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A single gene that promotes interaction of a phytopathogenic bacterium with its insect vector, *Drosophila melanogaster*

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Insects are major vectors of plant and animal disease, and bacterial phytopathogens are often disseminated by flies. We have previously reported that some isolates of the phytopathogenic bacterial species *Erwinia carotovora* infect *Drosophila* and activate an immune response. Using a genetic screen, we have now identified two genes that are required by *E. carotovora* to infect *Drosophila*. One of these genes has a regulatory role whereas the other, *evf*, confers an infectious phenotype: its transfer to non-infectious *Erwinia* strains or to several enterobacteria improves survival in the gut and triggers the immune response. Overexpression of *Erwinia virulence factor* (*evf*) allowed bacteria to colonize the apical side of the gut epithelium and in some cases to spread to the body cavity. Our results demonstrate a specific interaction between plant pathogens and flies that promote their dissemination.

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INTRODUCTION

Flies have often been thought to be involved in the transmission of animal and plant diseases. They live on decaying media, enriched in microorganisms, and are ideal vectors for microbial dissemination through food contamination because of their close association with animal or plant communities. Although the potential hazard of flies towards humans is generally accepted, little is known about the interactions between bacteria and potential fly vectors (Grubel *et al.*, 1997; Kobayashi *et al.*, 1999). In contrast with the situation observed for plague bacteria and fleas (Hinnebusch *et al.*, 2002) or for many protozoan parasites that infect humans and mosquitoes (Beerntsen *et al.*, 2000) in which the insect is an obligate host, there is no molecular report describing a specific interaction of a bacterial pathogen with a fly that allows its persistence. As a consequence of our ignorance, it is generally assumed that flies are passive vectors and transmit microbes by three potential means that might not involve specific interactions between the two partners: carriage on the body, regurgitation and defecation.

The fruitfly *Drosophila melanogaster* lives in decaying fruit and has been occasionally implicated in the transmission of phytopathogens such as the enteric bacteria *Erwinia carotovora* (Molina *et al.*, 1974; Kloepper *et al.*, 1981). *E. carotovora* species induce the soft rotting of many fruits and potatoes through the production of a battery of pectinolytic enzymes (Barras *et al.*, 1994). Recently, we identified a strain of *Erwinia carotovora carotovora*, *Ecc*15, that—unlike most bacterial species—induced a systemic immune response in *Drosophila* larvae after natural ingestion (Basset *et al.*, 2000). Feeding of larvae with living *Ecc*15 induced a strong expression of the antimicrobial peptide genes in the larval fat body, a functional equivalent of mammalian liver. This bacterial strain is not pathogenic for its host, but its capacity to induce a systemic immune response suggests that it has infectious properties and can be recognized by the *Drosophila* innate immune system. Interestingly, out of 16 *Ecc* strains originally tested, only 3 had the capacity to infect *Drosophila* larvae by natural infection, suggesting the existence of specific genes that allowed *Ecc*15 to interact with its insect host (Basset *et al.*, 2000).

Here, using a genetic screen, we have identified two genes that are required by *E. carotovora* to infect *Drosophila*. One gene has a regulatory role whereas the second, *evf*, confers an infectious phenotype not only on non-infectious *Ecc* strains, but also on various enterobacteria. The presence of *evf* increases bacterial persistence in the host, favouring the efficient dissemination of *Erwinia* by *Drosophila* even if it triggers the immune response. Bacteria overexpressing *evf* were able to colonize the entire midgut and to spread to the body cavity.

RESULTS

Two genes required by *Ecc*15 to infect *Drosophila*

A library of 5,500 *Ecc*15 derivatives carrying a randomly inserted NKBOR mini-transposon (Rossignol *et al.*, 2001) was screened for mutants that had lost the capacity to induce the expression of the antibacterial peptide encoding the gene *Diptericin* in the larval fat body. This screen was facilitated by the use of flies carrying a *Diptericin–green fluorescent protein* (*GFP*) reporter gene fusion that mimics the expression of the endogenous gene (Tzou *et al.*, 2000). Using this assay (Fig. 1A), we selected four mutants carrying NKBOR insertions in two different genes (see below) that prevented *Ecc*15

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from inducing *Diptericin–GFP* expression in the fat body. None of these mutants were impaired in growth (data not shown). Northern blot analysis demonstrated that all mutations had a strong effect on *Diptericin* expression (Fig. 1B). In addition to the fat body, *Ecc*15 also induces *Diptericin* expression in the cells of the anterior midgut (Tzou *et al.*, 2000). Figure 1A shows that the two *Ecc*15 mutants have also lost the capacity to induce a *Diptericin–lacZ* reporter gene in this tissue. The cloning by plasmid rescue and characterization of the regions flanking the NKBOR mini-transposon in the four mutants allowed the identification of two genes (Fig. 1C): *homologue of Rap* (*hor*), which has previously been characterized (Thomson *et al.*, 1997), and a new gene, *evf*; three independent insertions were found in this gene). In *E. carotovora*, Hor has previously been identified as a regulator of phytopathogenicity (Thomson *et al.*, 1997); as expected, the *hor* mutant that we isolated was affected in its ability to infect plants in a potato assay (Fig. 1A). In contrast, the *evf* mutant retained the capacity to infect plants. We cannot predict an activity for Evf because no similarities with known proteins or domains have been found. Interestingly, Southern blot analyses showed that the *hor* gene was found in all the *Ecc* isolates we tested, whereas *evf* was present only in strains *Ecc*15 and *Ecc*1488, two isolates that naturally infect *Drosophila* (Fig. 1D).

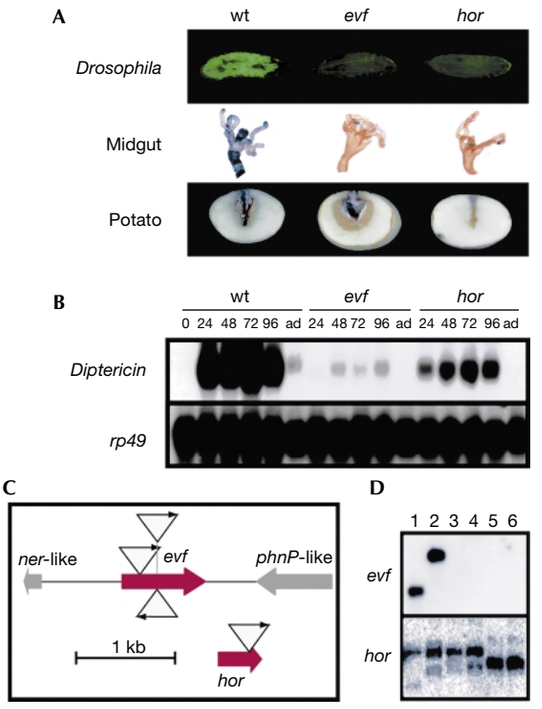

Fig. 1 | Identification of two genes of *Erwinia* required to infect *Drosophila* larvae. (**A**) *GFP* expression in larvae carrying a *Diptericin–GFP* reporter gene after infection by wild-type (wt) *Ecc*15, *evf* and *hor* mutants. Top, Local expression of a *Diptericin–lacZ* reporter gene by the same strains. Middle, LacZ staining (Tzou *et al.*, 2000) was performed on gut collected 1 d after infection. Bottom, Infection of potatoes by the same bacterial strains. Potatoes were infected as described previously (Jones *et al.*, 1993). (**B**) Northern blot analysis of *Diptericin* gene expression after natural infection of wild-type *Drosophila* with wild-type *Ecc*15, *evf* and *hor* mutants. RNA samples were extracted from *Drosophila* larvae collected at different time points after ingestion (24, 48, 72 and 96 h, and 'ad' for adults) and processed as described previously (Basset *et al.*, 2000). *rp49* hybridization was performed to normalize RNA samples. (**C**) Schematic representation of NKBOR insertion in regions containing *evf* and *hor* genes. Insertions of transposon NKBOR are represented by triangles with arrows. Genes located around *evf* are indicated. (**D**) Southern blot hybridizations of ^{32}P-labelled *evf* and *hor* genes with *Eco*RI- and *Pvu*II-cleaved total DNA from *Ecc*15 (lane 1), *Ecc*1488 (lane 2), *Ecc*1401 (lane 3), *Ecc*2140 (lane 4), *Ecc*2145 (lane 5) and *Ecc*2046 (lane 6).

Hor regulates *evf* expression

To analyse the relationships between *hor* and *evf*, we inserted *hor* and *evf* under the control of a constitutive promoter on a pSC101 derivative (pOM1; Espeli *et al.*, 2001). The resulting plasmids, called pOM1-hor and pOM1-evf, were able to rescue their respective mutations as monitored by measurement of the expression of a *Diptericin–lacZ* reporter gene (Fig. 2A). Constitutive expression of *evf* conferred the capacity to infect *Drosophila* on the *hor* mutant, indicating that Evf acts downstream of Hor. In contrast, the constitutive expression of *hor* was not able to rescue the lack of Evf. Strikingly, we observed that *Ecc*15 derivatives carrying pOM1-evf induced significant lethality in larvae (see below). This lethality was probably responsible for the reduced *Diptericin–lacZ* expression compared with that obtained with wild-type *Ecc*15. The regulation of *evf* expression by Hor was confirmed by reverse transcription–polymerase chain reaction (RT–PCR) experiments done on total RNA extracted from dissected gut, obtained from larvae infected by the wild type, and by the *evf* and *hor* mutants (Fig. 2B): neither *evf* expression nor *hor* expression was detected in *hor* mutants, and only *evf* expression was affected in *evf* mutants. These results indicated that the expression of *evf* is under the control of Hor. Regulation of *evf* expression by Hor is reminiscent of the situation encountered in other bacteria in which Hor homologues directly regulate the expression of virulence factors (see Discussion) and suggested a critical role for Evf in the interaction with *Drosophila*.

Bacteria expressing *evf* trigger the immune response

The importance of Evf in *Drosophila* infection was further evaluated by transferring the *evf* gene to a non-infectious *Ecc* strain, *Ecc*2046. Larvae carrying a *Diptericin–lacZ* fusion infected by *Ecc*2046 carrying pOM1-evf strongly expressed the reporter gene, whereas the strain containing the vector alone did not induce any immune response (Fig. 3A). In contrast, larvae infected by *Ecc*2046 carrying pOM1-hor did not express *Diptericin* (data not shown). This experiment indicated that the presence of *evf* was sufficient to explain the difference in infectious properties observed between the *Ecc*2046 and *Ecc*15 strains. This clearcut result prompted us to test the effect of *evf* expression in other enteric bacteria. *Escherichia coli*, *Salmonella typhimurium* and *Serratia marcescens* are three Gram-negative bacterial species that do not trigger any immune response in *Drosophila* after natural infection. The presence of pOM1-evf in these three bacteria induced a strong antibacterial response, whereas the presence of the vector alone had no effect (Fig. 3A). Taken together, our results indicated that the presence of Evf was sufficient by itself to promote a specific interaction between enteric bacteria and *Drosophila* larvae, resulting in the synthesis of antibacterial peptides.

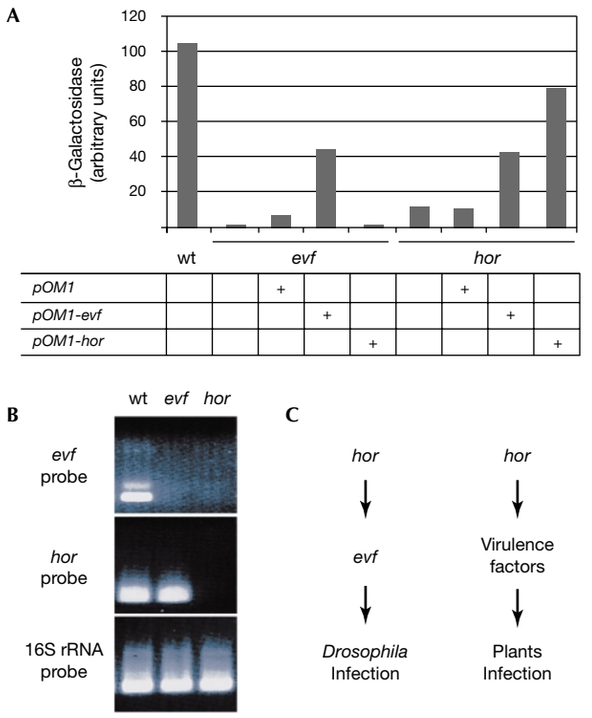

Fig. 2 | Epistatic relationship between *evf* and *hor*. (**A**) Quantitative measurements of β-galactosidase activity were performed with *Drosophila* larvae carrying the *Diptericin–lacZ* reporter gene collected 24 h after natural infection (Basset *et al.*, 2000) by *Ecc*15 derivatives. Each mutant was transformed by pOM1, pOM1-evf and pOM1-hor. Bars represent mean results of three samples of five *Drosophila* larvae. wt, wild type. (**B**) RNA expression was monitored by RT–PCR with RNA isolated from the gut of 30 *Drosophila* larvae 6 h after infection by wild-type (wt) *Ecc*15, *evf* mutant and *hor* mutant. *evf* mRNA, *hor* mRNA and 16S rRNA were analysed as indicated; 16S rRNA was used as a positive control. (**C**) Genetic networks required for *Drosophila* and plants infection by *Ecc*15.

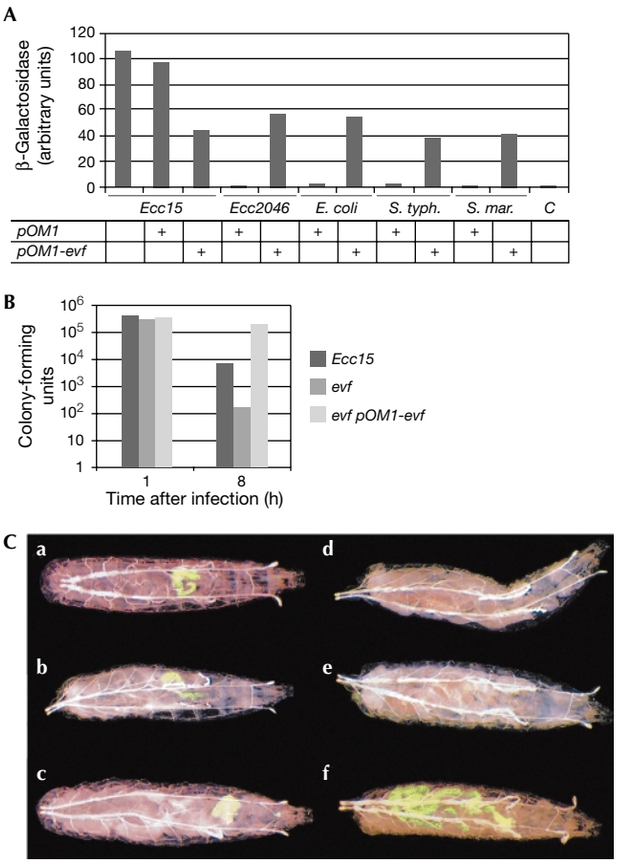

Fig. 3 | Immune response and persistence of bacteria expressing *evf*. (**A**) β-Galactosidase assay revealing the immune response in *Ecc*15, *Ecc*2046, *E. coli* MG1655, *Salmonella typhimurium* LT2 and *Serratia marcescens* expressing *evf* constitutively. Wild-type *Ecc*15 and *Ecc*15 carrying pOM1 were used as positive controls; *Ecc*2046, *E. coli* MG1655, *Salmonella typhimurium* LT2 and *Serratia marcescens* carrying pOM1 plasmid were used as negative controls. Lane C, banana alone. (**B**) Bacterial persistence was measured in wild-type *CantonS Drosophila* larvae (Basset *et al.*, 2000). The number of colony-forming units per larva obtained at each time point after infection is the mean of 120 larvae infected. (**C**) Visualization of *Ecc*15 derivatives carrying a *GFP* marker gene in *Drosophila* larvae. *Drosophila* larvae were infected with *Ecc*15 carrying pOM1–GFP (**a**, **d**), with *evf* mutant carrying pOM1–GFP (**b**, **e**), and with *evf* mutant carrying pOM1-evf–GFP (**c**, **f**). Pictures were taken 2 h (**a**–**c**) and 8 h (**d**–**f**) after infection. Exposure times were shorter in (**a**–**c**) than in (**d**–**f**). Magnification × 10.

Expression of *evf* enhances colonization in the host

The selective advantage that *evf* might confer on *Ecc*15 was assessed by analysing its effects on bacterial persistence and pathogenicity in larvae. By using wild-type *Ecc*15, the *evf* mutant or the *evf* mutant carrying pOM1-evf, we observed that the persistence of *Ecc*15 derivatives 8 h after ingestion was correlated with the level of expression of *evf* (Fig. 3B); for the *evf* mutant the number of bacteria was very much reduced in the gut compared with that of wild-type *Ecc*15. In contrast, the constitutive expression of *evf* resulted in a more than tenfold increase in the number of bacteria present in the larvae. This effect can be detected directly by using the same strains expressing *GFP* constitutively (Fig. 3C). For all strains, bacteria were detected in the anterior midgut only during the first hours after ingestion. By 8 h after ingestion, the bacteria were visible only in the midgut of larvae infected by strains expressing *evf* constitutively, and these bacteria were often able to colonize the entire midgut. We used immunostaining to analyse more precisely the localization of the *evf* mutant, or the *evf* mutant carrying pOM1-evf, in larvae 6 h after ingestion. We observed that the *evf* mutant did not persist, and rare bacteria remained associated with food in the lumen of the gut (Fig. 4A). In contrast, the same mutant overexpressing *evf* often colonized the gut edge (Fig. 4B). In 30% of the larvae tested, bacteria were also found in the haemolymph (Fig. 4C), indicating that overexpression of *evf* allowed the spreading of bacteria across the gut barrier.

As described above, we observed a strong lethality (up to 60%) in larvae infected by *Ecc*15 overexpressing *evf* that was apparent after 3–12 h (see Supplementary Information). It is important to note that all *Ecc*15 derivatives carrying pOM1-evf behaved identically to *Ecc*15 after direct injection into the body cavity of *Drosophila* adults (data not shown); they were not pathogenic for wild-type *Drosophila*, whereas all these derivatives kill *imd* mutants that do

not synthesize antibacterial peptides (Lemaitre *et al.*, 1995). Taken together, these results showed that *evf* expression was specifically required to establish the interaction between *Ecc*15 and *Drosophila* larval gut.

DISCUSSION

The strain *Ecc*15 was initially identified for its capacity to induce an immune response in *Drosophila* larvae after natural infection. The use of this strain has been a determinant in revealing the ability of *Drosophila* to activate a systemic immune response adapted to its aggressors (Vidal *et al.*, 2001) and to induce local immune responses (Tzou *et al.*, 2000). The high specificity of the *Drosophila–Ecc*15 interaction suggested that this strain possesses a unique mechanism to infect *Drosophila* and that this infection can induce the expression of antimicrobial peptide-encoding genes.

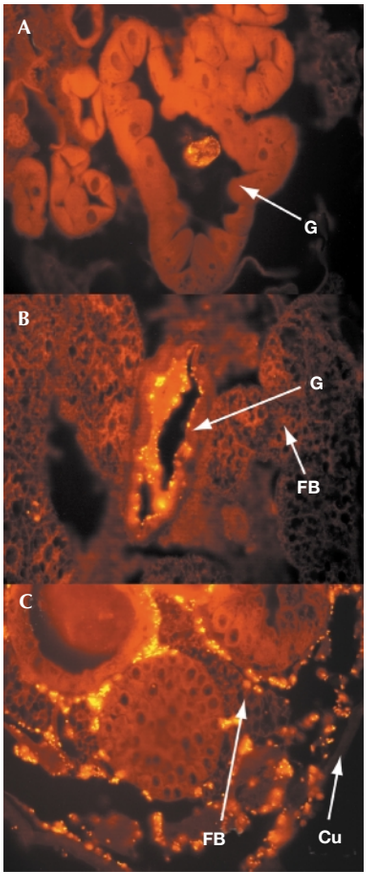

Fig. 4 | Representative immunostained sections of *Drosophila* larvae infected with *evf* mutant carrying pOM1–GFP (**A**) and with *evf* mutant carrying pOM1-evf–GFP (**B**, **C**). Thirty larvae infected with *evf* mutant carrying pOM1–GFP or with *evf* mutant carrying pOM1-evf–GFP were sectioned and bacteria were detected by anti-GFP antibodies. The *evf* mutant did not persist and rare bacteria can be detected with food in the lumen of the anterior midgut (**A**). Similar results were obtained with wild-type *Ecc*15 (data not shown). The *evf* mutant overexpressing *evf* colonized the epithelium of the entire midgut (**B**). In 30% of the larvae, bacteria carrying pOM1-evf were detected in the haemolymph (**C**, a case of major systemic infection). FB, fat body; G, gut; Cu, external cuticle. Magnification × 40.

Here, we have identified a single gene, *evf*, which is sufficient to specify an interaction between *Erwinia* species and the fruitfly *Drosophila* and to trigger the immune response. Our study demonstrated that flies are not passive vectors for bacteria, and that specific interactions allow persistence in larvae that might ensure effective dissemination of the bacteria. The observation that *evf* was found in only a limited number of *Erwinia* isolates suggests that this gene has been acquired recently by horizontal transfer. Interestingly, one *Ecc* strain, *Ecc*1401, does not possess the *evf* gene but still induces an immune response in *Drosophila*, suggesting that *Ecc* species might have selected other strategies to persist in *Drosophila*.

Our data showing that *evf* is controlled by the *hor* gene indicate that, in *Ecc*15, Hor is a key regulator, able to transmit signals from different environments to at least two types of effector: those involved in plant pathogenesis and those involved in *Drosophila* infection (Fig. 2C). Proteins strongly related to Hor (a homologue of RovA, also called Rap and SlyA) have been identified in several Gram-negative pathogens (*Yersinia pseudotuberculosis*, *Y. enterolitica*, *Serratia marcescens*, *Salmonella typhimurium* and *E. coli*) and control the expression of virulence genes such as tissue-specific adhesin, invasin, haemolysin and protease (Nagel *et al.* (2001) and references therein). Our data now extend the function of this class of regulators to the regulation of genes involved in *Drosophila* infection.

We observed that Evf promoted the adhesion of bacteria to the gut cells and was associated with crossing of the intestinal barrier. The change in properties induced by Evf results in the activation of the immune response either by a signal sent by gut cells or by the presence of bacteria in the haemolymph. It is possible that a function of Evf is to disrupt the peritrophic membrane, a chitinous membrane that lines the insect gut and prevents bacteria from entering the gut cells. Alternatively, Evf might permit the proliferation of bacteria in this environment, or it might act as a toxin that disturbs the physiology of the gut cells. Further studies are required to analyse the molecular activity of Evf. Interestingly, the presence of *evf* conferred infectious properties on the three enterobacteria tested, indicating that the way in which Evf ensures their survival in *Drosophila* can be effective in enterobacteria in general. This opens up the possibility of using *Drosophila* as a host model for Gram-negative bacterial pathogens.

Speculation

The exact nature of the *Drosophila–Ecc*15 interaction in nature requires further investigation. However, it is striking to observe that the level of *evf* expression modulates the outcome of the *Drosophila–Erwinia* interaction: in the absence of *evf*, the bacterium does not persist in larvae, whereas when *evf* is overexpressed, the bacterium becomes highly pathogenic. It is tempting to speculate that the level of *evf* expression in *Ecc*15 results from a beneficial co-evolution between the two partners: *Ecc*15 uses *Drosophila* as a vector for spreading, whereas *Drosophila* might benefit from transmitting bacteria that induce rotting of fruit and facilitate the life cycle of the larvae. In contrast with many other phytopathogenic bacteria, *Erwinia* species do not persist in the soil (Agrios, 1997), and the acquisition of a gene increasing survival in an insect that can be used as a vector might be important in spreading the bacteria from one plant to another. In agreement with this hypothesis, we have indeed observed that bacteria expressing *evf* were preferentially disseminated by *Drosophila* larvae; such larvae were still able, 24 h after infection, to propagate soft rot development in carrots (data not shown). Finally, we speculate that such interactions are not restricted

to *Erwinia* and *Drosophila* but that other bacteria, including human pathogens, have developed ways of persisting in housefly vectors, as suggested previously (Douglas & Beard, 1996; Kaslow & Welburn, 1996; Kobayashi *et al.*, 1999).

METHODS

Isolation and characterization of *Ecc*15 mutants. Random mutagenesis of *Ecc*15 was performed as described by Rossignol *et al*. (2001). A total of 5,500 KanR colonies were screened: pellets obtained from 10-ml cultures were used to feed *Diptericin–GFP Drosophila* larvae (Tzou *et al.*, 2002). Candidates that were unable to induce the expression of the *Diptericin–GFP* fusion after 48 h of infection were tested again by the same method and subsequently tested for the lack of induction of a *Diptericin–lacZ* reporter fusion. DNA from each mutant was extracted and digested by *Bgl*II, ligated and transformed into a DH5α pir strain. This allowed us to select a plasmid containing NKBOR with the flanking regions. Sequencing of one flanking region was performed by using the oligonucleotide 5′-ATTTTGAGTGACACAGGAAC-3′. The rescued plasmid was digested with *Bgl*II and *Bam*HI to delete the IS*10* inverted repeat close to R6K ori (see Rossignol *et al*. 2001), ligated and transformed in DH5α pir. The sequence of the second flanking region was determined by using the oligonucleotide 5′-GGATCATATGACAAGATGTG-3′.

Construction of plasmids. pOM1-evf was obtained by cloning a 967-bp *Bam*HI–*Hin*dIII PCR fragment containing *evf*, obtained by using oligonucleotides 5′-AGTGGATCCTGTAACCCCCCCAATAGG-3′ and 5′-AAGCCCAAGCTTAAAATCGAATGATTTAGA-3′. pOM1-hor was obtained by cloning a 479-bp fragment containing *hor* by using oligonucleotides 5′-AGTGGATCCTAACAATAAGGAGAGGTG-3′ and 5′-AGTAAGCTTCCTCTGCGTAACCCAAAT-3′. The pOM1–GFP and pOM1-evf–GFP plasmids were obtained by cloning a 1.5-kbp *Eco*RI fragment amplified from pFVP25.1 into pOM1 and pOM1-evf, respectively, linearized by *Eco*RI (Valdivia & Falkow, 1996). The PCR fragment was obtained by using the oligonucleotides 5′-ATTGTCTCATGAGCGGAT-3′ and 5′-ATCGACGAATTCCGCAGTTATTTGTACAA-3′.

RNA extraction and RT–PCR analysis. Total RNA from dissected guts were extracted with the kit Gram-cracker Reagents and RNAwiz (Ambion). RT–PCRs were performed on 1 μg of total RNA with the Titan-one RT–PCR system (Roche): *evf* mRNA was detected by using oligonucleotides 5′-ATTCAAGATGTGGATCTG-3′ and 5′-AGTAAGCTTGGTAATTGAATTTGCTTGG-3′, whereas *hor* mRNA was detected by using oligonucleotides 5′-GGAATTGCCATTAGGATC-3′ and 5′-GCCAATATATTTTTCTCAAGACGCG-3′. The positive control 16s rRNA was detected by the same method using oligonucleotides 5′-TAGCGATTCCGACTTCA-3′ and 5′-AACGCGAAGAACCTTAC-3′.

Immunostaining. Third-instar larvae were infected with *Ecc*15 derivatives carrying a GFP-expressing plasmid. Larvae were fixed for 2 h in Carnoy (ethanol:chloroform:acetic acid, 6:3:1 by vol.), washed three times (30 min each) in ethanol, incubated overnight in methyl benzoate and embedded in paraffin blocks. Animals were cut into 0.6-μm serial transverse sections that were deposited on slides and rehydrated by standard procedures. Samples were blocked for 20 min with 10% normal goat serum in Tris-buffered saline plus 0.3% Triton (TBT), hybridized with a 1:50 dilution of mouse anti-GFP antibody (Roche) for 2 h and then washed three times (5 min each) in TBT. Samples were incubated for 1 h with a 1:250 dilution of Alexa-594-conjugated anti-mouse antibody, washed again and mounted with aqueous mounting medium (DAKO Paramount). All antibodies were diluted in TBT plus 10% normal goat serum.

Supplementary data are available at *EMBO reports* Online (http://www.emboreports.org).

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Supplementary figure 1: Bacteria overexpressing *evf* induce a strong lethality in larvae. This figure shows the survival rate of larvae infected by wt *Ecc*15, *evf* mutant and *evf* mutant carrying the *pOM1-evf* plasmid. Uninfected larvae were used as control. Larvae were incubated in a mixture of banana (2/3) and bacteria (1/3 OD_{600} =200) for 30 min in a sealed centrifuge vial, transferred on a fly medium for 30 min, then washed in water and transferred individually in apple juice agar plate. Survival was determined at 29°C on 200 hundreds third instar larvae.