of the type VI secretion system corresponds to the accession number YP_606298.1. Monalysin amino-acids sequence analysis was performed using the HHpred software (Homology detection & structure prediction by HMM-HMM comparison http://toolkit.tuebingen.mpg.de/hhpred).

Fly stocks and infection assays

Oregon R flies were used as a standard wild-type strain and were maintained at 25°C. Adherens junctions were visualized using ubiquitin-DE-cadherin-GFP flies [14,31]. Upd3 expression in unchallenged gut and following infection, was monitored using upd3-Gal4, UAS-GFP flies (Buchon et al., 2009). Fly natural infections were carried out at 29°C on 4- to 8- day-old adult females as previously described. All the infections, except when specified, were carried out with bacterial preparation adjusted to an OD600 = 100 which correspond to 6.5E10 colony forming units per ml [11]. Monalysin was injected in the body cavity of fly using a Nanodrop microinjector (Nanoject). Virulence assays were performed at least three times in triplicate.

Monalysin expression, purification, and analysis

All cloning steps were performed as described earlier [33]. The sequence of Monalysin (from residue 1 to 271, accession number pseen3174) was PCR-amplified from genomic DNA (isolated from P. entomophila) and cloned into pDONR201 (Invitrogen). The ORF was then subcloned into the pETG-20A E. coli (a generous gift from Dr A. Geerlof, EMBL) destination vector to generate a constructs encoding Monalysin with an N-terminal fusion composed of the thioredoxin (TRX) protein, followed by a 6xHis-tag and a Tobacco Etch Virus (TEV) protease cleavage site. The construct was sequenced verified. The production and purification were performed as described earlier [34]. Briefly, the pETG-20A-Monalysin was transformed into Rosetta (DE3)

Reverse transcriptase quantitative PCR analysis

Total RNA was extracted from whole flies (5 for each assay) or from dissected guts without Malpighian tubules (14 for each assay) using TRIzol (Invitrogen). RT-qPCR was performed using SYBR Green I (Roche) on a Lightcycler 2.0 (Roche) as previously described [32]. Data represent ratio of the amount of mRNA detected normalized to the amount of the control rpl32 mRNA. Experiments were performed at least three times independently. Averages of more than three experiments are shown.

Cell cultures, treatments, cytotoxicity assays and live imaging

The macrophage-like lineage S2 cells derived from D. melanogaster embryos where grown in Schneider’s medium (Invitrogen). The S09 cells (Invitrogen) derived from Spodoptera frugiperda (Lepidoptera) pupal ovarian tissue were cultured in complete TNM-FH (Invitrogen). The mammalian cell lines Hela and the Retinal Pigmented Epithelial (RPE1) were grown in a humidified incubator with 5% CO2 at 37°C. Hela cells were cultured in MEM media supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, 1% glutamine and 1% NEAA (Gibco). RPE1 cells were cultured in DMEM media supplemented with 10% fetal calf serum, 1% penicillin-streptomycin and 1% glutamine (Gibco). Cell viability was observed using the LIVE/DEAD Viability/Cytotoxicity Assay Kit (Invitrogen) according to the provider instruction. Briefly cells are simultaneously labeled with calcine AM that reveals intracellular esterase activity in live cells and ethidium homodimer (EthD-1) that reveals plasma membrane damages. LDH release from damaged cells was measured following the instructions of the CytoTox-One Homogeneous Membrane Integrity Assay kit (Promega). In Situ Nick Translation was performed to detect fragmented DNA in nuclei. In situ DNA synthesis was performed by a DNA polymerase I (150 units/ml) (Takara) in the presence of a dNTP mix in which dUTP is tetramethylrhodamine-conjugated (Roche). The reaction was carried out for 90 min at room temperature. Live imaging and immunofluorescence were performed as previously described [8]. After treatment, cells were recovered, fixed with 4% PFA and permeabilized with 0.3% Triton X-100. Dead cells were detected using acridine orange staining (Invitrogen). Dead cells quantification was performed as follows: 16 hours after infection, guts were dissected and stained with acridine orange and DAPI. Pictures were taken using a fluorescent microscope. From these pictures, groups of 100 hundred DAPI stained nuclei were randomly defined and the number of acridine orange positive nuclei (ie dead cells) was determined. Three parcels per guts were analyzed. The results are the mean of four independent experiments. Nuclei were stained by DAPI (Sigma). All the images were performed using a Zeiss AxioImager Z1.
production of the antibody anti-monalysin
The Guinea pig antibody anti-Monalysin was provided by Eurogentec.

Circular dichroism, molecular weight and hydrodynamic radius determination
Far-UV Circular Dichroism (CD) spectra (Figure S2) were recorded with a JASCO J-810 spectropolarimeter (JASCO Corporation) equipped with a Peltier temperature control and using 1-mm thick quartz cells. The molecular weight of recombinant pro-monalysin and Monalysin was determined by MALDI-TOF/TOF. Molecular weight and hydrodynamic radius determination was performed by the ASTRA V software (Wyatt Technology). Proteins were loaded at a final concentration of 0.02 mM.

Edman sequencing
After SDS-PAGE electrophoresis and Coomassie blue staining, protein bands were excised. Proteins were extracted from gel and blotted onto polyvinylidene difluoride membranes with the ProSoB system (Applied Biosystems). The N-terminal sequences of proteins were determined by automated Edman degradation by introducing the blots into a Precise P494 automated protein sequencer (Applied Biosystems). The sequences obtained were compared to sequences in public protein sequence databases.

Statistical analysis
Survival assays have been performed at least three times in triplicate. The Kaplan-Meier log rank test was used to determine statistical significance. Dashed brackets represent the significance between the different infections (*: p<0.05, **: p<0.01, ***: p<0.001, ns = not significant). RT-qPCR analysis and cell death quantification using acidine orange staining are averages of at least 4 independent experiments. Error bars indicate standard errors. Statistical analysis was performed using a Wilcoxon test, and letters indicate significantly different values (*P<0.05).

Supporting Information
Figure S1 Identification of two putative Monalysin orthologs in Pseudomonas putida F1. Alignment of Monalysin amino-acids sequence and the sequence of its putative orthologs in Pseudomonas putida F1 encoded by the ORF Pput_1063 and Pput_1064. (TIF)

Figure S2 Far-UV CD spectra of Monalysin. The far-UV CD spectra were recorded with a JASCO J-810 spectropolarimeter (JASCO Corporation) equipped with a Peltier temperature control and using 1 mm thick quartz cells. CD spectra were averaged on three accumulations using a scanning speed of 50 nm/min. Measurements were performed between 190 and 260 nm at 20°C in 10 mM Hepes buffer pH 7.5, NaCl 150 mM with a protein concentration of 1 mg/ml. Circular dichroism of Monalysin reveals a spectrum of a protein with alpha-helix and beta-sheets. (TIF)

Figure S3 Recombinant Monalysin is processed by a proteolytic cleavage. (A) SDS-PAGE analysis of recombinant Monalysin. Lane 1: fresh sample, lane 2: old sample. (B) Silver staining of a SDS-PAGE fresh monalysin samples untreated (lane 1) or treated with trypsin (lane 2). (C) Native gel electrophoresis shows that Monalysin migrates at a high molecular weight. (TIF)
Figure S4 Absolute molecular weight determination of the Monalysin oligomer by Static Light Scattering analysis. The molar mass (left axis, dotted lines) and the UV_{280nm} absorbance (right axis, solid lines) are plotted as a function of the column elution volume. Monalysin measured mass and hydrodynamic radius are 546.5 KDa and 7.52 nm, respectively.

(TIF)

Figure S5 Western blot analysis of Monalysin. (A) Western-blot analysis of proteins from crude cell extracts and filtrate supernatant. Pe = P. entomophila wild-type strain. Annl = the monalysin deficient strain, Annl-pPSVmnl = the monalysin-deficient strain carrying a plasmid expressing a wild-type copy of the monalysin gene, Annl-pPSV = the monalysin-deficient carrying the plasmid pPSV53 without any insert. (B and C) Western-blot of the serum anti-monalysin recognition of pro-monalysin and monalysin. (A) Coomassie staining and (B) Western-blot of the serum recognition of pro-monalysin and monalysin. (C) Comparison of the serum anti-monalysin recognition of pro-monalysin and monalysin. The number next to the bacterial strains indicates the same amounts of predominantly the mature form Monalysin (line 2 and 3 respectively) and a sample containing the mature form Monalysin (line 1 and 3 respectively) as shown by coomassie staining. The western blot shows that the serum recognizes more epitopes in pro-monalysin than in monalysin. Indeed, monalysin (Figure S5C, lane 4) could be detected only when the exposure time was increased, which resulted in a saturating signal for pro-monalysin.

(TIF)

Figure S6 Survival analysis of wild-type Oregon flies following oral infection with various concentrations of bacteria. Survival curves of flies infected with various concentrations of (A) the P. entomophila wild-type strain (Pe), (B) the mnl deficient strain (Annl), (C) the gacA-deficient strain (AgacA). UN = unchallenged. The number next to the bacterial strains indicates the concentration (Optical Density measured at 600nm) of the bacterial sample used for the infection. Survival assays have been performed at least three times in triplicate.

(TIF)

Figure S7 Cell death in guts of infected flies monitored by acridine orange staining. Guts were dissected from unchallenged female Oregon flies (A) or infected for 16 h with wild-type P. entomophila (B), a mnl mutant (C) or a gacA mutant (D), and stained with acridine orange. Scale bars represent 50 μm.

(TIF)

Table S1 Bacterial strains used in this study.

(TIF)

Table S2 Monalysin cytotoxicity towards insect and mammalian cells. Different culture cell lines were treated with the indicated concentration of Monalysin. Sensitivity (+) or resistance (−) to Monalysin was determined by phase contrast microscopy observation performed at 4 and 24 h. For HRBC (human red blood cells) the sensitivity was monitored by hemolytic activity as described in (TIF).

(TIF)

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Author Contributions

Conceived and designed the experiments: OO IVG BL FGVDG AR. Performed the experiments: OO IVG RV CK II MRG. Analyzed the materials/analysis tools: BL FGVDG AR. Wrote the paper: OO BL.

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