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A secondary metabolite acting as a signalling molecule controls *Pseudomonas entomophila* virulence

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Summary

Pseudomonas entomophila **is an entomopathogenic bacterium that is lethal to** ***Drosophila melanogaster*** **within 1–2 days of ingestion of high doses. Flies orally infected with** ***P. entomophila*** **rapidly succumb despite the induction of both local and systemic immune responses. Recent studies suggest that its virulence relies on its ability to cause irreversible damages to the intestinal epithelium, in contrast to what is observed with milder pathogenic bacteria such as** ***Erwinia carotovora carotovora*** **Ecc15 or** ***Pseudomonas aeruginosa*** **PA14. The GacS/GacA two-component system plays a key role in** ***P. entomophila*** **pathogenicity. Here, we report the identification of the** ***pvf*** **genes, whose products are involved in production of a secondary metabolite involved in** ***P. entomophila*** **virulence. A** ***pvf*** **mutant is impaired in its ability to persist within the gut, to trigger the fly immune responses and to inflict gut damages. The expression of several genes is affected in a** ***pvf*** **mutant, independently of the Gac system. Moreover, growing a** ***pvf*** **mutant in medium supplemented with supernatant extracts from either the wild-type strain or a** ***gacA*** **mutant restore its pathogenicity. Collectively, our results indicate that we identified genes involved in the synthesis of a signalling molecule that controls** ***P. entomophila*** **virulence independently from the Gac system.**

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Introduction

Entomopathogenic bacteria have evolved many strategies to interact with and kill insects (Waterfield *et al*., 2004; Vallet-Gely *et al*., 2008). Previous studies have largely focused on the production of insecticidal toxins by entomopathogens, due to their potential use as biological control agents. However, little is known about the physiology of the infectious process during insect colonization by entomopathogenic bacteria. *Pseudomonas entomophila* was recently isolated from flies sampled in Guadeloupe. It is closely related to *Pseudomonas putida*, but differs from several *Pseudomonas* species in that it is highly pathogenic to *Drosophila melanogaster* after ingestion (Vodovar *et al*., 2005). The interaction between *P. entomophila* and *Drosophila* provides a useful model to decipher the interplay between bacterial virulence and insect immune defences, as both the insect host and entomopathogen are genetically amenable.

Pseudomonas entomophila persists in the *Drosophila* gut after ingestion, which induces the production of antimicrobial peptides, via the Imd pathway, both locally in the intestinal epithelium and systemically in the haemolymph (Liehl *et al*., 2006; Vodovar *et al*., 2006). *P. entomophila* counteracts the local immune response of *Drosophila* through secretion of a protease, AprA, which degrades antimicrobial peptides produced by the intestinal epithelium, thus preventing bacterial clearance from the gut (Liehl *et al*., 2006). However, an AprA-deficient mutant remains mildly pathogenic, indicating that *P. entomophila* virulence is multi-factorial. *P. entomophila* virulence is under the control of the GacS/GacA two component system (Vodovar *et al*., 2005; 2006; Vallet-Gely *et al*., 2010), a global regulatory system that is known to control secondary metabolite production, protein secretion and virulence determinants in gammaproteobacteria (Lapouge *et al*., 2008; Yang *et al*., 2008).

Recent studies have demonstrated that gut repair through increased epithelium renewal is an important component of *Drosophila* host defence to bacterial pathogens. This response is induced through the production of the secreted signalling product Unpaired3 by stressed enterocytes, which induces the JAK–STAT pathway in intestinal stem cells to promote their division

and differentiation (Jiang *et al*., 2009; Buchon *et al*., 2009a). In our experimental settings, a high infectious dose of *P. entomophila* does not induce epithelial renewal (Buchon *et al*., 2009a), in contrast to what is observed upon infection with *Pseudomonas aeruginosa* PA14, *Erwinia carotovora Ecc15* or *Serratia marcescens db11* (Apidianakis *et al*., 2009; Cronin *et al*., 2009; Buchon *et al*., 2009b) or ingestion of gut-damaging detergents (Amcheslavsky *et al*., 2009; Chatterjee and Ip, 2009). It is thought that the damage inflicted by *P. entomophila* is too severe to be repaired, which is supported by the observation that infecting flies with a sublethal dose of *P. entomophila* induces significant epithelial renewal (Buchon *et al*., 2009a; Jiang *et al*., 2009)

Taking advantage of *P. entomophila* genome sequence availability, we undertook a systematic inactivation of genes encoding putative virulence factors (Vodovar *et al*., 2006). Interestingly, *P. entomophila* may be able to synthesize a number of secondary metabolites that could contribute to its virulence. Indeed, many of them have been shown to be involved in host–pathogen interactions, like many phytotoxins produced by *Pseudomonas syringae* strains (Bender *et al*., 1999; Gross and Loper, 2009). Interestingly, pyocyanine [a redox active compound playing an important role in *P. aeruginosa* virulence in mammals (Lau *et al*., 2004)] was also shown to be one of the factors stimulating intestinal renewal in *Drosophila* after an oral infection by *P. aeruginosa* (Apidianakis *et al*., 2009). Also relevant to interactions with insect hosts are a *Photorhabdus* produced antibiotic that was shown to be able to suppress insect host defences (Eleftherianos *et al*., 2007), and a secondary metabolite required for the establishment of *Mycobacterium ulcerans* (the causative agent of Buruli ulcer) in the salivary glands of its insect host (Marsollier *et al*., 2005).

Moreover, several bacterial secondary metabolites act as signalling molecules: for instance, homoserine lactones are the key components of quorum sensing in Gram-negative bacteria (Fuqua and Greenberg, 2002; Shiner *et al*., 2005); γ-butyrolactones control secondary metabolites and cellular differentiation in *Streptomyces* sp. (Takano, 2006), and 2,4-diacetylphloroglucinol, which is a important contributor to biological control of plant diseases by many plant-associated *Pseudomonas* sp., can induce its own production (Maurhofer *et al*., 2004). These data prompted us to investigate the possible role of non-ribosomal peptide synthetase (NRPS) and polyketide synthetases (PKS) in the virulence of *P. entomophila* towards *Drosophila*.

Here we report the identification of a gene cluster involved in the synthesis of a secondary metabolite that plays a pleiotropic regulatory role in *P. entomophila* virulence towards *Drosophila*, acting as a signalling molecule.

Results

Identification of an NRPS involved in P. entomophila *virulence*

In order to investigate the role of secondary metabolite in *P. entomophila* virulence, genes encoding NRPS and PKS were inactivated (see Table 1 and Vodovar *et al*., 2006).

Mutants disrupted in genes *pseen0132*, *pseen2149*, *pseen2153*, *pseen3301*, *pseen2342*, *pseen2716*, *pseen2718*, *pseen5522*, *pseen3234*, *pseen2503*, *pseen3044* and deleted for the PKS cluster (from *pseen5523* to *pseen5536*) were tested for their ability to kill adults *Drosophila*. Only one mutant, carrying a disruption of the *pseen0132* gene, presented a defect in its pathogenicity when compared with wild-type *P. entomophila* (data not shown).

Characterization of the pvf *gene cluster*

The *pseen0132* gene is part of a four gene cluster (Fig. 1A), predicted to form a single transcriptional unit (Mao *et al*., 2009). It encodes an NRPS composed of an adenylation domain and a thiolation domain without a condensation domain (for review see Marahiel *et al*., 1997; Mootz *et al*., 2002), suggesting that it is able to recognize and to bind an unknown amino acid. Its C-terminal part shows similarity with dehydrogenases. Both *pseen0134* and *pseen0133* are predicted to code for proteins of unknown function. The *pseen0131*-encoded protein shows similarities to polyketide cyclases/dehydratases. In addition, *pseen0136* codes for a putative thioesterase that could also participate in secondary metabolite synthesis (Fig. 1A), and *pseen0137* encodes a putative transcriptional regulator. In order to identify the role of these genes in *P. entomophila* virulence, precise deletion mutants of the *pseen0131*, *pseen0132*, *pseen0133* and *pseen0134* genes were engineered, as well as insertion mutants of *pseen0136* and *pseen0137*. Results presented in Fig. 1 show that only the *pseen0133*, *pseen0132* and *pseen0131* mutants are less efficient than the wild-type strain to kill *Drosophila*, as was the *pseen0132* insertion mutant. It is worth noting that these mutants are only partially defective in their pathogenicity, in contrast to a *gacA* mutant that is completely devoid of virulence. Complementation experiments confirmed that *pseen0133*, *pseen0132* and *pseen0131* are indeed required for *P. entomophila* full pathogenicity (data not shown). Therefore, we named these genes the *pvf* genes (for *P. entomophila* virulence factor). As *pseen0134* is predicted to be part of the same transcriptional unit, we included it as part of the *pvf* gene cluster even if it does not seem to play a significant role in *P. entomophila* virulence (Fig. 1B). Interestingly, this *pvf* gene cluster does not seem to be widespread amongst bacterial species. It

Table 1. *P. entomophila* genes predicted to be involved in secondary metabolite synthesis.

Gene	Predicted function	Secondary metabolite	Reference
pseen0132	NRPS	Unknown	Vodovar *et al.* (2006)
pseen1815	NRPS	Pyoverdine	Matthijs *et al.* (2009)
pseen2149	NRPS	Unknown	Vodovar *et al.* (2006)
pseen2150	NRPS	Unknown	Vodovar *et al.* (2006)
pseen2153	PKS	Unknown	Vodovar *et al.* (2006)
pseen2154	NRPS	Unknown	Vodovar *et al.* (2006)
pseen2342	NRPS	Unknown	Vodovar *et al.* (2006)
pseen2500	NRPS	Pseudomonine	Matthijs *et al.* (2009)
pseen2503	NRPS	Pseudomonine	Matthijs *et al.* (2009)
pseen2716	NRPS	Unknown	Vodovar *et al.* (2006)
pseen2717	NRPS	Unknown	Vodovar *et al.* (2006)
pseen2718	PKS	Unknown	Vodovar *et al.* (2006)
pseen3044 (etlC)	NRPS	Entolysin	Vallet-Gely *et al.* (2010)
pseen3045 (etlB)	NRPS	Entolysin	Vallet-Gely *et al.* (2010)
pseen3229	NRPS	Pyoverdine	Matthijs *et al.* (2009)
pseen3230	NRPS	Pyoverdine	Matthijs *et al.* (2009)
pseen3231	NRPS	Pyoverdine	Matthijs *et al.* (2009)
pseen3232	NRPS	Pyoverdine	Matthijs *et al.* (2009)
pseen3234	NRPS	Pyoverdine	Matthijs *et al.* (2009)
pseen3301	NRPS	Unknown	Vodovar *et al.* (2006)
pseen3332 (etlA)	NRPS	Entolysin	Vallet-Gely *et al.* (2010)
pseen5520 (hcnC)		HCN	Ryall *et al.* (2009)
pseen5521 (hcnB)		HCN	Ryall *et al.* (2009)
pseen5522 (hcnA)		HCN	Ryall *et al.* (2009)
pseen5528	PKS	Unknown polyketide	Vodovar *et al.* (2006)
pseen5530	PKS	Unknown polyketide	Vodovar *et al.* (2006)
pseen5531	PKS	Unknown polyketide	Vodovar *et al.* (2006)
pseen5535	PKS	Unknown polyketide	Vodovar *et al.* (2006)
pseen5536	PKS	Unknown polyketide	Vodovar *et al.* (2006)

is only conserved in some other *Pseudomonas* strains (the three sequenced *Pseudomonas syringae* strains, *Pseudomonas fluorescent* PfO-1 and SBW25, *P. aeruginosa* PA7) and in *Burkholderia cenocepacia* (present in the four sequenced strains). In PA7 and the *B. cenocepacia* strains, an additional gene is found between *pvfA* (*pseen0134*) and *pvfB* (*pseen0133*). It encodes a small protein predicted to contain a cupin domain (Dunwell *et al.*, 2004).

A pvfC *mutant is affected in its abilities to persist in the* Drosophila *gut*

It was previously shown that *P. entomophila* virulence towards *Drosophila* is linked to its ability to persist in the gut, which results in the induction of a strong immune response both locally and systematically (Vodovar *et al.*, 2005; Liehl *et al.*, 2006). In order to better characterize the role of the *pvf* genes in the infectious process, we first compared the persisting abilities of a *pvfC* mutant to those of the wild-type strain and a *gacA* mutant. Adult flies were infected by feeding, and bacterial amounts were quantified at two time points following ingestion. Figure 2A shows that after 3 h, no significant differences in bacterial loads could be detected between flies infected by the wild-type strain, a *gacA* mutant and a *pvfC* mutant. As was already shown, wild-type *P. entomophila*, but not the *gacA* mutant, is able to persist, as indicated by the amount of bacteria still present in the *Drosophila* gut after 24 h (Liehl *et al.*, 2006). Interestingly, the amount of the *pvfC* mutant remaining in the *Drosophila* gut after 24 h was significantly reduced compared with wild-type *P. entomophila*. This indicates that a *pvfC* mutant is affected in its capacity to persist in the *Drosophila* gut.

A pvfC *mutant failed to trigger the fly immune responses*

One striking feature of *P. entomophila* is that its ingestion induces both a local immune response in the gut and a systemic one in the fat body of infected flies (Vodovar *et al.*, 2005; Liehl *et al.*, 2006). This immune response is mediated by the Imd pathway, which orchestrates *Drosophila* defences against Gram-negative bacteria (Lemaitre and Hoffman, 2007). We compared activation of the Imd pathway after infection by the wild-type *P. entomophila* strain, a *gacA* mutant, and a *pvfC* mutant. We used reverse transcriptase quantitative PCR (RT-qPCR) to measure the expression of the *Diptericin* (*Dpt*) gene, a target of this Imd pathway, specifically in the gut (local response) or in whole flies (reflecting mostly the systemic expression of *Diptericin* by the fat body) (Fig. 2B and C respectively). Four hours after infection, an increase in

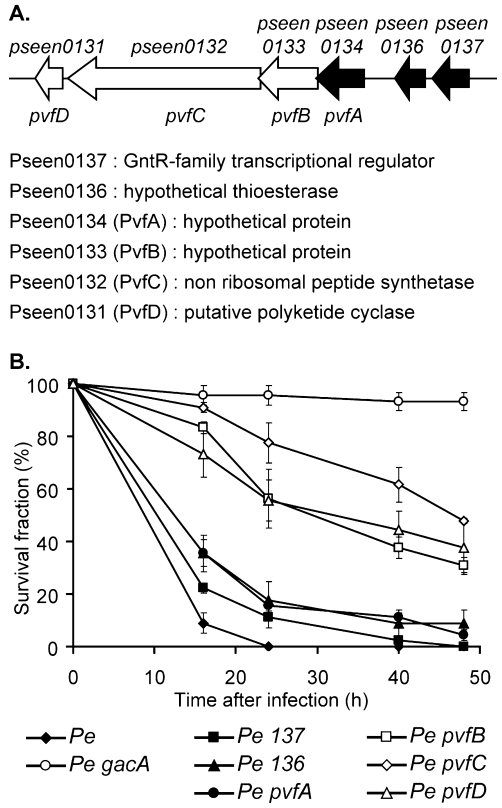

Fig. 1. Implication of the *pvf* genes in *P. entomophila* pathogenicity.
A. Schematic representation of the *pvf* gene cluster and surrounding ORFs in the *P. entomophila* genome. White arrows indicate genes involved in *P. entomophila* virulence. Prediction of the encoded protein function based on homology to other characterized proteins is indicated.
B. Wild-type flies (carrying a *Diptericin–lacZ* reporter construct) were infected by wild-type *P. entomophila* (*Pe*, closed diamonds), a *gacA* mutant (*Pe gacA*, opened circles), a *pseen0137* mutant (*Pe 137*, closed squares), a *pseen0136* mutant (*Pe 136*, closed triangles), a *pseen0134* mutant (*Pe pvfA*, closed circles), a *pseen0133* mutant (*Pe pvfB*, opened squares), a *pseen0132* mutant (*Pe pvfC*, opened diamonds) and a *pseen0131* mutant (*Pe pvfD*, opened triangles).

Diptericin expression upon infection by *P. entomophila* was already visible, at both the local and the systemic level. This increase was stronger after 16 h, whereas no specific activation of the Imd pathway by a *gacA* mutant could be detected even at this later time point, as previously described (Vodovar *et al.*, 2005; Liehl *et al.*, 2006). Compared with flies infected with wild-type *P. entomophila*, flies infected by a *pvfC* mutant displayed a significantly reduced *Diptericin* expression, in both their gut and fat body. This was especially marked at the 16 h time point. These results indicate that in contrast to wild-type *P. entomophila*, infection with a *pvfC* mutant does not trigger the fly immune response, at both the local and the systemic levels. This is consistent with the decrease in its persistence abilities.

A pvfC *mutant is affected in its ability to inflict damages to the* Drosophila *gut*

Pseudomonas entomophila pathogenicity has been linked to its ability to cause irreversible damage to the *Drosophila* gut, preventing intestinal stem cell proliferation and intestinal renewal (Buchon *et al.*, 2009b). In order to investigate the role of the *pvf* genes in these phenomena,

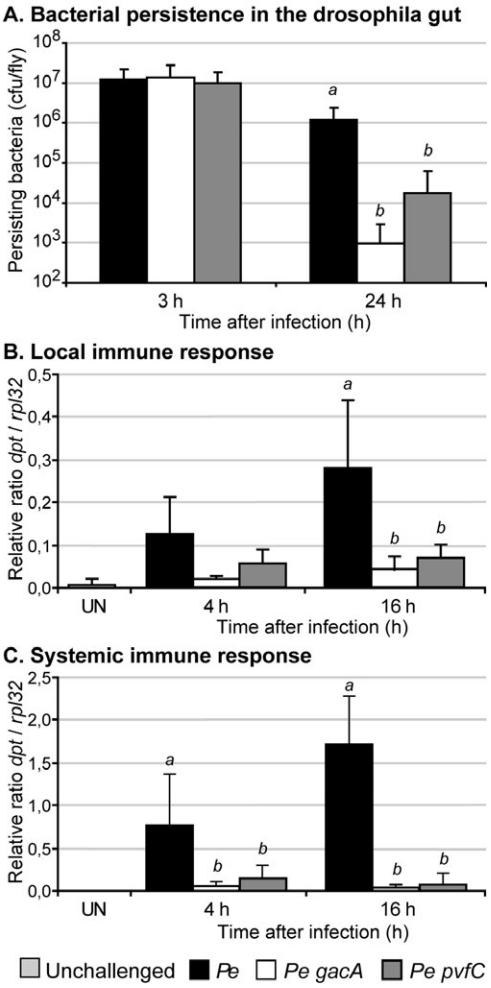

Fig. 2. Contribution of the *pvf* genes to *P. entomophila* ability to persist in the *Drosophila* gut and to trigger the Imd immune pathway.
A. Bacterial persistence was measured in living wild-type Oregon flies by plating appropriate dilutions of homogenates of five surface-sterilized adults on PIA medium. The flies had been previously orally infected with wild-type *P. entomophila* (*Pe*, black bars), a *gacA* mutant (*Pe gacA*, white bars) and a *pvfC* mutant (*Pe pvfC*, dark grey bars). Experiments were performed at least three times in triplicate.
B and C. Time-course analysis of *Diptericin* (Dpt) expression measured by RT-qPCR in guts (B) or whole body (C) of females flies, following infection with wild-type *P. entomophila* (*Pe*, black bars), a *gacA* mutant (*Pe gacA*, white bars) and a *pvfC* mutant (*Pe pvfC*, dark grey bars). Unchallenged flies (light grey bars) are indicated as controls.
Statistical analysis was performed using a Wilcoxon test, and letters indicate significantly different values ($P < 0.05$).

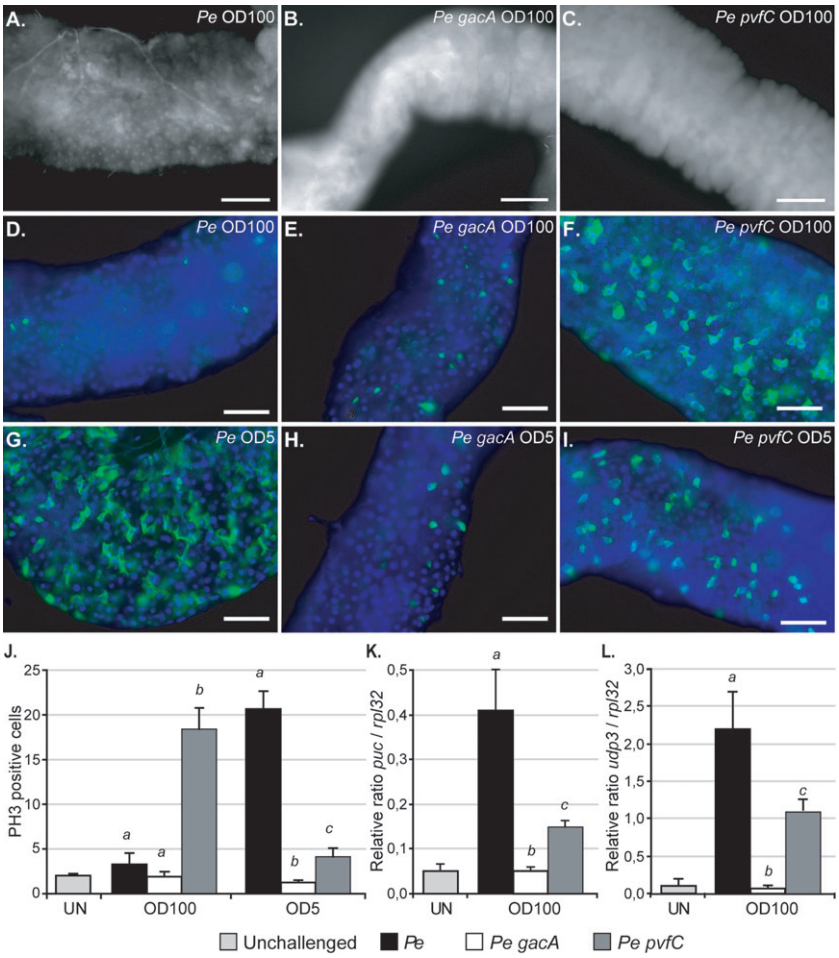

Fig. 3. Contribution of the *pvf* genes to *P. entomophila*-induced damage to the *Drosophila* gut.
A–C. Cell death was monitored in guts of infected flies by acridine orange staining. Guts were dissected from female Oregon flies infected for 16 h with wild-type *P. entomophila* (A), a *gacA* mutant (B) or a *pvfC* mutant (C), and stained with acridine orange. Scale bars represent 20 μm.
D–I. Stem cell proliferation was monitored using flies expressing an *esg-Gal4; UAS-GFP* reporter. Flies were infected for 16 h with wild-type *P. entomophila* (D and G), a *gacA* mutant (E and H) or a *pvfC* mutant (F and I), using standard (D, E and F) or sublethal (G, H and I) conditions. Nuclei are stained with DAPI. Scale bars represent 20 μm.
J. Stem cell proliferation in the midgut was quantified by counting the number of dividing PH3-positive cells. Flies were infected for 16 h, as described above, and dissected guts were detected by immunofluorescence staining with anti-PH3-specific antibody. For each experiment, at least six guts were analysed. Data represent the average of four independent experiments.
K. Analysis of *puckered* (*puc*) expression measured by RT-qPCR in guts extracted from flies infected for 16 h. For each assay, 14 guts were extracted. Results represent the average of four independent experiments.
L. Analysis of *unpaired3* (*upd3*) expression measured by RT-qPCR in guts extracted from flies infected for 16 h. For each assay, 14 guts were extracted. Results represent the average of four independent experiments.
Statistical analysis was performed using a Wilcoxon test, and letters indicate significantly different values ($P < 0.05$).

we first looked at cell death induction upon bacterial ingestion using acridine orange staining. We could detect a high number of dead cells in guts from flies infected by wild-type *P. entomophila* (Fig. 3A), but not in gut from flies infected by either a *gacA* mutant or a *pvfC* mutant (Fig. 3B and C respectively). This indicates that a *pvfC* mutant fails to cause damages to intestinal epithelial cells. We then investigated the effect of a *pvfC* mutation on intestinal epithelium renewal using two approaches described before (Jiang *et al*., 2009; Buchon *et al*., 2009a). Epithelium renewal requires ISC proliferation, and hence can be visualized using the *esgGal4*, *UAS-GFP* marker, which labels ISC, enteroblasts and newly synthesized enterocytes. The other approach involves counting the number of dividing cells along the midgut using an anti-phosphohistone H3 (anti-PH3) antibody as an indicator of mitotic activity (Buchon *et al*., 2009b). We used these two methods to compare epithelial renewal upon infection with wild-type *P. entomophila*, a *gacA* mutant and a *pvfC* mutant, under standard (Fig. 3D–F) or sublethal (Fig 3G–I) conditions. Quantifications of dividing cells are presented in Fig. 3J. As was already shown (Buchon

et al., 2009a), no epithelium renewal was observed after infection with wild-type *P. entomophila* (Fig. 3D and J) or a *gacA* mutant (Fig. 3E and J) at high infecting dose (our standard conditions). Under sublethal conditions, wild-type *P. entomophila* induced stem cell proliferation whereas a *gacA* mutant did not (Fig. 3G–J). Interestingly, many escargot-positive cells could be seen after infection with a high dose of the *pvfC* mutant, indicating a high level of epithelium renewal (Fig. 3F and J). Under sublethal conditions, the *pvfC* mutant induced epithelium renewal, albeit to a much lesser extent than wild-type *P. entomophila* (Fig. 3I and J).

Previous studies showed that ingestion of *P. entomophila* activates both JAK-STAT and another stress related pathway, the Jun N-terminal kinase (JNK) pathway, in the *Drosophila* gut (Jiang *et al*., 2009; Buchon *et al*., 2009a). The JNK pathway is a MAPK-type kinase cascade that is activated in response to cellular stress (McEwen and Peifer, 2005). The activation of both pathways can be monitored by measuring the expression of *puckered* (*puc*) (a direct downstream target of JNK signalling) or *upd3* (a target of JAK-STAT signalling) by RT-qPCR. Figure 3K and L shows that a *pvfC* mutant is less efficient than wild-type *P. entomophila* to activate the JNK pathway and the JAK-STAT pathway, respectively, but more efficient than a *gacA* mutant. This indicates that even if a *pvfC* mutant does not cause intestinal cell death, it is able to induce some level of cellular stress and epithelium renewal, in contrast to a *gacA* mutant. Altogether, these data indicate that even if the *pvfC* mutant retains some ability to cause damages to the intestinal cells, this ability is diminished compared with wild-type *P. entomophila*. This correlates with the overall pathogenicity of a *pvfC* mutant.

The pvf *genes do not allow a heterologous host to persist in the* Drosophila *gut*

In order to reveal the activity of proteins encoded by the *pvf* genes, we tested whether the *pvf* gene cluster could enhance the infectious properties of other bacterial strains towards *Drosophila*. Indeed, previous experiments showed that some virulence factors, such as Evf from *E. carotovora carotovora 15* (Basset *et al*., 2003) or Mcf (make caterpillar floppy) from *Photorhabdus luminescens* (Daborn *et al*., 2002) can confer virulence when expressed in non-pathogenic bacteria (Daborn *et al*., 2002; Acosta Muniz *et al*., 2007). We reasoned that if the *pvf* genes were introduced into another *Pseudomonas* strain, looking at this strain pathogenicity could tell us if the *pvf* genes are a key determinant for *P. entomophila* striking efficiency to kill *Drosophila*, especially when compared with other *Pseudomonas* strains. Thus, we cloned the *pvf* cluster in a replicative plasmid [from *pvfA* (*pseen0134*) to *pvfD* (*pseen0131*), see *Experimental procedures*], under the control of the *placUV5* promoter. The construct was sequenced, and was able to complement the pathogenicity defect of *pseen0133*, *pseen0132* and *pseen0131* mutants (Fig. 4). We then introduced this construct in *P. aeruginosa* PAO1 and *P. putida* KT2440, and compared the virulence of these strains to PAO1 and KT1440 harbouring the vector alone. No difference could be seen in pathogenicity (Fig. 4 and data not shown), indicating that the *pvf* genes could not confer by themselves the ability to persist and to cause irreversible damages to the *Drosophila* gut. The fact that this absence of effect is not due to an expression defect in the heterologous host is demonstrated downstream (Fig. 6B).

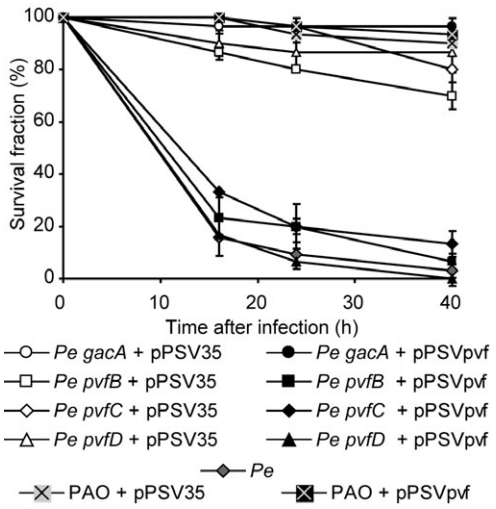

Fig. 4. Expression of the *pvf* genes in *trans* does not confer virulence in other *Pseudomonas* species. Wild-type flies (carrying a *diptericin–lacZ* reporter construct) were infected by wild-type *P. entomophila* (*Pe*, dark grey diamonds), mutants containing either the pPSV35 vector (opened symbols) or the pPSVpvf plasmid (closed symbols), a *gacA* mutant (circles), a *pvfB* mutant (squares), a *pvfC* mutant (diamonds), a *pvfD* mutant (triangles), or the *P. aeruginosa* PAO1 strain containing either the pPSV35 vector (PAO pPSV35, black cross in light square) or the pPSVpvf plasmid (PAO pPSVpvf, light cross in black square). Cultures were grown with gentamicin and 1 mM IPTG, to allow full expression of the *pvf* genes from the plasmid.

A pvfC *mutation leads to reduced expression of several genes*

We could not exclude that *P. entomophila* persistence ability relies on several independent factors, the *pvf* genes encoding only one of them. But another hypothesis that could explain the results above was that the *pvf* gene products could participate to an undefined regulatory system, controlling factors involved in the different steps of the infectious process. We chose to investigate this hypothesis, especially as some secondary metabolites have been shown to be able to interfere with global

regulatory system such as quorum sensing (Holden *et al*., 1999; Degrassi *et al*., 2002).

The GacS/GacA two component system is a main regulator of *P. entomophila* virulence. Upon phosphorylation by the GacS sensor, the GacA transcriptional regulator positively controls the expression of *rsmY* and *rsmZ*, specifying small non-coding RNAs. These RNAs bind small RNA-binding proteins called RsmA that act as translational repressors of target mRNAs (Lapouge *et al*., 2008; Vallet-Gely *et al*., 2010). Using β-galactosidase reporter fusions, we showed that the expression levels of *rsmZ* and *rsmY* were similar in a *pvfC* mutant and wild-type *P. entomophila* (Fig. 5A and data not shown). This indicates that the phenotypes of a *pvfC* mutant are not due to a global impairment of the GacS/GacA two-component system. However, when we measured the expression of nine translational fusions known to be regulated by the GacS/GacA two-component system in a *pvfC* mutant (I. Vallet-Gely, unpubl. data), we observed that four of them were expressed at a lower level compared with wild-type *P. entomophila* (see Fig. 5B–F for representative examples). Interestingly, this is not the case of the *pseen1550* gene, encoding AprA, whose role in *P. entomophila* virulence was previously demonstrated (Liehl *et al*., 2006).

This confirms that a *pvfC* mutation has a pleiotropic effect, and that the expression of several genes is affected in a *pvfC* mutant. This effect does not occur through the GacS/GacA two-component system, suggesting that the *pvf* genes participate in another regulatory mechanism that could also control virulence gene expression.

The pvf *genes are involved in the synthesis of a signalling molecule*

The *pvf* gene products are predicted to be involved in secondary metabolite synthesis. Their participation in a regulatory system suggests that this secondary metabolite could act as a signalling molecule. We reasoned that if a signalling molecule was required for virulence gene expression, we should be able to isolate it, and to restore the virulence of a *pvfC* mutant by adding this molecule to the growth medium. We extracted molecules from the culture supernatant using dichloromethane. These extracts were dried and dissolved in methanol. In order to test the signalling molecule hypothesis, we added extracts in the growth medium of the different strains and assayed for their pathogenicity. Growing a *pvfC* mutant in medium supplemented with extract from wild-type *P. entomophila* restored its pathogenicity to a level comparable to wild-type *P. entomophila* (Fig. 6A). The same phenomenon was observed when a *pvfC* mutant was grown with extract from a *gacA* mutant, but not with an extract from a *pvfC* mutant itself. In contrast, when an extract from wild-type *P. entomophila* was added to the growth medium of a

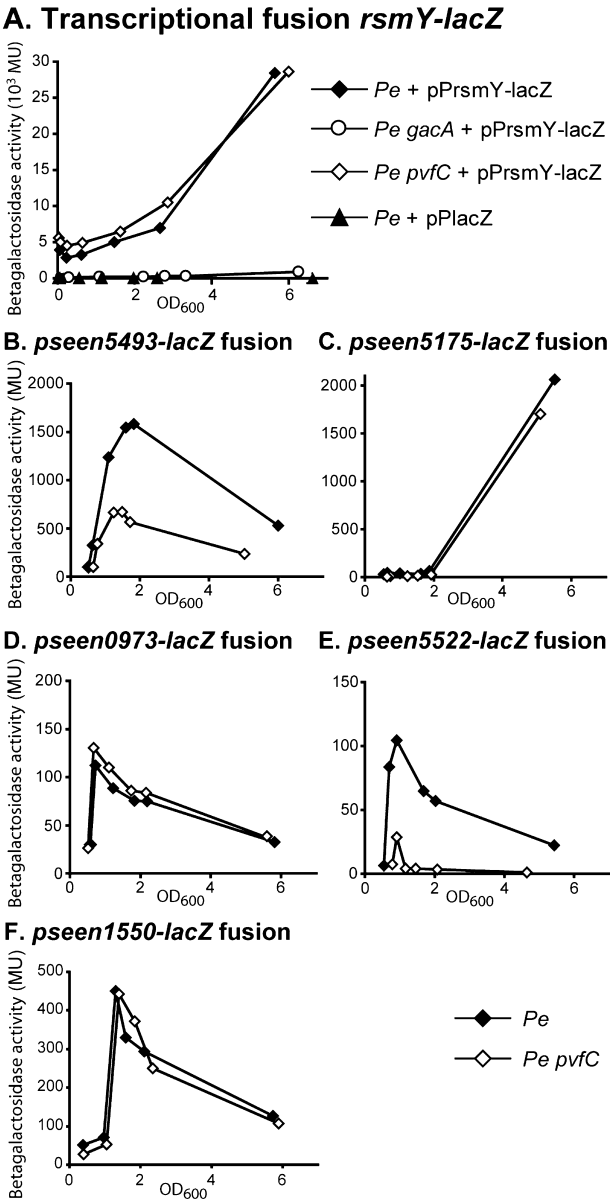

Fig. 5. A *pvfC* mutation affects expression of other *P. entomophila* genes. Quantification of the β-galactosidase activity of: (A) the transcriptional fusion *rsmY–lacZ*, (B) the translational fusion *pseen5493–lacZ*, (C) the translational fusion *pseen5175–lacZ*, (D) the translational fusion *pseen0973–lacZ*, (E) the translational fusion *pseen5522–lacZ* and (F) the translational fusion *pseen1550–lacZ*, as a function of bacterial growth in different genetic contexts. Closed diamonds represent expression of each fusion in wild-type *P. entomophila* (*Pe*), opened diamonds represent expression of each fusion in a *pvfC* mutant (*Pe pvfC*), opened circles represent expression of the *rsmY–lacZ* fusion in a *gacA* mutant, and closed triangles represent the basal expression level from the pPlacZ vector in wild-type *P. entomophila*.

gacA mutant, it remained innocuous. This confirms that a *pvfC* mutant is lacking a signalling molecule, which is produced by wild-type *P. entomophila* but not by a *pvfC* mutant. It is worth noting that the production of this

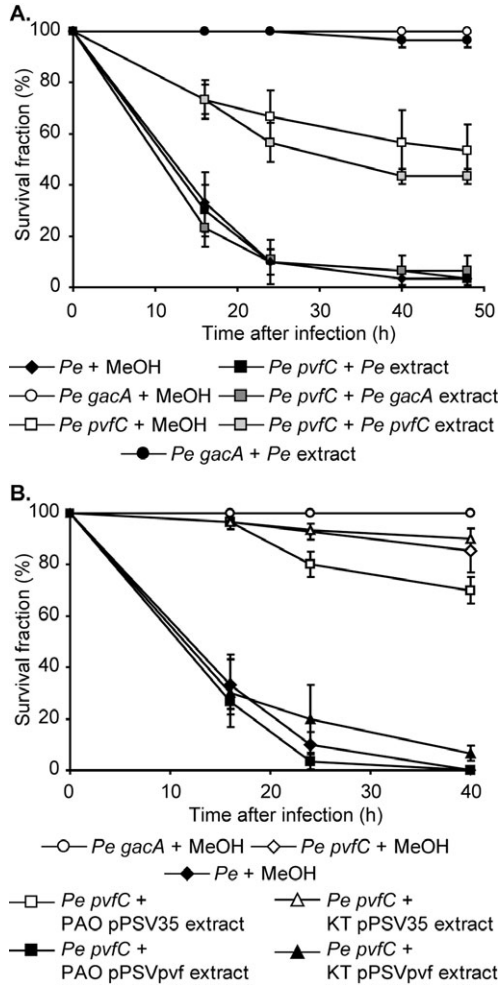

Fig. 6. Extracts of culture supernatants from bacteria expressing the *pvf* genes restores virulence of a *pvfC* mutant when supplemented in the growth medium.
A. Wild-type flies (carrying a *Diptericin–lacZ* reporter construct) were infected by wild-type *P. entomophila* grown in medium supplemented with methanol (*Pe* + MeOH, closed diamonds), a *gacA* mutant grown in medium supplemented with methanol (*Pe gac* + MeOH, opened circles) or extract from wild-type *P. entomophila* (*Pe gacA* + *Pe* extract, closed circles), a *pvfC* mutant grown in medium supplemented with methanol (*Pe pvfC* + MeOH, opened squares), extract from wild-type *P. entomophila* (*Pe pvfC* + *Pe* extract, closed squares), extract from a *gacA* mutant (*Pe pvfC* + *Pe gacA* extract, dark grey squares) or extract from a *pvfC* mutant (*Pe pvfC* + *Pe pvfC* extract, light grey squares).
B. Flies carrying a *Diptericin–lacZ* reporter construct were infected by wild-type *P. entomophila*, a *gacA* mutant or a *pvfC* mutant grown in medium supplemented with methanol (*Pe* + MeOH, closed diamonds; *Pe gacA* + MeOH, opened circles, and *Pe pvfC* + MeOH, opened diamonds respectively), or a *pvfC* mutant grown in medium supplemented with extract from PAO1 or KT2440 containing the pPSV35 plasmid (opened squares and triangles respectively) or the pPSVpvf plasmid (closed squares and triangles respectively),

signalling molecule is not controlled by the GacS/GacA two-component system. To determine if the *pvf* cluster was responsible for synthesis of the signalling molecule, we tested if the molecule was produced when the *pvf* genes were expressed in another *Pseudomonas* strain (PAO1 or KT2440). We supplemented the growth medium with extracts from strains containing the empty vector or expressing the *pvf* gene cluster. Extracts from *P. putida* KT2440 or *P. aeruginosa* PAO1 expressing the *pvf* cluster do restore the virulence of a *pvfC* mutant very efficiently, as compared with extracts from *P. putida* or *P. aeruginosa* containing the empty vector (Fig. 6B). This confirms that the *pvf* gene cluster is sufficient to synthesize a signalling molecule required for *P. entomophila* virulence.

Discussion

Only one of the NRPS/PKS encoding genes is mandatory in P. entomophila *virulence*

The systematic inactivation of NRPS/PKS-encoding genes involved in secondary metabolite synthesis revealed that only one among those selected plays a critical role in *P. entomophila* virulence. Surprisingly, secondary metabolites important for virulence of other bacteria do not seem necessary for *P. entomophila* interactions with *Drosophila*. For instance, cyanide, which is produced in high amounts by *P. entomophila* (Ryall *et al*., 2009), has been shown to be toxic for *Caenorhabditis elegans* (Gallagher *et al*., 2002) and *Drosophila* (Broderick *et al*., 2008), when produced by *P. aeruginosa*. However, these infection models are quite different from our study, as cyanide toxicity was evaluated after needle pricking of *Drosophila*, and not oral infection. Similarly, siderophores are often important for survival inside the host (Schryvers and Stojiljkovic, 1999; Ratledge, 2007; Mossialos and Amoutzias, 2009), but none of the two siderophores produced by *P. entomophila* (Matthijs *et al*., 2009) is required for its virulence (data not shown). However, we mostly looked at the overall pathogenicity of the secondary metabolite mutants; hence we can imagine that a closer look at intestinal damages might reveal subtler phenotypes. Moreover, redundancy might exist and inactivation of more than one cluster might be required to reveal a role in the infectious process.

Role of the pvf *genes in* P. entomophila *infectious process*

Infection of *Drosophila* by *P. entomophila* involves bacterial persistence in the intestinal tract, triggering of the local and systemic immune responses, and severe damages to the intestinal epithelium. Taking advantage of the genetic amenability of the host and pathogen, our goal is to elucidate the contribution of each virulence factor to the infectious process. Using this integrated approach, we previously identified a specific role of the AprA metalloprotease in bacterial protection against

antimicrobial peptides (Liehl *et al*., 2006). Here, we observed that like a *gac* mutant, a *pvf* mutant is affected in all aspects of the infectious process. Nevertheless, a careful analysis reveals that a *pvfC* mutant retains some virulence towards *Drosophila*, in contrast to a *gacA* mutant. While there was no significant difference between the *gac*A and *pvfC* mutants in terms of persistence or the induction of immune responses, there was a trend of the *pvfC* mutant always being higher. In addition, the *pvfC* mutant causes stress and damage to the intestinal epithelium that a *gacA* mutant does not. Indeed, even in the absence of cell death the *pvfC* mutant induces a strong epithelial renewal in the midgut, whereas a *gacA* mutant does not. In a similar manner, a *pvfC* mutant activates the JNK and JAK-STAT pathways, in contrast to a *gacA* mutant. The attenuated virulence of the *pvf* mutant is clearly shown by survival analysis, which monitors the global outcome of infection, as *pvfB*, *pvfC* and *pvfD* mutants retain the capacity to kill between 15% and 50% of infected flies. We conclude that inactivation of the *pvf* genes abolishes most, but not all, of the virulence towards *Drosophila*, indicating that it impacts important steps of the infectious process.

Wild-type *P. entomophila* does not induce stem cell proliferation under our standard experimental conditions that use a high infecting dose, despite activation of the JNK and JAK-STAT pathways (Buchon *et al*., 2009a; Jiang *et al*., 2009; our data). The observation that ingestion of paraquat or SDS, which also induces intestinal stress leading to epithelial renewal, failed to do so when applied in high amounts (Buchon *et al*., 2009a) suggests that the repair of gut damage is essential for fly survival and *P. entomophila* virulence factors disrupt epithelium renewal through excessive damage to the gut. From this point of view, it is tempting to speculate that a mutation in the *pvf* locus reduces the severity of cell damages, which would account for the ability of a *pvfC* mutant to trigger epithelial renewal. This supports the notion that excessive gut damage and suppression of gut repair has a primary role in the pathogenesis of *P. entomophila*. Future studies should analyse the relationship between *P. entomophila* persistence and ability to cause excessive damages, since it is possible that, contrary to expectation, *P. entomophila* persistence might be a consequence rather than a cause of its capacity to damage the gut.

The pvf *gene products could be responsible for the synthesis of a new signalling molecule*

Regulation through diffusible signalling molecules is well documented in *Pseudomonas* species (Withers *et al*., 2001; Juhas *et al*., 2005; Williams and Camara, 2009) and constitutes what is called quorum sensing regulation. Quorum sensing is reported to regulate virulence, production of secondary metabolites, establishment of symbiosis and biofilm formation in diverse bacteria and through several different types of signalling molecules. *N*-acylhomoserine lactones (AHLs), derived from *S*-adenosyl methionine, are synthesized by LuxI type AHL synthetases and are recognized by specific luxR type transcriptional regulators. Another family of signalling molecules is composed by 4-quinolones. The *Pseudomonas* Quinolone Signal (PQS) and its direct precursor molecule (HHQ) both act as signalling molecules controlling the expression of many genes (Diggle *et al*., 2006) and their regulatory effect is mediated by their binding to a specific transcriptional regulator (Xiao *et al*., 2006). Interestingly, quinolone signalling and AHL signalling both control virulence gene expression, but independently of one another (Deziel *et al*., 2005), indicating that expression of virulence genes may be under the control of several signalling molecules controlling independent sets of genes. The *P. entomophila* genome does not contain genes encoding a LuxI type AHL synthetase or any of the PQS biosynthesis enzymes, indicating that these signalling molecules are unlikely to be synthesized by *P. entomophila*. However, the GacS/GacA two-component system, another regulatory system involving a still unknown signalling molecule, plays a critical role in regulating *P. entomophila* virulence. This is of interest given our observation that the *pvf* gene products could synthesize a small molecule, which acts as a signalling factor. This is supported by the pleiotropic effect of a *pvfC* mutation, which impacts the expression of a subset of the Gac system target genes, and our observation that the pathogenicity of a *pvfC* mutant can be restored when extracts from culture supernatants of strains containing the *pvf* gene cluster are added to the growth medium. Considering that a transcriptional regulator is encoded by a neighbouring gene of the *pvf* cluster, it would have been tempting to imagine that the putative signalling molecule produced by the *pvf* gene products could exert its regulatory effect though this regulator. However, we demonstrated that this was not the case, as the *pseen0137* mutant is as virulent as the wild-type *P. entomophila* towards *Drosophila*. Either another transcriptional regulator is required, which would be localized somewhere else in the genome, or another regulatory mechanism is involved. Interestingly, both naturally produced and synthetically derived antibiotics can function as small molecules at subinhibitory concentrations, affecting transcription of many cellular functions (Yim *et al*., 2007). Their effect could be mediated by direct interactions with their target as antibiotics (ribosomal proteins or RNA polymerase), but precise mechanisms remain to be determined. Another putative regulatory mechanism could involve direct binding to mRNA secondary structures:

cyclic di-GMP, a secondary messenger known to be able to bind directly to proteins, was discovered to be sensed by a riboswitch class in mRNA that controls the expression of genes involved in numerous fundamental cellular processes (Sudarsan *et al*., 2008). Characterizing the structure of the signalling molecule synthesized by the *pvf* gene products should help us to better envision how it can exert its regulatory effect.

Collectively, our results indicate that we have identified a new type of signalling molecule, synthesized by the *pvf* gene products, which is involved in *P. entomophila* virulence, and does not interfere with the GacS/GacA signalization. Our data suggest that the target genes of the regulatory system involving the *pvf* genes and the target genes of the Gac system overlap. Identifying both these sets of target genes could help us to identify effectors important for interactions with *Drosophila*.

Conservation of the pvf *genes in other organisms*

The *pvf* genes are predicted to form a single transcriptional unit. Surprisingly, *pvfA* does not seem to play a major role in *P. entomophila* virulence, in contrast to *pvfB*, *pvfC* and *pvfD*. These three gene products are involved in the synthesis of a secondary metabolite that seems to act as a signalling molecule. It is tempting to speculate that PvfA could also be involved in this synthesis, but that the modification it introduces is not essential to the molecule activity. Another hypothesis could be that the effect of a *pvfA* mutation is smaller than a *pvfC* mutation because of some functional redundancy of PvfA. However, no other gene encoding a PvfA-like protein could be found in the *P. entomophila* genome.

In some bacteria (the four *B. cenocepacia* strains and *P. aeruginosa* PA7), the *pvf* gene cluster comprises an additional gene, predicted to encode a protein containing a cupin domain. Members of the cupin family are associated with secondary metabolite synthesis in *Streptomyces* (Dunwell *et al*., 2000). We can imagine that the small molecule synthesized by the *pvf* gene products in these bacteria may be somewhat different from the one synthesized in *P. entomophila*. It would be interesting to know if they share the same activity.

Remarkably, the *pvfC* homologue in *P. syringae* pv. *syringae* strain UMAF0158 (called *mgoA*) has also been associated with virulence (Arrebola *et al*., 2007). Indeed, a *mgoA* mutant is impaired in its ability to produce an antimetabolite toxin, called mangotoxin, which inhibits ornithine/arginine biosynthesis and contributes to virulence of tomato plants and epiphytic fitness (Arrebola *et al*., 2009). It is worth noting that the three sequenced strains of *P. syringae* do not produce mangotoxin, despite the presence of the *pvf* gene cluster in their genome (Arrebola *et al*., 2007). This suggests that MgoA is not directly involved in mangotoxin synthesis and could rather, as described here for PvfC, participate in the regulation of several products, one of them being the mangotoxin.

In conclusion, we have identified a new cluster of genes, termed *pvf* genes, which are involved in *P. entomophila* virulence. Our data indicate that these genes are required for the strong damage inflicted by *P. entomophila* to the *Drosophila* gut, and their absence allowed the repair of the intestinal epithelium and host survival. Therefore, this article sheds light on novel strategies used by entomopathogens to infect their host and suggests the existence of a new regulatory system specific of some *Pseudomonas* species.

Experimental procedures

Bacterial strains and culture conditions

Pseudomonas aeruginosa PAO1 (Gallagher *et al*., 2002) and *P. putida* KT2440 (Regenhardt *et al*., 2002) are part of the laboratory collection. *Escherichia coli* DH5α (Invitrogen) was used as the recipient strain for all plasmid constructs, and *E. coli* strain S17.1 (Simon *et al*., 1983) was used to conjugate plasmids into *P. entomophila*. *P. entomophila* was grown in LB for all experiments. *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^{-1} and tetracycline, 5 μg ml^{-1}. When *P. entomophila* was grown, antibiotics were used when necessary at the following concentrations: gentamicin, 50 μg ml^{-1} for liquid cultures and 150 μg ml^{-1} for solid media, tetracycline, 40 μg ml^{-1} and rifampicin, 30 μg ml^{-1}.

Construction of strains and plasmids

Construction of *P. entomophila* (Vodovar *et al*., 2005) deleted for the *gacA* gene (*Pe gacA*) or inactivated for the *pseen3044* (*etlC*) gene are described elsewhere (Vallet-Gely *et al*., 2010).

Insertion constructs for the *pseen0132*, *pseen2149*, *pseen2153*, *pseen3301*, *pseen2342*, *pseen2716*, *pseen2718*, *pseen5522*, *pseen3234*, *pseen2503*, *pseen0136* and *pseen0137* genes were generated by cloning an internal 500–800 bp fragment into the pINT non-replicative plasmid (Arne Rietsch, Case Western Reserve University), generating plasmids pINT132, pINT2149, pINT2153, pINT3301, pINT2342, pINT2716, pINT2718, pINT5522, pINT3234, pINT2503, pINT136 and pINT137. These constructs were conjugated into *P. entomophila* to inactivate the targeted genes by homologous recombination.

Deletion construct for the PKS cluster (from *pseen5523* to *pseen5536*) was generated by amplifying flanking regions by the PCR and then splicing the flanking regions together by overlap extension PCR. The resulting PCR products were then cloned into plasmid pEXG2 (Rietsch *et al*., 2005), yielding plasmids pEXΔPKS. This plasmid was then used to create strain *Pe ΔPKS*, containing a deletion of the genes from *pseen5523* to *pseen5536*, including the promoter sequence of *pseen5536*.

Deletion constructs for the *pseen0134*, *pseen0133*, *pseen0132* and *pseen0131* genes were generated using the

same principle, and this yielded plasmids pEXΔ134, pEXΔ133, pEXΔ132 and pEXΔ131, which allowed deletion of all but the last six codons of the *pseen0134* (*pvfA*) gene, deletion of the second half of the *pseen0133* (*pvfB*) genes, deletion of the last 843 codons of the *pseen0132* (*pvfC*) gene and removal of the first 18 codons (including the start codon) of the *pseen0131* (*pvfD*) gene. These plasmids were then used to create strains *Pe pvfA* (*Pe 134*), *Pe pvfB* (*Pe 1334*), *Pe pvfC* (*Pe 132*) and *Pe pvfD* (*Pe 131*), respectively, by allelic exchange. Deletions were confirmed by the PCR.

The *pseen5493–lacZ*, *pseen5175–lacZ*, *pseen0973–lacZ*, *pseen5522–lacZ* and *pseen1550–lacZ* translational reporter fusions were constructed according to (Suh *et al*., 2004). Approximatively 500 bp upstream of each gene were cloned into the pSS231 plasmid, in such a way that the ATG of each gene was fused in frame to the tenth codon of the *lacZ* gene. The EcoRI/AatII fragment was then subcloned into the Mini CTX lacZ (Hoang *et al*., 2000), for antibiotic compatibility purposes, generating plasmid Mini CTX F3045–lacZ. This plasmid was then conjugated into wild-type *P. entomophila*, a *gacA* mutant, and a *pvfC* mutant, creating strains *Pe* F5493–lacZ, *Pe* F5175–lacZ, *Pe* F0973–lacZ, *Pe* F5522–lacZ, *Pe* F1550–lacZ, *Pe gacA* F5493–lacZ, *Pe gacA* F5175–lacZ, *Pe gacA* F0973–lacZ, *Pe gacA* F5522–lacZ, *Pe gacA* F1550–lacZ, *Pe pvfC* F5493–lacZ, *Pe pvfC* F5175–lacZ, *Pe pvfC* F0973–lacZ, *Pe pvfC* F5522–lacZ and *Pe pvfC* F1550–lacZ by homologous recombination. These strains carry both the promoter fusion and the wild-type copy of each gene.

The plasmids were made by cloning PCR-amplified DNA fragments containing each of the *pvfB* (*pseen0133*), *pvfC* (*pseen0132*) and *pvfD* (*pseen0131*) from *P. entomophila* into the pPSV35 vector (Rietsch *et al*., 2005), generating plasmids pPSVpvfB, pPSVpvfC, pPSVpvfD. Cloning of the *pvf* gene cluster was performed in two successive steps; first, a DNA fragment containing both *pvfC* and *pvfD* was PCR amplified digested using EcoRI and HindIII, and cloned into the pPSV35 vector, yielding plasmid pPSVpvfCD. Then, a PCR-amplified fragment containing *pvfA*, *pvfB* and part of *pvfC* was digested using EcoRI and ScaI, and cloned into the pPSVpvfCD plasmid, yielding plasmid pPSVpvf.

A promoterless version of the pPSV35 was obtained after excision of the *placUV5* promoter on a DraIII/EcoRI fragment, end filling and religation. The *lacZ* gene was then cloned on a KpnI/HindIII fragment from the p18lacZ (A. Rietsch, unpubl. work), yielding plasmid pPlacZ. A fragment of approximately 500 bp containing the promoter region of *rsmY* and *rsmZ* was cloned into the Mini CTX lacZ (Hoang *et al*., 2000), and then subcloned in the pPlacZ plasmid on a KpnI/aatII fragment, yielding plasmid pPrsmY–lacZ and pPrsmZ–lacZ.

All primer sequences are available upon request.

β-Galactosidase activity

Cells were grown at 30°C in LB. Media were supplemented as needed with gentamicin (25 μg ml^{-1}) and IPTG at the concentration indicated. Cells were permeabilized with sodium dodecyl sulfate and $CHCl_3$ and assayed for β-galactosidase activity as described previously (Dove and Hochschild, 2004). Assays were performed at least three times in triplicate on separate occasions. Representative data sets are shown below. The values are averages based on one experiment.

Fly stocks

Flies carrying a *Diptericin–lacZ* reporter construct [*Dpt–lacZ* (Reichhart *et al*., 1992)] were used for all survival experiments. OregonR (OrR) flies were used as a standard wild-type strain for RT-qPCR analysis, live imaging and immunofluorescence. Flies carrying the *esg-Gal4*; *UAS-GFP* reporter construct (Yagi and Hayashi, 1997; Micchelli and Perrimon, 2006) were used to monitor intestinal stem cell proliferation.

Drosophila stocks were maintained at 25°C.

Virulence assay against D. melanogaster

To perform infection experiments, bacterial cultures were pelleted by centrifugation after 24 h of growth, and pellets were adjusted to OD_{600} = 100 for standard experiments, and OD_{600} = 5 for sub-lethal experiments. Pellets (120 μl) were applied to a paper filter disk that completely covered the agar surface of a standard fly culture vial. Thirty 4- to 8-day-old adult female flies were starved for 3 h at 29°C in empty vials prior to transfer to vials containing bacteria. Infections were maintained at 29°C and mortality was monitored daily for 4 days. Virulence assays were performed at least three times in triplicate on separate occasions. Representative data sets are shown. The values are averages based on one experiment.

RT-qPCR analysis

Total RNA was extracted from whole flies (five 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 (Romeo and Lemaitre, 2008). 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, giving similar results. Averages of three experiments are shown.

Live imaging and immunofluorescence

Imaging was performed as previously described (Buchon *et al*., 2009b). Briefly, GFP observation was performed using live imaging by dissecting guts in PBS and immediately mounting them in an anti-fading agent. Dead cells were detected using acridine orange staining (Invitrogen). For detection of PH3-positive cells by immunofluorescence, guts were dissected and fixed in PBS containing 0.1% Tween 20 (PBT) and 4% paraformaldehyde and rinsed in PBT. Guts were incubated with the primary antibody (anti-PH3) diluted in PBT + 1% BSA. Anti-PH3 was revealed with an Alexa594-coupled anti-mouse antibody (Invitrogen). Nuclei were stained by DAPI (Sigma). All the images were performed using a Zeiss Axioimager Z1.

Signal extraction from culture supernatants

Extraction of molecule from culture supernatant was performed essentially as described (Heeb *et al*., 2002; Dubuis *et al*., 2006). Briefly, 100 ml of bacterial cultures were pelleted by centrifugation after 24 h of growth. Supernatants were passed through a

0.2-μm-pore-size filter (Millipore Corporation, Bedford, MA), the pH was adjusted to 5.0 with HCl, and the preparation was extracted three times with one-third volume of dichloromethane. The extracts were pooled, dried with anhydrous Na_2SO_4, filtered through Whatman paper, evaporated to dryness and dissolved in 1 ml of methanol. For supplementation of growth medium with extracts, 150 μl of extract was added to 15 ml of bacterial cultures, which were subsequently allowed to grow for 20 h. Infection experiments were performed as previously described.

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