Cellular Microbiology (2007) **9**(1), 106–119
doi:10.1111/j.1462-5822.2006.00771.x
First published online 31 July 2006

Erwinia carotovora Evf antagonizes the elimination of bacteria in the gut of *Drosophila* larvae

Carlos Acosta Muniz,[1] Danielle Jaillard,[2] Bruno Lemaitre[1]* and Frédéric Boccard[1]*
[1]*Centre de Génétique Moléculaire du CNRS, Bâtiment 26, 1 Avenue de la Terrasse, 91198 Gif-sur-Yvette, France.*
[2]*Unité Mixte de Recherche 8080 du CNRS, Bât. 440 bis, Université Paris XI, 91405 Orsay, France.*

Summary

***Erwinia* Virulence Factor (Evf) has been identified in *Erwinia carotovora carotovora* 15 (*Ecc*15) as a virulence factor that promotes colonization of the *Drosophila* larval gut and provokes the triggering of a systemic immune response. Here we have analysed how Evf promotes persistence and colonization of bacteria inside the larval gut. *Erwinia evf* mutants do not persist in immune-deficient *Drosophila*, indicating that Evf does not act by counteracting immunity. The results indicated that Evf is not a toxin because various Gram-negative bacteria expressing *evf* can persist without affecting viability of *Drosophila* larvae. Evf did not appear to be a factor antagonizing a host-specific reaction because *in vitro* assays failed to reveal detoxifying enzymatic activities against various compounds thought to contribute to the hostile environment of the gut. These findings were corroborated by the observation that Evf is not required for survival in midgut organ cultures. By contrast, bacteria expressing *evf* allow persistence *in trans* of bacteria lacking *evf* indicating that Evf promotes the accumulation of Gram-negative bacteria in the anterior midgut by affecting gut physiology.**

Introduction

In recent years, a number of genetically amenable organisms have been used as models to study host/pathogen interactions. The fruit fly *Drosophila* has been very useful in characterizing signalling pathways and mechanisms used by the host to prevent and combat microbial infection. The *Drosophila* immune response consists of both cellular and humoral responses. Expression of immune effectors is mainly under the control of two signalling pathways designated Toll and Imd (Hultmark, 2003). The Toll pathway is predominantly activated by Gram-positive bacteria and fungi, and induces the synthesis of several peptides including the antifungal peptide drosomycin. On the other hand, the Imd pathway is activated predominantly by Gram-negative bacteria and induces the expression of different antimicrobial peptides encoding genes (e.g. diptericin). In addition, the Imd pathway controls the local expression of antimicrobial peptides in epithelia such as gut or trachea. Up to recently, most studies have involved the direct injection of microbes into the insect body cavity. In the last few years, a second approach called natural infection has been developed to mimic infections as they probably occur in nature (Basset *et al*., 2000). This method consists of feeding *Drosophila* larvae or adults with food containing a high bacterial titre. Isolation of bacteria that elicit an immune response after ingestion might reveal strategies that are used by microbes to persist in their host, especially the initial steps of infection (Vodovar *et al*., 2004).

Upon ingestion, most bacterial strains appear to be non-infectious, i.e. they do not persist in the fly and/or do not induce an immune response. Only a few microbes have been described as being able to trigger the immune response or to be pathogenic. These include *Serratia marcescens* (Flyg *et al*., 1980), a *qscR* mutant of *Pseudomonas aeruginosa* (Chugani *et al*., 2001), and *Pseudomonas entomophila* (Vodovar *et al*., 2005). In previous studies, we have shown that several *Erwinia* species were able to elicit the immune response (Basset *et al*., 2000). Among these, *Erwinia carotovora carotovora* 15 (*Ecc*15) is able to persist in the gut of larvae and induces both a local and systemic immune response while not killing the larvae. By using a genetic screen, we have identified two genes that were required by *Ecc*15 to infect *Drosophila* (Basset *et al*., 2003). The first gene encodes a global regulator, Hor, and seemed to exert its effect by regulating the second identified gene, *evf*. *Erwinia* Virulence Factor (Evf) may play a role in gut persistence as its transfer into different enterobacteria makes them infectious for *Drosophila*. No homologous genes were found in other organisms and no domains with predicted activity or signature could be discerned in Evf.

Received 16 April, 2006; revised 8 June, 2006; accepted 13 June, 2006. *For correspondence. E-mail lemaitre@cgm.cnrs-gif.fr; Tel. (+33) 1 69 82 32 27; Fax (+33) 1 69 82 43 86 or email boccard@cgm.cnrs-gif.fr; Tel. (+33) 1 69 82 32 11; Fax (+33) 1 69 82 31 50.

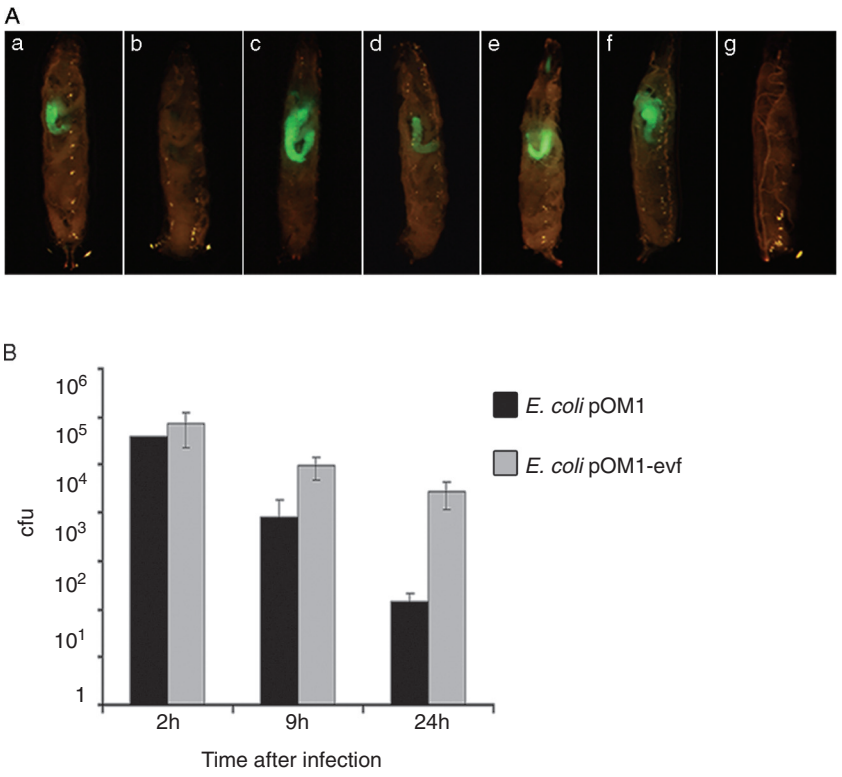


Fig. 1. Effect of Evf on the persistence of Gram-negative bacteria in the gut.
A. Wild-type (OrR) larvae were naturally infected with various bacteria expressing a GFP reporter gene. (a) *Ecc*15 carrying pOM1-GFP, (b) *Ecc*15 *evf* mutant carrying pOM1-GFP, (c) *Ecc*15 *evf* mutant carrying pOM1-evf-GFP, (d) *S. typhimurium* pOM1-evf-GFP, (e) *E. coli* pOM1-evf-GFP, (f) *P. aeruginosa* PAO1 carrying pX2-evf-GFP and (g) *P. aeruginosa* carrying pX2-gfp. Pictures were taken 6 h after infection.
B. Bacterial persistence was measured in wt larvae. Bacterial counts were obtained by plating, on LB medium containing spectinomycin (100 μg ml^{-1}), the larval homogenates of five surface-sterilized larvae that were naturally infected with *E. coli* carrying pOM1 and *E. coli* carrying pOM1-evf. The number of colony forming units (cfu) per larva obtained at each point after infection represents the mean of three independent measurements.

Here we describe a number of experiments performed to understand how Evf allows bacteria to persist in the *Drosophila* larval gut. We show that Evf activity relies on the presence of Evf in the cytoplasm of Gram-negative bacteria. Our results indicate that persistence of bacteria in the gut does not involve a detoxifying activity directed against the host immune system but rather leads to a modification of insect gut physiology that is under normal circumstances, responsible for the eradication of ingested bacteria.

Results

Evf allows persistence of Gram-negative bacteria in the gut of Drosophila

In a previous study, we have shown that transfer of *evf* to different enterobacteria, i.e. *Escherichia coli*, *Salmonella typhimurium* and *Serratia marcescens*, transformed these bacteria into infectious microbes that induced a strong antibacterial response upon ingestion (Basset *et al.*, 2003). To further characterize the interaction of these bacteria with the fly, *Drosophila* larvae were fed with bacteria expressing both *evf* and the gene encoding green fluorescent protein (GFP), *gfp*. Whereas overexpression of *evf* in *Ecc*15 induced a strong lethality in *Drosophila* 12 h after feeding, no lethality was apparent with the three other enterobacteria. All enterobacteria were present in the gut after 6 h and no fluorescence was apparent in other tissues of the fly (Fig. 1A). The persistence of bacteria in the gut was more precisely estimated by plating gut extracts. In the case of wild-type (wt) *E. coli* cells, the titre of bacteria decreased from 10^6 after 1 h to 10^4 after 9 h and 10^2 after 24 h. By contrast, the titre of *E. coli* cells expressing *evf* remained high, between 10^4 and 10^5 after 9 or 24 h (Fig. 1B). This level is similar to that obtained with *Ecc*15 overexpressing *evf* (Basset *et al.*, 2003).

The same experiment was performed with various *Pseudomonas* species, Gram-negative bacteria that are more distantly related to enterobacteria. In the absence of *evf*, *Pseudomonas* species such as *P. aeruginosa* PAO1 or *Pseudomonas putida* KT2440 did not persist and did not induce an immune response as previously described (Vodovar *et al.*, 2005). *P. aeruginosa* PAO1 (Fig. 1A) and *P. putida* KT2440 (data not shown) expressing *evf* persisted in the gut, induced a strong antibacterial response and provoked lethality after 12 h (data not shown).

The ability of Evf to confer infectivity was tested for different Gram-positive bacteria. *evf* was placed under the control of the promoter P_{SPAC} and inserted in the plasmid pDG148 expressing *gfp* (see *Experimental procedures*). This recombinant plasmid was transformed in *Bacillus megaterium*, *Bacillus subtilis* and *Streptococcus faecalis*. In all cases, whereas fluorescence was apparent in the gut upon ingestion, no fluorescence remained visible after 6 h and no Toll-dependent or Imd-dependent immune responses were detected (data not shown).

Whereas the bacterial entomopathogen *P. entomophila* is able to infect *Drosophila* but also additional species belonging to different insect orders (Vodovar *et al*., 2005), the host range of other entomopathogens such as *Bacillus thuringiensis* is more restricted (de Maagd *et al*., 2001). To determine the host range against which Evf can confer infectious properties, larvae of various *Drosophila* species (*D. virilis*, *D. busckii*, *D. bifascata*, *D. simulans*) and of two lepidopteran species (*Bombyx mori* and *Galleria mellonella*) were infected by *Ecc*15 carrying a pOM1-evf plasmid. *Ecc*15 expressing *evf* were able to persist in all of the tested *Drosophila* species and lethality was apparent after 12 h. By contrast, these bacteria were not able to persist in either *Bombyx* or *Galleria* (data not shown). Collectively, our data indicate that *evf* is a specific Gram-negative virulence factor that promotes colonization to a restricted niche, the gut of *Drosophila* larvae.

Regulation of synthesis and localization of Evf

We have previously shown that *evf* expression requires Hor (Basset *et al*., 2003), a general regulator of virulence in various enterobacteria (Thomson *et al*., 1997). However, no canonical promoter sequence could be identified in the upstream region of *evf*. In order to determine the extent of sequences required for *evf* expression, we fused different fragments of various length from that region (0, 150 and 300 nucleotides) to the *lacZ* gene (Fig. 2A). The activity of these constructs was tested in different genetic backgrounds, i.e. in wt *Ecc*15, in *hor* mutants and in an *E. coli* derivative with *lacZ* deleted. In the control with no fragment inserted upstream of *lacZ*, no β-gal activity was detected in *Ecc*15. While the construct carrying the region extending up to 150 bp gave rise to a low level of β-gal activity, constructs carrying the region extending up to 300 bp upstream of *evf* promoted the highest amount of β-gal activity. This activity was lost in a *hor* mutant indicating that the 300-bp-long region upstream of the *evf* coding sequence contains the information for promoter activity and sites required for Hor regulation. In *E. coli* cells that do not possess the *hor* gene, the level of β-gal activity was similar to that obtained with the *hor* mutant revealing a low Hor-independent promoter activity. As pOM1 derivatives are present at about 10 copies per cell, these results indicate that in *Ecc*15, the wt level of Evf corresponds to a level of about 60 units (u) of β-gal in the exponential growth phase (Fig. 2B) and reached 120 u in the stationary growth phase (data not shown). By extrapolation from results obtained in *E. coli* (Deng *et al*., 2004), these data indicate a steady state abundance of about three copies of RNA per DNA molecule in wt *Ecc*15. From the level obtained in stationary phase in the *hor* mutant carrying

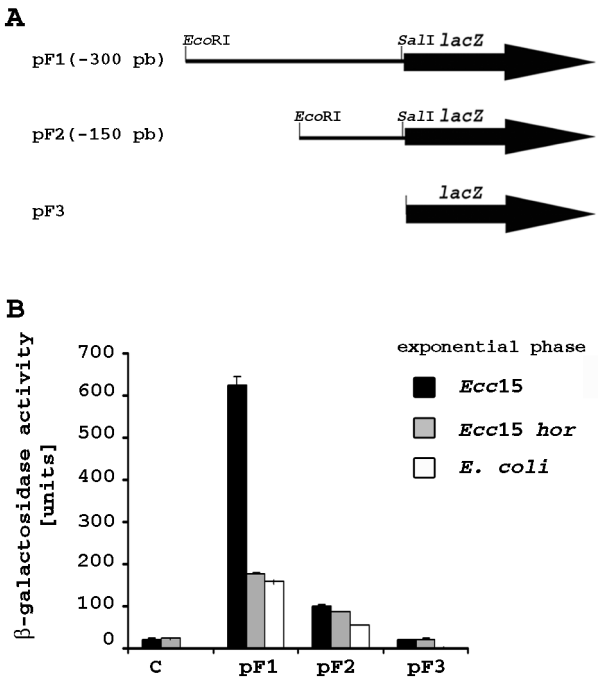


Fig. 2. Level of expression of *evf* required to infect *Drosophila*.
A. Analysis of the *evf* promoter region. Various lengths of the upstream region of *evf* were amplified by PCR and cloned upstream of a *lacZ* reporter gene. The numbers given in brackets denote the size of the *evf* upstream region in the different constructs pF1, pF2 and pF3.
B. Analysis of expression mediated by different regions of the *evf-lacZ* reporter gene in *Ecc*15, *Ecc*15 *hor* mutant and in an *E. coli* derivative deleted for *lacZ*. The β-galactosidase activities represent the mean ± SD of three independent experiments. Control activity (C) corresponds to the background level of the strains.

the construct with the 300-bp-long region upstream of the *evf* coding sequence (400 u, data not shown), we can deduce that a level of 40 u is synthesized from the chromosomal copy of *evf* in the *hor* mutant. As this mutant is not infectious, the minimal expression level required for infection should be greater than 40 u and less or equal to 120 u (the level calculated from a single copy in *Ecc*15 in stationary phase).

Evf activity could not be predicted from its primary sequence. A clue to the Evf action leading to bacterial persistence was the identification of its subcellular localization in the cell. Interestingly, Evf contains a putative transmembrane domain (residues 128–148) predicted to target the C-terminal domain of the protein to the periplasm and the N-terminal domain to the cytoplasm. To verify the predicted localization, we constructed two classes of gene fusions (Fig. 3); one class fuses various parts of *evf* to *lacZ*, the other one various parts of *evf* to *phoA* encoding phosphatase alkaline. β-Gal activity should be detected if Evf is present in the cytoplasm whereas no activity should be found if Evf is associated with the membrane or targeted to the periplasm. By

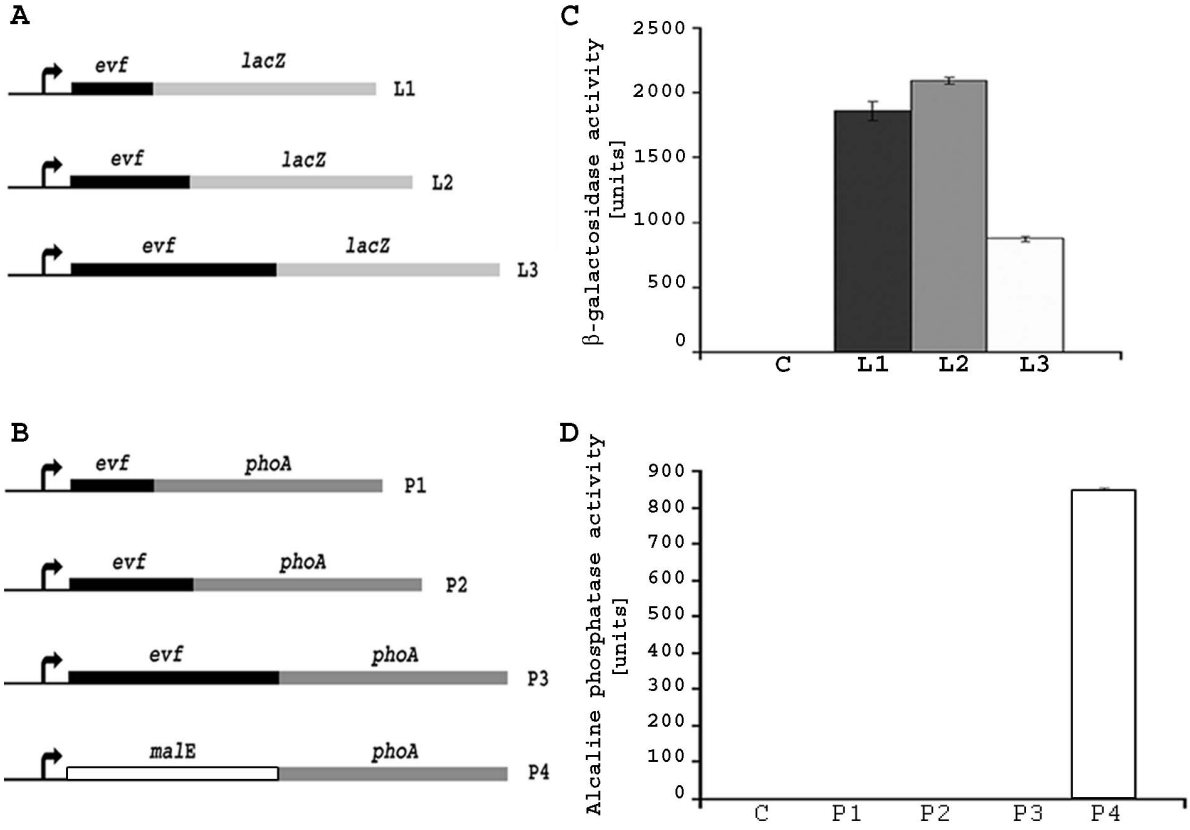


Fig. 3. Cellular localization of Evf.
A and B. Translational fusions of *evf* to *lacZ* (L) and *phoA* (P). L1 and P1 fusions carry the N-terminal region of Evf predicted to have a cytoplasmic localization. The L2 and P2 fusions code for the N-terminal region together with the predicted transmembrane domain of Evf. The L3 and P3 fusions carry the full-length *evf*. The P4 fusion is a control that contains the signal peptide of MalE responsible for the export of MalE into the periplasm. The vectors pMC1403 and pPHO7 were used for the *evf-lacZ* and *evf-phoA* fusions respectively.
C and D. β-Galactosidase and phosphatase alkaline activities of different fusions of *evf* indicate a cytoplasmic localization of Evf. The β-galactosidase and phosphatase alkaline activities represent the mean ± SD of three independent measurements. Control activity (C) corresponds to the background level of the strains carrying the plasmid control.

contrast, PhoA activity would indicate a periplasmic localization of the fusion (Manoil, 2000). *evf-lacZ* fusions all gave rise to β-gal activity regardless the site of fusion in *evf* (Fig. 3C). Conversely, no PhoA activity was detected with the different fusions. A control *malE-phoA* fusion containing the signal for periplasm localization of MalE gave rise to PhoA activity (Fig. 3D). Altogether, the genetic approach predicted that Evf is synthesized in the cytoplasm and no association with the periplasm was found. This result was further confirmed by Western blot analyses using protein extracts from different subcellular compartments and antibodies directed against Evf (data not shown).

Is Evf sufficient to confer infectivity to Gram-negative bacteria?

The ability of *evf* to confer infectivity to different, normally non-persistent, Gram-negative bacteria such as *Pseudomonas* or *Erwinia* species suggested at least two alternative modes of action. First we could imagine that Evf indirectly protects bacteria in the gut of larvae by modifying some metabolic pathways or by activating a transcriptional network that renders the bacterial cells refractory to elimination by the host immune system or other harmful molecules in the gut. Alternatively Evf could exert its action autonomously by modifying directly a host effector participating in bacterial clearance. If the first hypothesis is true, we would expect that several *E. coli* genes are involved in this metabolic pathway and we should be able to isolate *E. coli* mutants expressing *evf* that are unable to infect *Drosophila* larvae. To this end, we constructed an *E. coli* strain carrying a single *evf-lacZ* operon cloned downstream of the P_R promoter integrated in the chromosome (see *Experimental procedures*). The rationale behind the insertion of *lacZ* downstream of *evf* was to directly identify mutants resulting from Tn*10* transposition in *evf*. Three thousand *E. coli*

Table 1. Sensitivity of *Ecc*15 derivatives to various reactive oxygen and nitrogen intermediates.

		Diameter (mm) of growth inhibition zone (mean ± SD)[a]		
Compound	Concentration	*Ecc*15 pOM1	*Ecc*15 *evf* pOM1	*Ecc*15 *evf* pOM1-evf
Paraquat	2%	18.0 ± 0.5	18.0 ± 0.5	18.5 ± 1.0
H_2O_2	250 mM	21.0 ± 0.5	21.0 ± 0.5	21.0 ± 0.5
HOCl	5%	25.0 ± 0.1	25.0 ± 0.5	25.0 ± 0.5
GSNO	1 M	13.0 ± 0.2	12.5 ± 1.0	13.0 ± 0.3
SIN-1	1 M	9.0 ± 0.1	9.0 ± 0.1	9.0 ± 0.1
SNAP	500 mM	10.5 ± 0.2	10.0 ± 0.5	10.5 ± 0.2
Spermin/NONOate	1 M	15.0 ± 0.3	15.0 ± 0.6	17.0 ± 0.5
DETA/NONOate	1 M	20.0 ± 0.6	20.0 ± 0.6	20.0 ± 0.3

a. Growth inhibition zones around 6 mm diameter disks soaked with 10 μl of the different solutions were measured after overnight incubation. The values are the averages of three measurements.

variants were generated using pNKBOR, a mini-Tn*10* derivative (see *Experimental procedures*). These 3000 variants were individually tested in *Drosophila* larvae for the inability to induce a *Diptericin-gfp* fusion, a read-out that correlates with the capacity to infect the host. Among the 3000 variants, two non-infectious variants were identified, which were deficient for β-gal activity, i.e. NKBOR affected directly *evf*. All remaining 2998 lac+ variants were infectious. We concluded from this experiment that genes belonging to a pathway putatively affected by Evf could not be identified using this approach. To directly identify genes whose expression might be modified by the presence of Evf, we compared the transcriptome of *E. coli* cells expressing *evf* and that of wt *E. coli* cells. No specific and reproducible changes of gene expression were detected indicating that Evf does not significantly affect the bacterial transcriptome (data not shown).

We therefore considered the possibility that *evf* could exert its activity directly, for example by detoxifying or neutralizing harmful molecules present in the *Drosophila* gut. A set of tests was developed to determine whether Evf could confer resistance or allow adaptation to hostile conditions, more specifically to reactive oxygen species (ROS), acid or alkaline stress, ethanol stress, osmotic stress, or resistance to trypsin or lysozyme treatment. *Ecc*15 strains behaved similarly to all reactive nitrogen and oxygen intermediates conditions independently of the presence or of the absence of *evf* expression (Table 1). It is interesting to note that *Ecc*15 was more susceptible to paraquat, and as susceptible to H_2O_2 or HOCl as *P. putida* that does not persist in the larval gut. These results suggest that although oxidative stress plays an important role in clearance of bacteria in the gut of adult insects (Ha *et al*., 2005a,b), Evf does not act in larvae by counteracting this eradication process. Similarly, no significant differences were detected for the other types of stress (data not shown). Altogether, our results support the idea that Evf is a direct effector promoting persistence, while this effect cannot be explained simply by a protection against a chemical stress.

Nature of the activity of Evf

*Ecc*15 do not persist in wt *Drosophila* larvae and the number of bacteria after 24 h is reduced by a factor 10^3 (Basset *et al*., 2000). In contrast, the number of bacteria was reduced only 10-fold in *imd*-deficient *Drosophila* lines unable to induce an antibacterial response, remaining high at a level of 10^5 and 10^6 after 24 h (Basset *et al*., 2000 and Fig. 4A). This demonstrates a role for Imd pathway-dependant immune responses in the control of *Ecc*15. To determine whether Evf activity antagonizes early events of the host antibacterial response, we monitored the persistence of *Ecc*15 *evf* mutants in Imd-deficient *Drosophila* larvae (Fig. 4A). In the absence of a functional Imd pathway, *evf* mutants were not able to persist. This indicates that Evf does not promote persistence by directly counteracting the Imd-dependant immune response. Of note, *E. coli* cells lacking *evf* do not persist in *Relish* flies after oral infection (data not shown). Altogether, this indicates that Evf does not target the larval antimicrobial peptide defence.

It is generally assumed that the *Drosophila* gut constitutes a hostile environment unfavourable for bacterial persistence. To determine whether Evf affects persistence in this environment, we isolated the gut immediately after ingestion of bacteria synthesizing GFP, maintained them in a physiological buffer and followed the persistence of fluorescent bacteria up to 24 h. Strikingly, under these conditions, *evf* mutants persisted as well as bacteria expressing *evf* (Fig. 4B). These results therefore indicate that elimination of bacteria requires the maintenance of the gut in the body of the larvae and may involve complex physiological properties such as peristaltic flushing.

Antagonizing the peristaltic movements or other mechanical processes eliminating ingested microbes

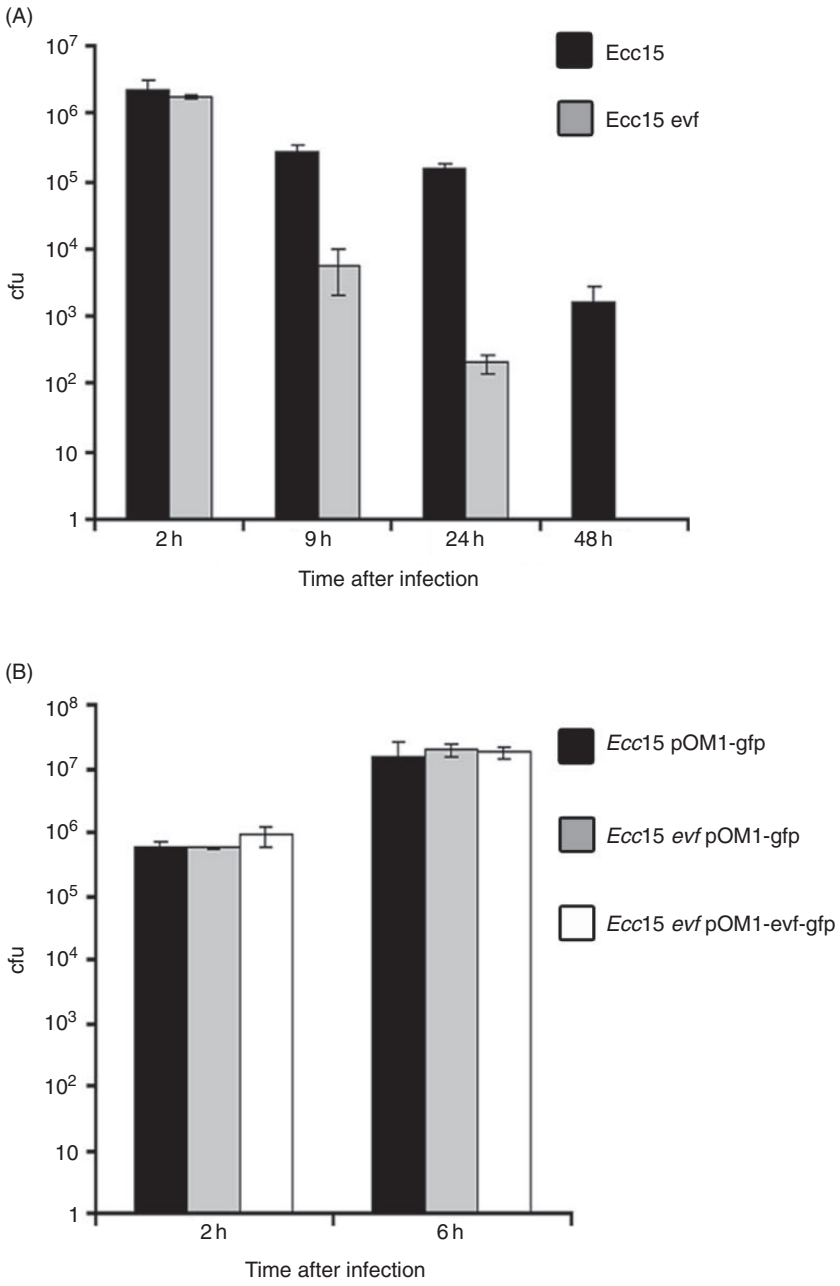
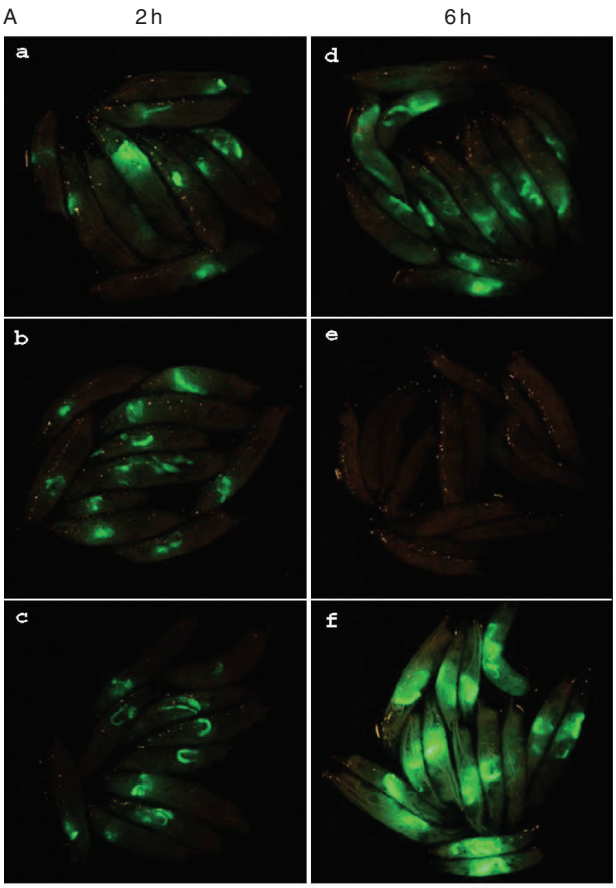


Fig. 4. Persistence of *evf* mutant bacteria in *Relish* larvae and isolated gut preparations.
A. Persistence of *Ecc*15 *evf* mutant in *Relish* larvae. Bacterial persistence was measured by plating appropriate dilutions of homogenates of five surface-sterilized larvae that were naturally infected with rifampicin-resistant strains of *Ecc*15 and *Ecc*15 *evf* mutant. Bacterial counts were obtained by plating the larval homogenates on LB agar containing 100 μg ml^{-1} of rifampicin. The number of colony forming units (cfu) per larva obtained at each point after infection represents the mean of three independent measurements.
B. Persistence of *Ecc*15 *evf* mutants in isolated gut. Bacterial persistence was measured in isolated guts of wt larvae by plating appropriate dilutions of homogenates of five isolated guts of larvae that were naturally infected with *Ecc*15 pOM1-gfp, *Ecc*15 *evf* pOM1-gfp and *Ecc*15 *evf* pOM1-evf-gfp. Larvae were orally infected by bacteria, and, at 2 h post infection, the gut of larvae were dissected and incubated in Schneider medium. Bacterial counts were obtained by plating the gut homogenates collected at 2 and 6 h post infection on LB agar containing 100 μg ml^{-1} of spectinomycin. The number of cfu per gut obtained at each point after infection represents the mean of three independent measurements.

should allow the persistence of other bacteria *in trans*. To test this hypothesis, we performed a set of oral infections using bacterial mixtures containing either fluorescent *Ecc*15 alone, fluorescent *Ecc*15 *evf* mutant alone, or *Ecc*15 together with fluorescent *Ecc*15 *evf* mutant bacteria (Fig. 5). As expected, fluorescence was still detected 6 h after ingestion of *Ecc*15 whereas no fluorescence was detected at the same time with fluorescent *evf*- bacteria (Fig. 5A). Remarkably, 6 h after ingestion, fluorescence was evident when fluorescent *evf* mutants were coinfected with *Ecc*15. These results indicate that Evf antagonizes directly or indirectly the processes responsible for bacterial eradication. Plating bacteria isolated from infected gut allowed direct counting of bacteria and confirmed observations of GFP fluorescence, because the titre of *Ecc*15 *evf* mutant bacteria was reduced by a factor 10^3 6 h post infection (Fig. 5B), while this level remained high in the presence of *evf*-expressing bacteria. Similar effects of Evf on persistence *in trans* were obtained when *Erwinia* strains were substituted by *E. coli* strains (data not shown). Altogether, our results indicate that *evf*-expressing bacteria



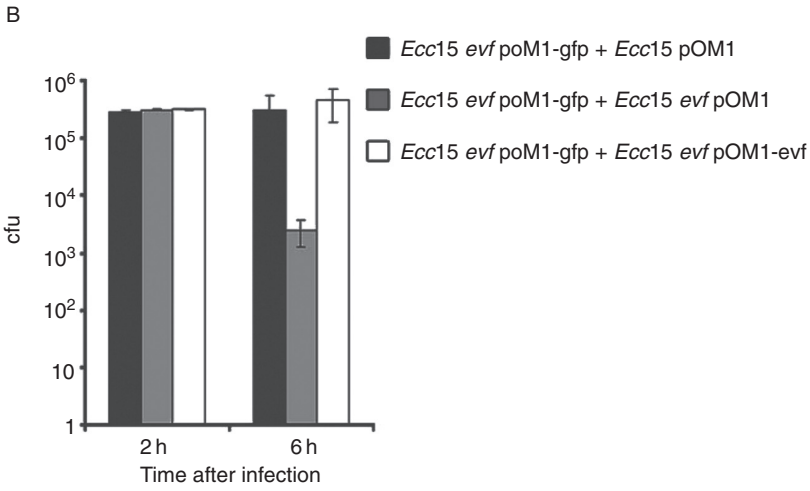


Fig. 5. Persistence of non-infectious bacteria by *evf*-expressing bacteria.
A. Persistence of *evf* mutants coinfected with bacteria expressing *evf* in wt larvae. Larvae were photographed at 2 h (a, b, c) and 6 h (d, e, f) post infection. (a and d) GFP expressing bacteria in larvae infected with a mixture of *Ecc*15 pOM1 + *Ecc*15 *evf* pOM1-GFP (b and e) larvae infected with a mixture of *Ecc*15 *evf* and *Ecc*15 *evf* carrying pOM1-gfp, and (c and f) larvae infected with a mix of *Ecc*15 *evf* pOM1-evf and *Ecc*15 *evf* pOM1-gfp.
B. Bacterial counts were obtained by plating the larval homogenates of five surface-sterilized larvae that were naturally infected with the mixtures of bacteria on LB medium containing spectinomycin (100 μg ml^{-1}). The number of colony forming units (cfu) per larva obtained at each point after infection represents the mean of three independent measurements.

promoted efficient persistence of non-infectious bacteria *in trans*.

Overexpression of evf *promotes bacterial accumulation in the gut and induces lethality*

*Ecc*15 bacteria carrying *evf* under the control of the promoter Pro3 in a pSC101 plasmid derivative (Espeli *et al*., 2001) express *evf* about 50 times more efficiently than wt *Ecc*15. Under these conditions, bacteria were able to persist for a longer period and at a higher level than wt *Ecc*15 (more than 10 times, Basset *et al*., 2003). By using a derivative expressing *gfp*, we were able to visualize bacterial accumulation predominantly in the gut (Fig. 1 and Basset *et al*., 2003). To observe in greater detail the consequences of accumulating *Ecc*15 derivatives in the

gut, we performed histological analyses at different time points following ingestion of bacteria. Electron and optic micrographs of transversal sections of larval midguts revealed a high accumulation of bacteria in the gut lumen delimited by the peritrophic matrix (Fig. 6C). At 2 h 30 min after infection, the mucus that protects the digestive epithelium was absent in larvae infected with *Ecc*15 pOM1-evf (Fig. 6C and D) compared with the non-infected control (Fig. 6A and B). At time 6 h, the gut lumen was filled with bacteria that appeared in regular arrangement, the peritrophic matrix lining the epithelial cells was intact, and high quantities of cellular material seemed to be present in the space between the epithelial cells and the peritrophic matrix (Fig. 6F–H).

The high numbers of bacteria in the gut revealed the ability of Evf to promote bacterial persistence at least in this part of the animal. To determine the effect of Evf on the outcome of an infection in other tissues, bacteria overexpressing *evf* were injected directly in the haemocoele of adult flies and larvae, which did not lead to apparent lethality (data not shown). These results therefore indicated that *evf*-conferred lethality relies on persistence in the gut.

Discussion

Evf was initially identified as a virulence factor that promotes bacterial colonization of the *Drosophila* gut, yet also triggers a systemic immune response. A number of reports have documented the role of insects in general and *Drosophilidae* in particular in the dissemination of phytopathogenic bacteria such as *Erwinia carotovora* (Kloepper, 1981). The finding that *evf* was found only in a subset of *Erwinia* strains that have infectious properties towards *Drosophila* suggested that *evf* is an example of a gene that promotes survival and dissemination of bacteria in their environment. The goal of the present study was to analyse how Evf promotes colonization and persistence of bacteria inside the *Drosophila* larval gut. Our data show that Evf is not a toxin or a factor that antagonizes a specific host reaction of *Drosophila*. Rather, expression of this gene promotes the accumulation of bacteria in the anterior midgut dramatically affecting gut physiology. Interestingly, our study reveals unexpected reminiscences between the mechanisms of Evf mediated colonization of the *Drosophila* gut and the flea gut blockage induced by the plague agent *Yersinia pestis* (Hinnebusch *et al*., 2002).

In most cases, Gram-negative bacteria do not persist in the gut of *Drosophila*, indicating that the intestine is a rather hostile environment for invading bacteria (Ha *et al*., 2005b). The mechanisms involved in bacterial clearance from the gut are poorly characterized, although recent studies have pointed towards a role of antimicrobial peptides and ROS in *Drosophila* adults. We have recently shown that the local expression of antimicrobial peptides in the gut, but not their systemic expression by the fat body, limits the growth of the entomopathogenic bacteria *P. entomophila* in *Drosophila* (Liehl *et al*., 2006). This illustrates the importance of Imd-mediated antibacterial responses in the gut against orally ingested bacteria. *P. entomophila* counteracts this response by secreting an zinc metalloprotease, AprA, which degrades antimicrobial peptides. A role for AprA in protection against the Imd-dependent immune response was supported by the observation that AprA was required to promote persistence in wt flies but was dispensable in a *Relish* background in which antimicrobial peptides active against Gram-negative bacteria are not produced. In contrast to the situation described above, we observed that *evf*-deficient bacteria did not persist in either wt or *Relish* mutant flies. This indicates that Evf does not provide any protection against antimicrobial peptides. This is corroborated by the observation that *evf* expressing bacteria did not resist against the *Drosophila* antimicrobial response when injected directly into the body cavity. Whereas a normal level of *evf* expression in *Ecc*15 does not induce larval lethality, overexpression on a multicopy plasmid (up to 50-fold) induces lethality within 6 h, a time frame too rapid for an efficient response involving antimicrobial peptides.

Natural gut infection has been associated with the rapid synthesis of ROS by the host, and the dynamics of ROS generation and elimination appears to be vital in *Drosophila* because flies that lack the capacity for ROS removal show an increased mortality after feeding with non-pathogenic bacteria (Ha *et al*., 2005b). Pathogenic bacteria are known to use specific responses to resist host ROS including the expression of detoxifying enzymes such as catalase (Harris *et al*., 2003). It was therefore tempting to predict a role for Evf in protection against ROS. However, *in vitro* assays failed to demonstrate a detoxifying activity against various chemicals including ROS or NO. The lethality induced upon infection by *Ecc*15 overexpressing *evf* was not suppressed in a fly line overexpressing a catalase (data not shown). It is interesting to note that *Ecc*15 bacteria do not appear to be more resistant to ROS species such as paraquat, hypochlorite or superoxide than naturally non-infectious bacteria, e.g. *P. putida* (C. Acosta Muniz, unpublished data).

Importantly, our observations that (i) *evf* mutants persisted in isolated guts equally well as wt *Ecc*15 and (ii) *evf*-expressing bacteria can exert an effect *in trans* on other bacteria do not support the hypothesis that Evf might be involved in the detoxification of compounds operative in gut bacterial clearance. Rather, persistence of *evf* mutants in isolated guts revealed that the elimination process requires the presence *in situ* of the gut in the

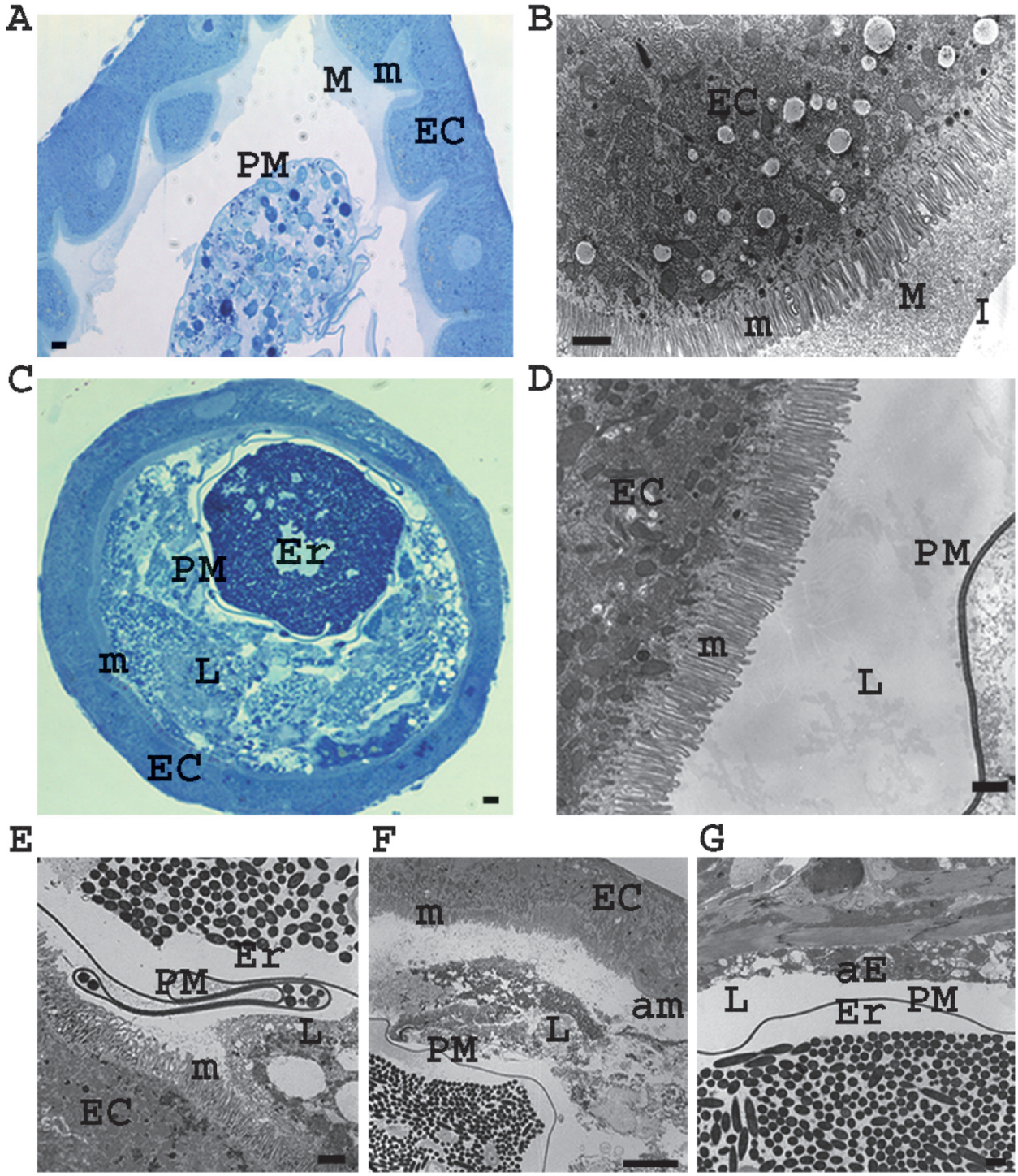


Fig. 6. e*vf* overexpression in *Ecc*15 provokes a strong perturbation of the *Drosophila* larval gut. Transversal sections of larval anterior midguts collected at 2 h 30 min (A and D) or 6 h (B–C, E–G) after natural infection with *Ecc*15 *evf* pOM1-evf (C–G) or control (A, B) were analysed. (A, C) Semi-thin sections were observed under bright field. (B, D–G) Ultra-thin sections were observed by TEM. At 2 h 30 min and 6 h after infection, the mucus that protects the digestive epithelium was absent (compare B with D and E). At 6 h after infection, the bacteria accumulated in the gut and the peritrophic matrix is not altered (C and E–G). At this time, the epithelial cells displayed abnormal microvilli and cellular material seemed to be present in the space between the epithelial cells and the peritrophic matrix (compare D with E and F). aE, epithelial cell absence; am, abnormal microvilli; EC, epithelial cell; Er, *Ecc*15 *evf* pOM1-evf; L, lumen; m, microvilli; M, mucus; PM, peritrophic matrix. (Scale bar; A, C and G, 10 μm; B- D, 3 μm; E, F and H, 2 μm).

animal body. In addition, the effect of Evf is specific for gut persistence in larvae as no lethality was apparent after direct injection into the body cavity of bacteria overexpressing *evf* (our unpublished data). This indicates that Evf mediated persistence is specific to the physiology and architecture of the *Drosophila* larval gut.

To gather insight into the activity of Evf, we took advantage of the strong effect provoked by the 50-fold overexpression of *evf*. This overexpression leads to a high level of bacterial accumulation inside the gut lumen. Although still being contained by the peritrophic matrix, a disappearance of mucus at the apical side of the epithelial cells and the appearance of cellular debris between epithelial cells and the peritrophic matrix can be observed. Ultimately, death of the larvae occur within 6–12 h following ingestion. Remarkably, microscopic analyses indicated that this large accumulation of bacteria does not break the peritrophic matrix and no direct contacts are apparent between bacterial and epithelial cells. The integrity of the peritrophic matrix despite the high bacterial load corroborates our previous observations that revealed spreading of bacteria across the gut barrier only in a minor fraction of larvae (Basset *et al*., 2003). We also noticed that the bacterial distribution was not random but rather seemed to follow a specific ordered arrangement that is reminiscent of an organized bacterial community such as a biofilm. Altogether, these results indicate that Evf activity may allow access of bacteria to a specific location, such as the proventriculus, by interfering locally with gut peristalsis. This colonization/proliferation hypothesis is reminiscent of the association of *Y. pestis* with its flea vector. Transmission of plague by fleas depends on infection of the proventriculus by a dense aggregate of *Y. pestis* cells organized in a biofilm that blocks normal blood feeding (Hinnebusch *et al*., 1996). From this point of view, it is striking that other entomopathogens such as *P. entomophila* and *Serratia entomophila* also induce an anti-feeding reaction that perturbs the physiology of the larval gut (Hurst *et al*., 2000; 2004; Vodovar *et al*., 2005). Together, these studies suggest that blockage of gut peristalsis, and thus food bolus movement, may be a common strategy used by entomopathogenic bacteria to circumvent elimination from the insect gut, although the molecular mechanisms may vary. Food movement through the gut is, of course, a necessary biological feature of the digestive process, yet may also be viewed as an important process for the elimination of potential pathogenic organisms. Microorganisms possessing a means to persist in the alimentary tract are thus at a natural advantage to avoid natural elimination.

In the absence of a direct functional homologue in another species or a tertiary structural protein signature, the nature of the molecular function of Evf still remains elusive. Our results indicate that Evf accumulates in the cytoplasm and its activity confers infectious properties only to Gram-negative bacteria. The ability of Evf to allow persistence in bacteria as diverse as *E. coli* or *Pseudomonas* species suggests that this protein plays a direct role in gut persistence of bacteria. It should be noted that only one similar open reading frame (ORF) can be identified in the databases upon a Psi BLAST search, which is *plu*2433 of the entomopathogen *Photorhabdus luminescens* TT01 (Duchaud *et al*., 2003). Although no evidence exists to date suggesting a role of PLU2433 in virulence, it is interesting that during the natural life-cycle of *P. luminescens,* the bacteria exist in a symbiotic relationship within the intestine of *Heterorhabditis* nematodes reinforcing the notion that these proteins may be involved in specific gut interactions.

In agreement with a direct role of Evf, the absence of any effects on the *E. coli* transcriptome and our inability to identify a suppressor gene should be noted. The localization of the protein in the cytoplasm as well as the low transcriptional level of *evf* detected in *Ecc*15 suggests that Evf does not function as a toxin. An attractive hypothesis is that Evf may interact with other proteins endogenous to many Gram-negative bacteria resulting in a modification of the bacterial cell structure that subsequently allows bacterial persistence and the formation of aggregates. This may promote the formation of a biofilm, as our microscopic data indicate, and/or the specific attachment to a receptor within the gut, such as chitin. The host range specificity of Evf for Gram-negative bacteria and the absence of a suppressor of *evf* indicate that Evf mediates its effect by itself or affect an essential cellular structure conserved among these types of bacteria such as components of the cell wall. Several colonization factors have been shown to be enzymes that modify the bacterial cell envelope. An example is the *pagP* locus of *Bordetella bronchiseptica* that encodes a palmitoyl transferase that modifies lipid A as part of the adaptation of this organism required for persistent infection (Preston *et al*., 2003).

Infectious strategies of several pathogenic bacteria involve the manipulation of the host immune response. Many human pathogenic bacteria species trigger excessive inflammatory reactions that damage host tissues. A specific feature of bacteria expressing *evf* is the induction of both local and systemic host immune responses. The mechanisms that link Evf to immune activation are not yet fully understood. However, recent studies suggest that this effect may be a consequence of bacterial colonization rather than a direct effect of Evf *per se*. In support of this hypothesis, neither ingestion nor injection of pure Evf protein has an effect on host viability or the immune response (data not shown). We and others have also recently shown that peptidoglycan recognition proteins (PGRPs) with amidase activity degrade the peptidoglycan of Gram-negative bacteria and prevent the host immune

Table 2. Bacterial strains and plasmids.

Strains, plasmids	Description	Source or reference
Bacterial strains		
Erwinia carotovora 15 (*Ecc*15)	Wild type	Basset *et al*. (2003)
*Ecc*15 *evf*	evf::Tn*10* (Kan^R)	Basset *et al*. (2003)
Escherichia coli K12 MG1655	Wild type	Lab collection
MG1656	Wild type Δ lac *MluI*	Espeli *et al*. (2001)
CC118	*araD139Δ(ara, leu)7697 ΔlacX74 phoA-20 galE galK thi rpsE rpoB argE(Am) recA1*	Manoil and Beckwith (1985)
Pseudomonas aeruginosa PAO1	Wild type	Gallagher *et al*. (2002)
Pseudomonas putida KT2440	Wild type	Laboratory collection
Salmonella typhimurium LT2	Wild type	Laboratory collection
Bacillus subtilis 168	Wild type	Laboratory collection
Streptococcus faecalis JH2-2	Wild type	Laboratory collection
Bacillus megaterium	Wild type	Laboratory collection
Plasmids		
pOM1	Cloning vector pSC101 derivative (Spc^R)	Espeli *et al*. (2001)
pOM3	pOM1 expressing *lacZ*	Espeli *et al*. (2001)
pOM1-GFP	pOM1 expressing *gfp*	Basset *et al*. (2003)
pOM1-evf-GFP	pOM1 expressing *evf* and *gfp*	Basset *et al*. (2003)
pX2-GFP	pX2 expressing *gfp*	Vodovar *et al*. (2005)
pX2-evf-GFP	pX2 expressing *gfp* and *evf*	This study
pDG148-GFP	pDG148 expressing *gfp*	Joseph *et al*. (2001)
pDG148-evf-GFP	pDG148 expressing *gfp* and e*vf*	This study
pMC1403	ColE1, *lacZ* gene fusion vector	Casadaban *et al*. (1980)
pPHO7	phoA gene fusion vector	Gutierrez and Devedjian (1989)
pNKBOR	R6K derivative carying a mini-Tn*10*-based transposon	Rossignol *et al*. (2001)
pHK11-Amp	HK022-based integrative vector	Rossignol *et al*. (2002)
pHK-int	pSC101 derivative expressing integrase	Rossignol *et al*. (2002)
pHK11-pR-evf-lacZ	pHK11-Amp carrying P_R-evf-lacZ	This study

response of flies to the presence of ingested bacteria in the gut (Bischoff *et al*., 2006; Zaidman-Rémy *et al*., 2006). This activity might be a natural host mechanism allowing the establishment of a tolerance threshold level of bacteria in the gut, presumably present in natural ingested food, thereby avoiding over-stimulation of the immune response under normal conditions. Moreover, it was also proposed that the systemic immune response induced by persistent and infectious bacteria such as *Ecc*15 is mediated by the translocation of small peptidoglycan fragments from the gut lumen to the haemolymph. Our data are compatible with a model in which bacterial persistence in the gut leads to a local increase of the peptidoglycan concentration that exceeds the host tolerance level and results in stimulation of the immune system. This is consistent with our observation that Evf does not promote crossing of the gut barrier by bacteria. As peptidoglycan is expected to be able to cross the peritrophic matrix, it remains intriguing that high titres of ingested Gram-negative bacteria like *E. coli* do not induce the immune response although high numbers of bacteria are still present in the gut several hours after ingestion. The paradoxical absence of immune response activation despite the presence of high numbers of cells at early time points after ingestion indicates that triggering of the immune response by infectious bacteria such as *Ecc*15 either requires bacterial proliferation and detection of *de novo* synthesized peptidoglycan compounds or alternatively depends on detection of peptidoglycan molecules in a specific compartment of the gut.

Further work will be necessary to identify the exact biological activity of Evf and to determine whether gut tissues in *Drosophila* constitute the site of initial bacterial colonization and which are the cell types involved in bacterial recognition.

Experimental procedures

Drosophila *stocks*

Oregon^R (Or^R) flies were used as a standard wt strain. *Relish^{E20}* flies carry a null mutation in *Relish* that encodes the transactivator regulated by the Imd pathway (Hedengren *et al*., 1999). *Drosophila* stocks were maintained at 25°C.

Bacterial strains

The strains used in this study are listed in Table 2. Bacteria were cultured in Lennox medium with the appropriate antibiotics (100 μg ml^{-1} rifampicin; 100 μg ml^{-1} ampicillin; 300 μg ml^{-1} carbenicillin; kanamycin 50 μg ml^{-1} and 100 μg ml^{-1} spectinomycin). The rifampicin-resistant *Ecc*15, *Ecc*15 derivatives and *Pseudomonas* were grown at 29°C. *E. coli* strains were grown at 37°C if not otherwise indicated.

Chemicals

Analytical grade H_2O_2, paraquat and NaClO, were purchased from Sigma. Diethylenetriamine (DETA) NONOate (±)-S-Nitroso-

N-acetylpenicillamine (SNAP), S-Nitrosoglutathione (GSNO), SIN-1 Hydrochloride and Spermine NONOate were a kind gift from Jean-Claude Drapier (Institut de Chimie des Substances Naturelles, CNRS, Gif-sur-Yvette) and purchased from Calbiochem.

Drosophila *natural bacterial infection*

Approximately 200 third-instar larvae were placed in a 2 ml tube containing 200 μl of concentrated bacteria pellet (OD_{600} = 200) from an overnight culture and 400 μl of crushed banana. The larva, bacteria and banana were thoroughly mixed in the microfuge tube; the tube was closed with a foam plug, incubated at room temperature for 30 min, and the mix was then transferred to a standard corn-meal fly medium and incubated at 29°C. For bacterial counting experiments, the infected larvae were first rinsed in water and transferred to a fresh fly medium at 2 h after infection. Counting procedures were performed with larvae rinsed in water and dipped in 70% ethanol (three times for 5 s) for external sterilization and then homogenized and spread onto Luria–Bertani (LB) plates containing the required antibiotic at each different time point. For bacterial counting in isolated guts, larvae were dissected 2 h after infection in *Drosophila* Schneider medium after ethanol sterilization, and the guts were placed in Schneider medium with 10% bovine serum, homogenized and spread onto LB plates containing antibiotics at each time point. Bacterial injections of adults were performed by pricking adults in the thorax with a thin needle previously dipped into a concentrated pellet of a bacterial culture (OD_{600} = 200).

Transmission electron microscopy (TEM)

Infected *Drosophila* larvae were dissected in Schneider medium, and the guts were immediately fixed with 2.5% glutaraldehyde, 1% paraformaldehyde, 1% potassium ferrocianide solution in 0.1 M cacodylate buffer, pH 7.4, for 80 min at room temperature. Dehydration of the guts was performed in an ascending series of ethanol concentrations, and then the samples were embedded in Epon 812. The guts were cut at 0.5 μm semi-thin sections for light microscopy or 60 nm ultra-thin sections for TEM with a Leica Ultramicrotome. Semi-thin sections were stained with methylene blue and Azur II and observed under an Axiophot Zeiss microscope. Ultra-thin sections were contrasted with uranyl acetate and lead citrate and observed with a Philips 208 electron microscope.

Microscopic observations of larvae

Live larvae infected with GFP expressing bacteria were anesthetized on ice and viewed under epifluorescent illumination (excitation filter 480/40 nm; dichroic filter 505 nm; emission filter 510 nm) with a Leica (Heerburg, Switzerland) MZFLIII dissecting microscope. Images were recorded with a charge-coupled device camera (Nikon).

Midgut organs cultures

Midguts were isolated from infected *Drosophila* and incubated in Schneider *Drosophila* Medium (Gibco) supplemented with 10% fetal bovine serum (Biomedia).

DNA manipulations

All DNA manipulations, restriction digestions, ligations and transformations were performed using standard genetic and molecular techniques (Sambrook *et al*., 1989; Miller, 1992). Plasmid DNA was purified using a Quiagen kit. Restriction and DNA modifying enzymes were obtained from Boehringer Mannheim or New England Biolabs and used according to the manufacturers' instructions. PCR reactions were performed in a 50 μl mix for 30 cycles using Phusion High-fidelity DNA polymerase (Finnzymes) according to the manufacturer's instructions in a DNA thermal cycler PTC-100 (MJ Research). PCR products were purified with the QIAquik kit (Qiagen) before and after digestion of the amplification product.

Plasmids used in this study are listed in Table 2. pF1 and pF2 were constructed by inserting PCR fragments harbouring different portions of the *evf* promoter region into pOM3 (Espeli *et al*., 2001) cut with EcoRI and SalI. Plasmid pF3 was constructed by deletion of the EcoRI-SalI fragment of pOM3 (Fig. 2A). The PCR products were generated using the downstream primer 5′-ATGCTAGTCGACAATCACTCCTATT GTGGTGG-3′ and the upstream primers 5′-ATGCTAGAATT CATTTACTCACGAAAAATT-3′ (pF1) and 5′-ATCGATGAATT CTATCTTTAATTATGGTTA-3′ (pF2) and cut with SalI and EcoRI.

Constructions of evf-lacZ *and* evf-phoA *gene fusions*

L1-L3 fusions (Fig. 3) were constructed by inserting different regions of *evf* gene amplified by PCR using the plasmid pOM1-evf (Basset *et al*., 2003) into pMC1403 (Casadaban *et al*., 1980) cut with EcoRI. The PCR products were generated using the upstream primer 5′-GGAATCTAGACATTCAGTTCGCTGC-3′ and the downstream primers 5′-ATCGACGAATTCCTTTGG CTACTTCAACGCCTTTTAC-3′ (L1), 5′-ATCGACGAATTCCGG TTTTATTCATTTCGGCACTTAAACC-3′ (L2) and 5′-ATCGAC GAATTCCATATACATAATTTTTATTTGG-3′ (L3) and cut with EcoRI (located in the upstream region of *evf* and in the downstream primers). P1-P3 fusions were constructed by inserting PCR fragments harbouring different portions of *evf* gene into pPHO7 (Gutierrez and Devedjian, 1989) cut by HindIII and using the plasmid pOM1-evf like template. The PCR products were generated using the upstream primer 5′-ATCGACAAG CTTGAATTCGAGCTCGGTACCCCC-3′ and the downstream primers 5′-ATCGACAAGCTTTTGGCTACTTCAACGCCTTT-3′ (P1), 5′-ATCGACAAGCTTTTATTCATTTCGGCACTTAA-3′ (P2) and 5′-ATCGACAAGCTTACATAATTTTTATTTGGCTT-3′ (P3). P4 fusion was constructed by inserting a PCR fragment of *mal*E gene, that contains the signal peptide for the exportation to the periplasm, in pPHO7 cut by HindIII. The PCR product was generated using the same upstream primer that in the other *phoA* fusions and the downstream primer 5′-ATCGACAAGCTTTT AGTCTGCGCGTCTTTCAGGGC-3′. The PCR products were digested with HindIII.

Integration of the P_R-evf-lacZ *operon in the* E. coli *chromosome*

First, the P_R-*evf-lacZ* construction was made as follows. The plasmid pOM1-evf was digested by EcoRI and HindIII, the 1 kb

fragment was ligated in the pHK11-Amp (Rossignol *et al*., 2002) digested with EcoRI and HindIII. The P_R promoter was cloned as a double strand oligonucleotide in the pHK11-evf digested with EcoRI and PstI. The *lacZ* gene was obtained from pOM3 digested with SalI, filled in with Klenow fragment and digested with HindIII. *lacZ* was subsequently cloned in the pHK11-P_R-evf digested with NcoI, filled in by Klenow fragment and digested with HindIII. For integration of P_R-*evf-lacZ* operon in the *E. coli* chromosome, we used the system which allows the insertion of DNA by site-specific integration into the bacteriophage HK022 bacterial attachment site (Rossignol *et al*., 2002).

Random mutagenesis of the 'E. coli pR-evf-lacZ'

For the construction of mutants we used a mini-Tn*10* based transposon NKBOR as described previously (Rossignol *et al*., 2001).

Transcriptome analysis of E. coli *strains carrying pOM1 and pOM1-evf*

Total RNAs of strains containing pOM1 and pOM1-evf were extracted from exponential cultures as described before (Espeli *et al*., 2001). cDNAs produced from total RNA isolated from *E. coli* MG1655 Δ*lacIZ* carrying pOM1 or pOM1-evf strains were Cy-3 and Cy-5 labelled respectively, and hybridized on *E. coli* DNA chips carrying the complete set of *E. coli* ORFs. DNA chips were prepared in the Gif/Orsay DNA Microarray Platform (GODMAP).

β-Galactosidase and alkaline phosphatase assays

β-Galactosidase and alkaline phosphatase activities were measured in permeabilized cells as described previously (Miller, 1992; Manoil, 2000). β-Galactosidase and alkaline phosphatase assays were performed in triplicate.

Acknowledgements

We thank I. Attree, S. Gruss, C. Manoil, Ph. Bouloc, P. Serror, J.-L. Drapier for providing bacterial strains and reagents, A. Jacq for helpful discussions, Christoph Scherfer, Peter Liehl and Sean Kennedy for critical reading of the manuscript and Brigitte Maroni and Michèle Valens for technical help. C.A. was supported by a doctoral fellowship from CONACYT (Mexico). The laboratories of F. B and B. L. were funded, respectively, by CNRS and by CNRS, the Agence Nationale de la Recherche, the Schlumberger and Bettencourt Foundations and the ACI Microbiologie.

References

Basset, A., Khush, R.S., Braun, A., Gardan, L., Boccard, F., Hoffmann, J.A., and Lemaitre, B. (2000) The phytopathogenic bacteria *Erwinia carotovora* infects *Drosophila* and activates an immune response. *Proc Natl Acad Sci USA* **97:** 3376–3381.

Basset, A., Tzou, P., Lemaitre, B., and Boccard, F. (2003) A single gene that promotes interaction of a phytopathogenic bacterium with its insect vector, *Drosophila* melanogaster. *EMBO Rep* **4:** 205–209.

Bischoff, V., Vignal, C., Duvic, B., Boneca, I.G., Hoffman, J.A., and Royet, J. (2006) Downregulation of the *Drosophila* immune response by peptidoglycan-recognition proteins SC1 and SC2. *PLoS Pathog* **2:** e14.

Casadaban, M.J., Chou, J., and Cohen, S.N. (1980) In vitro gene fusions that join an enzymatically active beta-galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translational initiation signals. *J Bacteriol* **143:** 971–980.

Chugani, S.A., Whiteley, M., Lee, K.M., D'Argenio, D., Manoil, C., and Greenberg, E.P. (2001) QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **98:** 2752–2757.

Deng, S., Stein, R.A., and Higgins, N.P. (2004) Transcription-induced barriers to supercoil diffusion in the *Salmonella typhimurium* chromosome. *Proc Natl Acad Sci USA* **101:** 3398–3403. Epub 2004 March 3391.

Duchaud, E., Rusniok, C., Frangeul, L., Buchrieser, C., Givaudan, A., Taourit, S., *et al.* (2003) The genome sequence of the entomopathogenic bacterium *Photorhabdus luminescens*. *Nat Biotechnol* **21:** 1307–1313. Epub 2003 October 1305.

Espeli, O., Moulin, L., and Boccard, F. (2001) Transcription attenuation associated with bacterial repetitive extragenic BIME elements. *J Mol Biol* **314:** 375–386.

Flyg, C., Kenne, K., and Boman, H.G. (1980) Insect pathogenic properties of *Serratia marcescens*: phage-resistant mutants with a decreased resistance to Cecropia immunity and a decreased virulence to *Drosophila*. *J Gen Microbiol* **120:** 173–181.

Gallagher, L.A., McKnight, S.L., Kuznetsova, M.S., Pesci, E.C., and Manoil, C. (2002) Functions required for extracellular quinolone signaling by *Pseudomonas aeruginosa*. *J Bacteriol* **184:** 6472–6480.

Gutierrez, C., and Devedjian, J.C. (1989) A plasmid facilitating in vitro construction of phoA gene fusions in *Escherichia coli*. *Nucleic Acids Res* **17:** 3999.

Ha, E.M., Oh, C.T., Bae, Y.S., and Lee, W.J. (2005a) A direct role for dual oxidase in *Drosophila* gut immunity. *Science* **310:** 847–850.

Ha, E.M., Oh, C.T., Ryu, J.H., Bae, Y.S., Kang, S.W., Jang, I.H., *et al.* (2005b) An antioxidant system required for host protection against gut infection in *Drosophila*. *Dev Cell* **8:** 125–132.

Harris, A.G., Wilson, J.E., Danon, S.J., Dixon, M.F., Donegan, K., and Hazell, S.L. (2003) Catalase (KatA) and KatA-associated protein (KapA) are essential to persistent colonization in the *Helicobacter pylori* SS1 mouse model. *Microbiology* **149:** 665–672.

Hedengren, M., Asling, B., Dushay, M.S., Ando, I., Ekengren, S., Wihlborg, M., and Hultmark, D. (1999) Relish, a central factor in the control of humoral but not cellular immunity in *Drosophila*. *Mol Cell* **4:** 827–837.

Hinnebusch, B.J., Perry, R.D., and Schwan, T.G. (1996) Role of the *Yersinia pestis* hemin storage (hms) locus in the transmission of plague by fleas. *Science* **273:** 367–370.

Hinnebusch, B.J., Rudolph, A.E., Cherepanov, P., Dixon, J.E., Schwan, T.G., and Forsberg, A. (2002) Role of *Yersinia* murine toxin in survival of *Yersinia pestis* in the midgut of the flea vector. *Science* **296:** 733–735.

Hultmark, D. (2003) *Drosophila* immunity: paths and patterns. *Curr Opin Immunol* **15:** 12–19.

Hurst, M.R., Glare, T.R., Jackson, T.A., and Ronson, C.W. (2000) Plasmid-located pathogenicity determinants of *Serratia entomophila*, the causal agent of amber disease of grass grub, show similarity to the insecticidal toxins of *Photorhabdus luminescens*. *J Bacteriol* **182:** 5127–5138.

Hurst, M.R., Glare, T.R., and Jackson, T.A. (2004) Cloning *Serratia entomophila* antifeeding genes – a putative defective prophage active against the grass grub *Costelytra zealandica*. *J Bacteriol* **186:** 5116–5128.

Joseph, P., Fantino, J.R., Herbaud, M.L., and Denizot, F. (2001) Rapid orientated cloning in a shuttle vector allowing modulated gene expression in *Bacillus subtilis*. *FEMS Microbiol Lett* **205:** 91–97.

Kloepper, J.W., Brewer, J.W., and Harrison, M.D. (1981) Insect transmission of *Erwinia carotovora* var. *carotovora* and *Erwinia carotovora* var. *atroseptica* to potato plants in the field. *Am Potato J* **58:** 165–175.

Liehl, P., Blight, M., Vodovar, N., Boccard, F., and Lemaitre, B. (2006) Prevalence of local immune response against oral infection in a *Drosophila/Pseudomonas* infection model. PLOS pathogen **2:** e56.

de Maagd, R.A., Bravo, A., and Crickmore, N. (2001) How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *Trends Genet* **17:** 193–199.

Manoil, C. (2000) Tagging exported proteins using *Escherichia coli* alkaline phosphatase gene fusions. *Methods Enzymol* **326:** 35–47.

Manoil, C., and Beckwith, J. (1985) TnphoA: a transponson probe for protein export signals. *Proc Natl Acad Sci U S A* **82:** 8129–8133.

Miller, J. (1992) *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Preston, A., Maxim, E., Toland, E., Pishko, E.J., Harvill, E.T., Caroff, M., and Maskell, D.J. (2003) *Bordetella bronchiseptica* PagP is a Bvg-regulated lipid A palmitoyl transferase that is required for persistent colonization of the mouse respiratory tract. *Mol Microbiol* **48:** 725–736.

Rossignol, M., Basset, A., Espeli, O., and Boccard, F. (2001) NKBOR, a mini-Tn10-based transposon for random insertion in the chromosome of Gram-negative bacteria and the rapid recovery of sequences flanking the insertion sites in *Escherichia coli*. *Res Microbiol* **152:** 481–485.

Rossignol, M., Moulin, L., and Boccard, F. (2002) Phage HK022-based integrative vectors for the insertion of genes in the chromosome of multiply marked *Escherichia coli* strains. *FEMS Microbiol Lett* **213:** 45–49.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Thomson, N.R., Cox, A., Bycroft, B.W., Stewart, G.S., Williams, P., and Salmond, G.P. (1997) The rap and hor proteins of *Erwinia*, *Serratia* and *Yersinia*: a novel subgroup in a growing superfamily of proteins regulating diverse physiological processes in bacterial pathogens. *Mol Microbiol* **26:** 531–544.

Vodovar, N., Acosta, C., Lemaitre, B., and Boccard, F. (2004) *Drosophila*: a polyvalent model to decipher host–pathogen interactions. *Trends Microbiol* **12:** 235–242.

Vodovar, N., Vinals, M., Liehl, P., Basset, A., Degrouard, J., Spellman, P., *et al.* (2005) *Drosophila* host defense after oral infection by an entomopathogenic *Pseudomonas* species. *Proc Natl Acad Sci USA* **102:** 11414–11419.

Zaidman-Rémy, A., Hervé, M., Poidevin, M., Pili-Floury, S., Kim, M.-S., Blanot, D., *et al.* (2006) The *Drosophila* amidase PGRP-LB modulates the immune response to bacterial infection. *Immunity* **24:** 463–473.

