Title:

Quorum sensing regulation in *Erwinia carotovora* affects development of *Drosophila melanogaster* infected larvae

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Running Title: Quorum sensing regulates *evf* in *Drosophila*

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Abstract

Multi-host bacteria must rapidly adapt to drastic environmental changes, relying on integration of multiple stimuli for an optimal genetic response. *Erwinia spp.* are phytopathogens that cause soft-rot disease in plants. *Erwinia carotovora Ecc15* is used as a model for bacterial oral-route infection in *Drosophila melanogaster* as it harbors a gene, the *Erwinia* virulence factor (Evf), which has been previously shown to be a major determinant for infection of *D. melanogaster* gut. However, the factors involved in regulation of *evf* expression are poorly understood. We investigated whether *evf* could be controlled by quorum sensing since, in the *Erwinia* genus, quorum sensing regulates pectolytic enzymes, the major virulence factors needed to infect plants. Here, we show that transcription of *evf* is positively regulated by quorum sensing in *Ecc15 via* the acyl-homoserine lactone (AHL) signal synthase ExpI, and the AHL receptors ExpR1 and ExpR2. Moreover, we demonstrate that the GacS/A two-component system is partially required for *evf* expression. We also show that the load of *Ecc15* in the gut depends upon the quorum sensing-mediated regulation of *evf*. Furthermore, we demonstrate that larvae infected with *Ecc15* suffer a developmental delay as a direct consequence of the regulation of *evf via* quorum sensing. Overall, our results show that *Ecc15* relies on quorum sensing to control production of both pectolytic enzymes and Evf. This regulation influences the interaction of *Ecc15* with its two known hosts, indicating that quorum sensing and GacS/A signaling systems may impact bacterial dissemination *via* insect vectors that feed on rotting plants.

Significance

Integration of genetic networks allows bacteria to rapidly adapt to changing environments. This is particularly important in bacteria that interact with multiple hosts. *Erwinia carotovora Ecc15* is a plant pathogen that uses *Drosophila melanogaster* as a vector. To interact with these two hosts, *Ecc15* uses two different sets of virulence factors: plant cell wall-degrading enzymes to infect plants and the *Erwinia* virulence factor (*evf*) to infect *Drosophila*. Our work shows that, despite the virulence factors being different, both are regulated by homoserine lactone quorum sensing and the two component GacS/A system. Moreover, we show that these pathways are essential for *Ecc15* loads in the gut of *Drosophila* and that this interaction carries a cost to the vector in the form of a developmental delay. Our findings provide evidence for the importance of quorum sensing regulation in the establishment of multi-host interactions.

Introduction

Insects play an important role in the dissemination of microorganisms that cause both human and plant diseases. This dissemination may be an active process whereby microbes develop strategies to interact with insects and use them as vectors (1, 2). To do so, bacteria must have the ability to persist within the host (either lifelong or transiently), evading or resisting its immune system in order to abrogate their elimination (3, 4). The host vector will respond with a battery of innate defenses, such as production of antimicrobial peptides and reactive oxygen species as well as behavioral strategies (e.g. avoidance), and physiological responses (e.g. increased peristalsis) (5–9). The successful

60 establishment of these interactions, from the bacterial perspective, ultimately depends on
61 maximizing the fitness of the microorganism and minimizing the impact on the fitness of
62 the vector host (1). Phytopathogenic bacteria such as *Phytoplasma sp., Xylella fastidiosa*,
63 *Pantoea stewartii* (formerly *Erwinia stewartii*), or *Erwinia carotovora* (also known as
64 *Pectobacterium carotovorum*), are among those known to establish close associations
65 with insects and to rely on these hosts as vectors, presumably to facilitate rapid
66 dissemination among plants (10–13). Thus, understanding the molecular mechanisms
67 governing the establishment of these interactions is crucial to prevent insect-borne
68 diseases.

69 Bacteria from the *Erwinia* genus produce pectolytic enzymes that degrade plant
70 tissue, causing soft root-disease (14). These bacteria survive poorly in soil, overwinter in
71 decaying plant material (14), and use insects, including *Drosophila* species (12, 15) as
72 vectors. Specifically, the non-lethal interaction between the phytopathogen *Erwinia*
73 *carotovora* (strain *Ecc15*) and *Drosophila melanogaster* has been used as a model to study
74 bacteria-host interactions. Oral infections with *Ecc15* lead to a transient systemic
75 induction of the immune system in *D. melanogaster* and consequent production of
76 antimicrobial peptides (7, 16). These responses are strain-specific and highly dependent
77 on the expression levels of the *Erwinia* virulence factor gene (*evf*) (17), which promotes
78 bacterial infection of the *Drosophila* gut (18). Additionally, expression of *evf* requires the
79 transcriptional regulator Hor (17), but the signals required for the activation of this
80 regulator remain unknown.

Quorum sensing has recently been shown to be important in the regulation of bacterial traits that affect the persistence and/or virulence of bacteria in insects (19–22). Many bacteria use quorum sensing to regulate gene expression as a function of population density (23, 24). This cell-cell signaling mechanism relies on the production, secretion, and response to extracellular signaling molecules called autoinducers (24–26). Bacteria from the *Erwinia* genus produce a mixture of plant cell wall-degrading enzymes (PCWDE), which are the major virulence factors used to degrade plant tissues and potentiate bacterial invasion of the plant host (27–30). In these bacteria, expression of these PCWDE is tightly regulated by two main signaling pathways: the acyl-homoserine lactone (AHL) quorum sensing system, and the GacS/A two-component system (31–34). Typically, the AHL quorum sensing system present in *Erwinia spp.* includes the AHL synthase ExpI (35), and two AHL receptors, ExpR1 and ExpR2 (36), which are homologues to the canonical LuxI/R quorum sensing system first identified in *Vibrio fischeri* (37–39). The GacS/A two-component system is also activated at high cell density, and, like the AHL quorum sensing system, regulates virulence in many Gram-negative pathogenic bacteria (40–45). Given the importance of these two signal transduction pathways for the expression of the major plant virulence factors in *Erwinia spp.,* we investigated whether quorum sensing and the GacS/A system also regulate *evf* expression in *Ecc15*. Additionally, we tested whether these signaling pathways are important for *Ecc15* infection, and determined the consequences of this interaction for the insect host. Our results show that PCWDE and *evf* expression in *Ecc15*, which are required for the interactions with plants and insects, respectively, are both regulated by the same quorum sensing signaling

pathway. Moreover, we demonstrate that *evf* expression has a negative effect on the insect host as it leads to a developmental delay in larvae infected with *Ecc15*.

Results

The expression of *evf* is regulated by both AHL-dependent quorum sensing and the GAC system

We first investigated whether activation of the production of PCWDE in *Ecc15* requires both the AHL quorum sensing system and the GacS/A two-component system (GAC), as occurs in other members of the *Erwinia* (or *Pectobacterium*) genus (32, 35, 46). We constructed deletion mutants of *expI* and *gacA,* the genes encoding homologues of the AHL-synthase and the response regulator of the GAC system, respectively. We determined whether any of these mutations cause a growth defect in *Ecc15*, and observed no difference in growth compared to the WT strain (Fig. S1). We then measured pectate lyase activity in supernatants of cultures from *Ecc15* WT, *expI* or *gacA* mutants, as this is one of the PCWDE typically secreted by *Erwinia spp.*. As shown in Fig. 1a (and replicate experiments in Fig. S2), both the *expI* and the *gacA* mutants exhibit pronounced reductions in pectate lyase activity when compared to the WT (TukeyHSD test, $p<0.001$, Fig. S2C). Addition of a mixture of exogenous 3-oxo-C6-HSL and 3-oxo-C8-HSL, the major AHLs produced by *Erwinia carotovora* (46), to an *expI* mutant culture was sufficient to restore production of this PCWDE to higher levels than the WT (Fig. 1A, TukeyHSD test $p<0.001$, Fig. S2C). In addition, both the *expI and gacA* mutants are impaired in virulence to the plant host, which we tested by measuring the mass of macerated tissue in potato

tubers inoculated with these genotypes (Fig. 1B, TukeyHSD test $p<0.001$, Fig. S2F). In contrast, the *evf* mutant shows no significant difference in maceration with respect to the WT (Fig. 1B and Fig. S2D-F). Altogether, these results show that production of pectate lyase, as well as plant host-virulence, are regulated by both the AHL and GAC systems in *Ecc15*, as occurs in other *Erwinia spp.,* where *expI* and *gacA* mutants have been shown to be avirulent (34, 47, 48). Moreover, we show that *evf* is not necessary for plant infection (Fig. 1B and Fig. S2D-F).

To investigate whether *evf* expression is also regulated by these two systems, we analyzed the expression of a transcriptional reporter consisting of a Green Fluorescent Protein (GFP) fused to the promoter of *evf* (P_{evf}::*gfp*) in mutants of either AHL quorum sensing or GAC signaling systems. We observed that the expression of the P_{evf}::*gfp* is reduced in the *expI* mutant when compared to the WT (TukeyHSD test, $p<0.001$), and that this expression can be restored if exogenous AHLs are supplied to the culture (Fig. 1C, Fig. S2G-I). In the *gacA* mutant, expression of the *evf* promoter is also reduced compared to the WT, but not as much as in the *expI* mutant (Fig. 1C, TukeyHSD test $p<0.001$, Fig. S2G-I). Since it was previously shown that mutants in the GAC system produce less AHLs (34), we asked if the difference observed between the WT and the *gacA* mutant could be solely explained by the lower levels of AHLs produced by the latter. However, addition of exogenous AHLs to the cultures of a *gacA* mutant did not restore the levels of P_{evf}::*gfp* expression to WT levels (Fig. S3). Therefore, we conclude that the *gacA* phenotype regarding *evf* expression is mostly independent of AHLs. Overall, these results show that

full activation of both *evf* expression and PCWDE activity is dependent on quorum sensing regulation *via* AHLs, and, to a lesser extent, on activation of the GAC system.

In the absence of AHLs, the AHL receptors ExpR1 and ExpR2 lead to repression of virulence traits such as PCWDE (35, 49). These receptors are DNA binding proteins that act as transcriptional activators of *rsmA,* which encodes a global repressor of quorum sensing-regulated genes in *Erwinia spp.* (36, 49, 50). Upon AHL binding, these receptors lose their ability to bind DNA, resulting in decreased expression of *rsmA* and, consequently, increased expression of virulence traits (51, 52). To determine whether ExpR1 and ExpR2 also mediate AHL-dependent regulation of *evf* expression, we constructed deletions of these two genes in the *expI* background. We measured expression of the P_{evf}::*gfp* reporter in this *expI expR1 expR2* triple mutant, with or without exogenous AHLs. Because AHLs block activation of RsmA *via* ExpR1 and ExpR2 (51, 52), deletion of *expR1 and expR2* in the *expI* background is expected to result in the de-repression of *evf*. Consistent with this prediction, P_{evf}::*gfp* expression is higher in the *expI expR1 expR2* than in the *expI* single mutant (Fig. 2A, TukeyHSD test $p<0.001$, Fig. S4A-C). However, the expression levels of P_{evf}::*gfp* are lower in the *expI expR1 expR2* than those of the WT (Fig. 2A, TukeyHSD test, $p<0.001$, Fig. S4A-C). The fact that deletion of these two receptors in the *expI* background is not sufficient to fully restore expression of *evf* to WT levels indicates that additional regulators control the expression of *evf*. Nonetheless, while addition of exogenous AHLs to a culture of an *expI* mutant increases P_{evf}::*gfp* expression, it remains unaltered in the triple *expI expR1 expR2* mutant (Fig. 2A, TukeyHSD test $p=1$, Fig. S4A-C). Therefore, AHL-

dependent regulation of *evf* expression is mediated by *expR1* and *expR2,* as is also the case for the regulation of PCWDE in other *Erwinia spp.* (34, 36, 49).

Regulation of *evf* by AHL quorum sensing is mediated by *hor*

It was previously shown that Hor, a global regulator of diverse physiological processes in many animal and plant bacterial pathogens (53), is a positive regulator of *evf* (17) and that, as in other *Erwinia spp.*, *hor* is regulated by quorum sensing (54). Therefore, we asked if AHL-dependent regulation of *evf* is *via hor*. We analyzed the expression of the P_{evf}::*gfp* reporter in a *hor* mutant, and found that it is lower than in the WT, and as low as in the *expI* mutant (Fig. 2A). Moreover, we observed that addition of exogenous AHLs to a *hor* mutant does not restore the expression of *evf* (Fig. 2A, TukeyHSD test p=1, Fig. S4A-C). We next cloned the *hor* gene under the control of a *lac* promoter in the plasmid containing the P_{evf}::*gfp* fusion, and measured *evf* expression levels in the *expI* and *gacA* mutants expressing or not the *hor* gene. We observed that expression of *hor* in either the *expI* or the *gacA* mutants restores *evf* expression to levels similar to those of the WT (Fig. 2B, TukeyHSD test p<0.001, Fig. S4D-F). Therefore, regulation of *evf* is mediated by both the AHL and the GAC systems and occurs via *hor*. Next, we asked whether these systems regulate *hor* itself by analyzing the expression of a *hor* promoter fusion (P_{hor}::*gfp*) in *expI* and *gacA* mutants. As for the *evf* reporter, we observed that, P_{hor}::*gfp* expression is lower in an *expI* mutant when compared to the WT (Fig. 2C, TukeyHSD test p<0.001, Fig. S4G-I). Moreover, this expression can be complemented to WT levels by the addition of exogenous AHLs to the growth medium of the *expI* mutant (Fig. 2C, TukeyHSD test p=0.08,

Fig. S4G-I). These data demonstrate that *hor* expression is regulated by AHLs and is necessary for the increase of *evf* expression mediated by AHLs.

Infection by *Ecc15* causes a developmental delay in *D. melanogaster* larvae dependent on quorum sensing and GAC regulation of *evf* expression

It is known that Evf promotes infection in the *D. melanogaster* gut (18, 19). To examine the effects of down-regulation of *evf* on quorum sensing and GAC mutants we measured *Ecc15* loads upon oral infection. We inoculated *Ecc15* WT, *evf*, *expI* or *gacA* into *D. melanogaster* L3 stage larvae, and assessed the dynamics of bacterial loads by counting the number of colony forming units (CFU) of *Ecc15* over time. As previously reported, *Ecc15* infection is transient and larvae are able to clear it after 24 hours (Fig. 3 and (18)). Additionally, we observed that the rate of elimination of the bacteria from the larval gut is not significantly different between the WT and the *evf*, *gacA*, and *expI* mutants (Fig3, lmm, Chi-square test p=0.27). However, we also observed that *Ecc15* WT loads were approximately ten times higher compared to the loads of the *evf* mutant when considering the entire infection period (Fig. 3, TukeyHSD test p<0.001, Fig. S5), confirming that *evf* is required for optimal infection of the larval gut by *Ecc15*. Importantly, a similar trend was observed when comparing the WT to either of the two mutants impaired in *evf* expression: *gacA* or *expI* (Fig. 3. TukeyHSD test p<0.001, Fig. S5), revealing the importance of quorum sensing-regulation and the GAC system in the infection process. Taken together, our data show that *evf* provides *Ecc15* with the ability to reach high loads in the insect gut, but does not increase its capacity to survive inside it.

Next, we asked if infection of *D. melanogaster* larvae by *Ecc15* has an effect on larval development. To investigate this possibility, we infected *D. melanogaster* L3 stage larvae orally with *Ecc15* WT or an *evf* mutant and followed their development over time. We found that infection by WT *Ecc15* delays *D. melanogaster* larvae passage to pupal stage an average of 49 hours, when compared to non-infected larvae (Fig. 4A and FigS6, TukeyHSD test $p<0.001$, Fig. 4B). Moreover, we show that this strong delay is *evf*-dependent, since larvae exposed to an *evf* mutant only show a delay of 8 hours when compared to non-infected larvae (TukeyHSD test, $p<0.001$, Fig4B). We then asked if the mutants in the quorum sensing pathway and GAC system, which have low expression of *evf*, would show a similar phenotype. We observed that larvae exposed to the *expI* mutant, which has very low expression of *evf*, also show only a 4 hour delay with respect to non-infected larvae, similar to the *evf* mutant (TukeyHSD test, $p<0.001$, Fig4B). Interestingly, larvae infected with the *gacA* mutant, which has intermediate levels of *evf* expression, show an intermediate developmental delay, taking an average of 26 hours longer than non-infected larvae to reach the pupal stage (TukeyHSD test, $p<0.001$, Fig4B). Since the developmental delay correlated with the levels of *evf* expression in the strains tested, we next examined whether constitutive overexpression of *evf* would exacerbate the phenotype. We observed that larvae infected with a WT *Ecc15* overexpressing *evf* died before reaching the pupal stage (Fig. 4C-D). These results show that *Ecc15* has a negative impact on larval development and this effect requires both *evf* and the quorum sensing and GAC regulatory systems.

DISCUSSION

Erwinia spp. are phytopathogenic bacteria thought to depend on insects to spread among plant hosts (1, 12, 13). To interact with both plants and insects, *Ecc15* relies on different traits that seem to be specific for the interaction with each host. In this bacterium, PCWDE are the major virulence factors required for plant infection (40) and Evf is required to infect *D. melanogaster,* but not necessary to infect potato tubers (Fig. 1B and (16, 17)). It was not known whether *Ecc15*, which relies on multiple hosts for survival, regulates host-specific traits using the same or different signal transduction networks. Here we showed that the AHL-dependent ExpI/ExpR system, which regulates plant virulence factors (33, 35, 36, 49) is also essential for the expression of the insect virulence factor *evf*, suggesting that the signal transduction networks regulating traits required across hosts are the same. An *expI* mutant had lower levels of *evf* expression than the WT which could be restored by addition of exogenous AHLs to the growth medium. We also demonstrated that the GAC system, that is thought to respond to the physiological state of the cell (42) and is involved in regulation of plant virulence factors (41, 55) is also necessary for full expression of *evf*. Additionally, we showed that regulation by these two networks occurs through *hor*, a conserved transcriptional regulator of the SlyA family (56), previously found to be regulated by quorum sensing in another *E. carotovora* strain (54). ExpR1 and ExpR2 AHL receptors function as activators of *rsmA*, the global repressor of the AHL-regulon; therefore, we expected the *expI expR1 expR2* mutant to have the same levels of *evf* expression as the *expI* mutant supplemented with AHLs. However, we found that the *expI expR1 expR2* mutant has lower levels of *evf* expression than both the *expI*

supplemented with AHLs and the WT. Moreover, we showed that complementation of the *expI expR1 expR2* mutant with AHLs does not change the level of *evf* expression. These results show that *expR1* and *expR2* are required for *Ecc15's* response to AHLs, but also indicate that an additional AHL-independent regulator, is playing a role in the regulation of *evf* in this bacterium. One possibility is that *Ecc15* has additional orphan *luxR* genes, DNA binding proteins homologous to LuxR that lack a cognate AHLs synthase. These orphan genes are divided in two categories, those that have both a LuxR DNA and an AHL binding domain, such as ExpR2, and those that have only the typical LuxR DNA binding domain (57), such as *vqsR* in *Pseudomonas aeruginosa.* In this bacterium, in response to an unknown signal, *vqsR* has been found to downregulate expression of virulence through binding to the promoter region of the quorum sensing receptor *qscR,* inhibiting its expression without responding to AHLs (58). Because addition of exogenous AHLs to the *expI expR1 expR2* mutant does not change the level of *evf* expression, this unknown regulator is more likely to lie within the second category of orphan LuxR receptors. Our data also suggests that this unknown regulator could be repressed by *rsmA,* since the *expI* mutant shows lower levels of *evf* expression than *expI expR1 expR2*. In *Erwinia spp.* another layer of regulation required for PCWDE expression is the detection of external environmental signals like pectin, a component of the plant cell wall (34, 55, 59, 60, 35, 51, 47, 48). In the absence of plant signals, transcription of PCWDE is repressed. Unlike in the regulation of PCWDE in *Erwinia spp.*, in our experimental setting we have no evidence for the need of a host signal since we can detect *evf* expression in cells grown in LB without the need for other signals. However, this does not exclude the possibility that

277 environmental signals, perhaps related to insect derived compounds, have a role in the
278 overall levels of *evf* expression.

279 It has been hypothesized that *evf* was horizontally acquired by *Ecc15* and a few
280 other *Erwinia spp*. As these phytopathogens often use insects as vectors, one hypothesis
281 for the selective benefit of acquiring *evf* is that this gene might be important to favor
282 bacterial transmission by strengthening the interaction of *Ecc15* with *Drosophila*. This
283 hypothesis is supported by our results showing that *evf* allows *Ecc15* to have higher loads
284 at the initial stage of *Drosophila* larval infection. However, the rate of *Ecc15* elimination
285 post-infection was the same in WT and an *evf* mutant. This suggests that *evf* is promoting
286 transmission of *Ecc15* by increasing the overall number of bacteria that reach the gut.
287 Moreover, we show here that larvae infected with *Ecc15* are developmentally impaired
288 when compared to non-infected larvae, and this developmental delay is dependent on
289 *evf.* These results are in agreement with previous reports showing that larvae infected
290 with WT *Ecc15* were smaller due to inhibition of the larval proteolytic activity promoted
291 by *Drosophila*-associated *Lactobacillus* species (61). Additionally, infection of *Drosophila*
292 adults and larvae with WT *Ecc15* causes cell damage, which induces epithelial cell death,
293 leading to activation of immunity, stem cell regeneration programs and
294 differentiation/modification of the cellular structure of the gut, essential for its repair (7,
295 16, 62). These studies, together with our results, show that *evf* expression in *Ecc15* has an
296 overall deleterious effect on the host, and thus acquisition of *evf,* which enables higher
297 host loads and is presumably beneficial for bacterial transmission, seems to have resulted
298 in a tradeoff for host fitness.

Due to a lack of genetic information, tracing the evolutionary history of this protein is challenging. It was previously reported that, besides *Ecc15*, *evf* was only identified in strain *Ecc1488* (16, 17). By comparing the amino acid sequence of Evf to recent genome databases, we found only a few more candidate ortholog proteins with amino acid sequence identity higher than 60% (Table S3). The highest sequence similarities found, besides those of other *Erwinia spp.*, corresponded to proteins from *Cedecea neteri, Enterobacter AG1, Rahnella sp., Klebsiella aerogenes* and *Escherichia coli* (Table S3). *K. aerogenes and E. coli* are ubiquitous bacterial species that can colonize the gut of different animals, particularly mammals, but also insects (63–65). Similarly to *Erwinia spp.*, *Rahnella sp*. and *C. neteri* are bacterial species often isolated from plants that also establish gut associations with insects (64, 66, 67). Enterobacter AG1 is a bacterial species isolated from the gut of mosquitos that has been shown to decrease the ability of *Plasmodium falciparum* to colonize the gut (68, 69). Since the structural fold of Evf is unique (70) and that protein structure is more conserved than sequence identity (71), we predicted the secondary structures of these ORFs using phyre2 (72). We found that the predicted secondary structure of all five ORFs is identical to Evf (Table S3). Importantly, the cysteine residue (position 209), which in *Ecc15* Evf is palmitoylated, a post-translational modification essential for its function (70), is conserved in all the five ORFs. Interestingly, *evf*-like genes with low amino acid sequence identity (lower than 40%), but with a predicted secondary structure highly similar to that of the Evf (72), can be found in other bacteria such as *Vibrio sp.* or the major insect pathogen *Photorhabdus luminescens* ((18) Locus PLU2433). *P. luminescens* colonizes the gut of *Heterorhabditis bacteriophora,* an

321 insect-preying nematode (73, 74). The nematode enters through the insect's respiratory
322 and/or digestive tract and regurgitates the bacteria into its hemolymph. Once in the
323 hemolymph, *Photorhabdus* produces a battery of toxins that kills the insect allowing the
324 nematode to feed on the corpse, favoring *Photorhabdus* recolonization (75–77).
325 *Photorhabdus* possesses several genes possibly involved in the establishment of the
326 interaction with the host, many of which are regulated by quorum sensing (78, 79). Thus,
327 it is possible that the Evf ortholog from *Photorhabdus* is involved in the mechanisms
328 required for colonization of the nematode, or in the pathogenicity towards the insect. Our
329 results indicate that Evf orthologs can be found in bacteria with apparently different
330 lifestyles. However, all of these bacteria encounter multiple hosts mainly through the gut,
331 including insects, and undergo rapid environmental changes. It is possible that Evf has a
332 conserved role in host transition mainly through insect colonization or pathogenesis.
333 Quorum sensing regulation is associated with tight control of density dependent
334 activation of genes encoding functions that are often essential for the establishment of
335 host-microbe interactions (26). For instance, in the interaction between the squid
336 *Euprymna scolopes* and *V. fischeri,* mutants in the quorum sensing system are less
337 efficient in persisting in the light organ, being outcompeted by other strains (80, 81). Here
338 we show that in *Ecc15*, besides regulating PCWDE in plant infections, employs quorum
339 sensing for the *evf*-mediated increased bacterial loads in *Drosophila* larvae. Our study also
340 demonstrates that the quorum sensing and GAC regulatory pathways have a strong effect
341 in the Evf-mediated developmental delay caused by *Ecc15.* Moreover, overexpression of
342 *evf* leads to a complete developmental arrest of larvae, eventually killing them. Therefore,

one possible benefit of having *evf* expression under the control of these networks might be to minimize the detrimental effect that the *evf*-dependent infection has on the insect host while still enabling a transient infection. On the other hand, insects are attracted to rotten plant tissue, and if *evf* is important for promoting the interaction of *Ecc15* with its insect vector (*Drosophila)*, synchronization of the expression of *evf* and the PCWDE might have been selected as advantageous for bacterial dissemination. This phenomenon, called predictive behavior, is particularly common in symbiotic relationships where the microbe often experiences a predictable series of cyclic environments (82). In mammalian hosts, a very predictable change when transitioning from the outside environment to the oral cavity is the immediate increase in temperature followed by a decrease in oxygen. This phenomenon has been described for *E. coli* gut colonization where, coupled to an increase in temperature, downregulation of genes related to aerobic respiration is observed (83). In the case of *Ecc15* it is possible that control of PCWDE and *evf* expression is intertwined so that following colonization of the plant, *evf* expression is triggered, anticipating the appearance of the insect vector which is attracted to rotten plant tissue, and thus maximizing the probability of establishing the interaction with this host vector.

Our results show that, in *Ecc15*, the regulatory networks responding to self-produced quorum sensing signals and physiological cues sensed by the GAC system are used to control expression of traits required to infect different hosts. Thus, the signal transduction mechanisms are the same even though the functions involved in the interactions with each plant or insect host are largely different. Therefore, our findings

reinforce the central role of quorum sensing in the regulatory circuitry controlling the array of traits used by bacteria to interact with diverse hosts.

Materials and Methods

Bacterial strains, plasmids, and culture conditions.

The strains and plasmids used in this study are listed in Table S1 of the supplementary material. All bacterial strains used are derived from wild type (WT) *Ecc15* strain (7). *Ecc15* and mutants were grown at 30°C with aeration in Luria-Bertani medium (LB). When specified, medium was supplemented with 0.4% polygalacturonic acid (PGA; Sigma P3850), to induce the expression of PCWDEs. *E. coli* DH5α was used for cloning procedures and was grown at 37°C with aeration in LB. When required, antibiotics were used at the following concentrations (mg liter^{-1}): ampicillin (Amp), 100; kanamycin (Kan), 50; spectinomycin (Spec), 50; chloramphenicol (Cm), 25. To assess bacterial growth, optical density at 600 nm (OD_{600}) was determined in a Thermo Spectronic Helios delta spectrophotometer.

Genetic and molecular techniques.

All primer sequences used in this study are listed in Table S2 in supplemental material. *P. carotovorum Ecc15* deletion mutants listed in Table S1 were constructed by chromosomal gene replacement with an antibiotic marker using the λ-Red recombinase system (84). Plasmid pLIPS, able to replicate in *Ecc15* and carrying the arabinose-inducible λ-Red recombinase system was used (34). Briefly, the DNA region of the target gene,

386 including approximately 500 bp upstream and downstream from the gene, was amplified
387 by PCR and cloned into pUC18 (85) using restriction enzymes. These constructs, containing
388 the target gene and its flanking regions, were divergently amplified by PCR, to introduce a
389 *Xho*I restriction site in the 5' and 3' regions and to remove the native coding sequence of
390 the target gene. The kanamycin cassette from pkD4 was amplified with primers also
391 containing the *Xho*I restriction site. The fragment containing the kanamycin cassette was
392 then digested with *Xho*I and was introduced into the *Xho*I-digested PCR fragment carrying
393 the flanking regions of the target gene. The final construct, containing the kanamycin
394 cassette flanked by the upstream and downstream regions of the target gene was then
395 amplified by PCR, and approximately 2 micrograms of DNA were electroporated into the
396 parental strain (FDV31) expressing the λ-Red recombinase system from pLIPS, to favour
397 recombination. To construct the plasmid carrying the promoter *evf* fused to GFP
398 (pFDV54), a fragment of 503 bp containing the *evf* promoter was amplified from WT *Ecc15*
399 DNA with the primers P1194 and P1195. This fragment was then digested with *Hin*dIII and
400 *Sph*I and ligated to pUC18. GFP was amplified from the pCMW1(86) vector using primer
401 P0576 and P0665. Both the GFP and pUC18-P_{evf} were digested with *Sph*I and *Bam*HI,
402 ligated and 2 µl of the ligation reaction were used to transform Dh5α (pFDV54). The same
403 procedure was used for the P_{hor}::*gfp* fusion using primers P1351 and P1352 for promoter
404 amplification (493 bp) and primers P1353 and P1354 for GFP amplification. Digestions
405 were made with enzymes *Hin*dIII/*Pst*I and *Pst*I/*Xba*I (pFDV84). For *hor* overexpression, a
406 *Nco*I site was introduced in pOM1-P_{evf}::*gfp* with primers P1309 and P1310. *hor* was
407 amplified using primers P1311 and Primers 1312 from WT template DNA. Then both the

plasmid and the fragment carrying *hor* were digested with *Nco*I and *Sac*I and subsequently ligated (pFDV104).

PCR for cloning purposes was performed using the proofreading Bio-X-ACT (Bioline) enzyme. Other PCRs were performed using Dream Taq polymerase (Fermentas). Digestions were performed with Fast Digest Enzymes (Fermentas), and ligations were performed with T4 DNA ligase (New England Biolabs). All cloning steps were performed in either *E. coli* DH5α or WT *Ecc15*. All mutants and constructs were confirmed by PCR amplification and subsequent Sanger sequencing performed at the Instituto Gulbenkian de Ciência sequencing facility.

Pectate lyase activity assay.

Ecc15 and mutants were grown overnight in LB with 0.4% PGA, inoculated into fresh media to a starting OD_{600} of 0.05 and incubated at 30°C with aeration. After 6 hours of incubation, aliquots were collected to evaluate growth and to analyse pectate lyase (Pel) activity in cell-free supernatants, using the previously described procedure (55) based on the thiobarbituric acid colorimetric method (87). Each experiment included at least 5 independent cultures per genotype, and was repeated on 3 independent days.

Plant virulence assay.

Plant virulence was analysed by assessing the maceration of potato tubers with the protocol adapted from (34, 88). Potatoes were washed and surface sterilized by soaking for 10 min in 10% bleach, followed by 10 min in 70% ethanol. Overnight cultures in LB

broth were washed twice and diluted to an OD600 of 0.05 in phosphate-buffered saline (PBS). Thirty-microliter aliquots were then used to inoculate the previously punctured potatoes. Potato tubers were incubated at 28°C at a relative humidity above 90% for 48 h. After incubation, potatoes were sliced, and macerated tissue was collected and weighed.

Promoter expression assays.

Ecc15 carrying the different plasmid-borne promoter reporter fusions were grown overnight in LB supplemented with Spectinomycin (LB + Spec), inoculated into fresh medium at a starting OD_{600} of 0.05 and incubated at 30°C with aeration. At the indicated timepoints, aliquots were collected to assess growth and the expression of the reporter fusion. For the analyses of reporter expression, aliquots of the cultures were diluted 1:100 in PBS and expression was measured by flow cytometry (LSRFortessa; BD) and analysed with Flowing Software v 2.5.1, as previously described (55). A minimum of 10,000 green fluorescent protein (GFP)-positive single cells were acquired per sample. Expression of the promoter-*gfp* fusions is reported as the median GFP expression of GFP-positive single cells in arbitrary units. Each experiment included at least 5 independent cultures per genotype, and was repeated on 3 independent days.

***Drosophila* Stocks**

DrosDel w^{1118} isogenic stock (w^{1118} *iso*) was used in all experiments (89, 90). Stocks were maintained at 25°C in standard corn meal fly medium composed of 1.1 L water, 45 g molasses, 75 g of sugar, 10 g agar, 70 g cornmeal, 20 g yeast. Food was autoclaved and

cooled to 45°C before adding 30 mL of a solution containing 0.2 g of carbendazim (Sigma) and 100 g of methylparaben (Sigma) in 1 L of absolute ethanol. Experiments were performed at 28°C

Developmental delay and bacterial CFUs assays

Egg laying was performed in cages containing adult flies at a ratio of 3 females to 1 male. To synchronize the embryo stage, flies were initially incubated for 1 hour at 25°C to lay prior fertilized eggs. After this initial incubation, flies were transferred to new cages where eggs were laid for 4 to 6 hours in the presence of standard corn meal fly medium. After this period, eggs were removed and incubated at 25°C for 72 hours to obtain L3-stage larvae. For bacterial infections, third-instar larvae were placed in a 2 ml Eppendorf containing 200 µl of concentrated bacteria pellet (OD_{600} = 200) from an overnight culture and 400 µl of standard corn meal fly medium. Larvae, bacteria and food were then thoroughly mixed with a spoon, the Eppendorf was closed with a foam plug and incubated at room temperature for 30 min. The mix was then transferred to a 25 ml plastic tube containing 7.5 ml of standard corn-meal fly medium and incubated at 28°C. To assess development of the larvae post-infection pupa were count every 12 hours for 5 days. For CFU counts, larvae were inoculated as described above. At each time point, 5 larvae were randomly collected, surface sterilized for 10 seconds in ethanol 70% and washed with miliQ water. Individual larvae were then transferred to Eppendorfs containing 300µl of 1x PBS and homogenized with a blender. The homogenate was diluted 100-fold and serial dilutions were plated in LB. Plates were incubated overnight at 30°C.

474

475 **Statistical analysis**

476 Statistical analyses were performed in R(91) and graphs were generated using the
477 package ggplot2(92) and GraphPad. All experiments were analysed using linear mixed-
478 effect models [package lme4(93)]. Significance of interactions between factors was tested
479 by comparing models fitting the data with and without the interactions using analysis of
480 variance (ANOVA). Models were simplified when interactions were not significant.
481 Multiple comparisons of the estimates from fitted models were performed with a Tukey
482 HSD (honestly significant difference) test (packages lmerTest(94) and multicomp(95)). To
483 each statistical group a letter is attributed, different letters stand for significant statistical
484 difference.

485

486 **Data availability**

487 Data will be fully available and without restriction upon request.

488

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494

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References

1. Eigenbrode SD, Bosque-Pérez NA, Davis TS. 2018. Insect-Borne Plant Pathogens and Their Vectors: Ecology, Evolution, and Complex Interactions. Annu Rev Entomol 63:169–191.

2. Nadarasah G, Stavrinides J. 2011. Insects as alternative hosts for phytopathogenic bacteria. FEMS Microbiol Rev 35:555–575.

3. Vallet-Gely I, Lemaitre B, Boccard F. 2008. Bacterial strategies to overcome insect defences. Nat Rev Microbiol 6:302–313.

4. Matthews KR. 2011. Controlling and Coordinating Development in Vector-Transmitted Parasites. Science 331:1149–1153.

5. Lemaitre B, Hoffmann J. 2007. The host defense of *Drosophila melanogaster*. Annu Rev Immunol 25:697–743.

6. Buchon N, Broderick NA, Lemaitre B. 2013. Gut homeostasis in a microbial world: insights from *Drosophila melanogaster*. Nat Rev Microbiol 11:615–626.

7. Buchon N, Broderick NA, Poidevin M, Pradervand S, Lemaitre B. 2009. Drosophila Intestinal Response to Bacterial Infection: Activation of Host Defense and Stem Cell Proliferation. Cell Host Microbe 5:200–211.

8. Leulier F, Parquet C, Pili-Floury S, Ryu J-H, Caroff M, Lee W-J, Mengin-Lecreulx D, Lemaitre B. 2003. The *Drosophila* immune system detects bacteria through specific peptidoglycan recognition. Nat Immunol 4:478–484.

9. Bae YS, Choi MK, Lee W-J. 2010. Dual oxidase in mucosal immunity and host–microbe homeostasis. Trends Immunol 31:278–287.

10. Redak RA, Purcell AH, Lopes JRS, Blua MJ, Mizell RF, Andersen PC. 2004. The biology of xylem fluid-feeding insect vectors of Xylella fastidiosa and their relation to disease epidemiology. Annu Rev Entomol 49:243–270.

11. Menelas B, Block CC, Esker PD, Nutter FW. 2006. Quantifying the Feeding Periods Required by Corn Flea Beetles to Acquire and Transmit *Pantoea stewartii*. Plant Dis 90:319–324.

12. Kloepper JW, Brewer JW, Harrison MD. 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.

13. Molina JJ, Harrison MD, Brewer JW. 1974. Transmission ofErwinia carotovora var.atroseptica byDrosophila melanogaster Meig. I. Acquisition and transmission of the bacterium. Am Potato J 51:245–250.

14. Perombelon MCM, Kelman A. 1980. Ecology of the Soft Rot *Erwinias*. Annu Rev Phytopathol 18:361–387.

15. Shapiro L, De Moraes CM, Stephenson AG, Mescher MC. 2012. Pathogen effects on vegetative and floral odours mediate vector attraction and host exposure in a complex pathosystem. Ecol Lett 15:1430–1438.

16. Basset A. 2000. The phytopathogenic bacteria Erwinia carotovora infects Drosophila and activates an immune response. Proc Natl Acad Sci 97:3376–3381.

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

18. Muniz CA, Jaillard D, Lemaitre B, Boccard F. 2007. Erwinia carotovora Evf antagonizes the elimination of bacteria in the gut of Drosophila larvae. Cell Microbiol 9:106–119.

554 19. Kamareddine L, Wong ACN, Vanhove AS, Hang S, Purdy AE, Kierek-Pearson K, Asara
555 JM, Ali A, Morris Jr JG, Watnick PI. 2018. Activation of Vibrio cholerae quorum
556 sensing promotes survival of an arthropod host. Nat Microbiol 3:243–252.

557 20. Enomoto S, Chari A, Clayton AL, Dale C. 2017. Quorum Sensing Attenuates Virulence
558 in Sodalis praecaptivus. Cell Host Microbe 21:629–636.e5.

559 21. Perchat S, Talagas A, Poncet S, Lazar N, Li de la Sierra-Gallay I, Gohar M, Lereclus D,
560 Nessler S. 2016. How Quorum Sensing Connects Sporulation to Necrotrophism in
561 Bacillus thuringiensis. PLOS Pathog 12:e1005779.

562 22. Park S-J, Kim S-K, So Y-I, Park H-Y, Li X-H, Yeom DH, Lee M-N, Lee B-L, Lee J-H. 2014.
563 Protease IV, a quorum sensing-dependent protease of Pseudomonas aeruginosa
564 modulates insect innate immunity. Mol Microbiol 94:1298–1314.

565 23. Fuqua WC, Winans SC, Greenberg EP. 1994. Quorum sensing in bacteria: the LuxR-
566 LuxI family of cell density-responsive transcriptional regulators. J Bacteriol 176:269–
567 275.

568 24. Bassler BL. 1999. How bacteria talk to each other: regulation of gene expression by
569 quorum sensing. Curr Opin Microbiol 2:582–587.

570 25. Waters CM, Bassler BL. 2005. Quorum sensing: cell-to-cell communication in
571 bacteria. Annu Rev Cell Dev Biol 21:319–346.

572 26. Mukherjee S, Bassler BL. 2019. Bacterial quorum sensing in complex and dynamically
573 changing environments. Nat Rev Microbiol 17:371–382.

574 27. Mattinen L, Tshuikina M, Mäe A, Pirhonen M. 2004. Identification and
575 Characterization of Nip, Necrosis-Inducing Virulence Protein of *Erwinia carotovora*
576 subsp. *carotovora*. Mol Plant Microbe Interact 17:1366–1375.

577 28. Saarilahti HT, Henrissat B, Tapio Palva E. 1990. CelS: a novel endoglucanase identified
578 from Erwinia carotovora subsp. carotovora. Gene 90:9–14.

579 29. Marits R, Koiv V, Laasik E, Mae A. 1999. Isolation of an extracellular protease gene of
580 Erwinia carotovora subsp. carotovora strain SCC3193 by transposon mutagenesis and
581 the role of protease in phytopathogenicity. Microbiology 145:1959–1966.

582 30. Mäe A, Heikinheimo R, Palva ET. 1995. Structure and regulation of the Erwinia
583 carotovora subspecies carotovora SCC3193 cellulase gene celV1 and the role of
584 cellulase in phytopathogenicity. Mol Gen Genet MGG 247:17–26.

585 31. Pirhonen M. 1991. Identification of Pathogenicity Determinants of *Erwinia*
586 *carotovora* subsp. *carotovora* by Transposon Mutagenesis. Mol Plant Microbe
587 Interact 4:276.

588 32. Eriksson ARB, Andersson RA, Pirhonen M, Palva ET. 1998. Two-Component
589 Regulators Involved in the Global Control of Virulence in *Erwinia carotovora* subsp.
590 *carotovora*. Mol Plant Microbe Interact 11:743–752.

591 33. Andersson RA, Eriksson AR, Heikinheimo R, Mäe A, Pirhonen M, Kõiv V, Hyytiäinen H,
592 Tuikkala A, Palva ET. 2000. Quorum sensing in the plant pathogen *Erwinia carotovora*
593 *subsp. carotovora*: the role of *expR*(Ecc). Mol Plant-Microbe Interact MPMI 13:384–
594 393.

595 34. Valente RS, Nadal-Jimenez P, Carvalho AFP, Vieira FJD, Xavier KB. 2017. Signal
596 Integration in Quorum Sensing Enables Cross-Species Induction of Virulence in
597 *Pectobacterium wasabiae*. mBio 8.

598 35. Pirhonen M, Flego D, Heikinheimo R, Palva ET. 1993. A small diffusible signal
599 molecule is responsible for the global control of virulence and exoenzyme production
600 in the plant pathogen Erwinia carotovora. EMBO J 12:2467–2476.

601 36. von Bodman SB, Ball JK, Faini MA, Herrera CM, Minogue TD, Urbanowski ML, Stevens
602 AM. 2003. The quorum sensing negative regulators EsaR and ExpR(Ecc), homologues
603 within the LuxR family, retain the ability to function as activators of transcription. J
604 Bacteriol 185:7001–7007.

605 37. Nealson KH. 1977. Autoinduction of bacterial luciferase. Occurrence, mechanism and
606 significance. Arch Microbiol 112:73–79.

607 38. Nealson KH, Platt T, Hastings JW. 1970. Cellular control of the synthesis and activity
608 of the bacterial luminescent system. J Bacteriol 104:313–322.

609 39. Tsai C-S, Winans SC. 2010. LuxR-type quorum-sensing regulators that are detached
610 from common scents. Mol Microbiol 77:1072–1082.

611 40. Rutherford ST, Bassler BL. 2012. Bacterial Quorum Sensing: Its Role in Virulence and
612 Possibilities for Its Control. Cold Spring Harb Perspect Med 2:a012427–a012427.

613 41. Heeb S, Haas D. 2001. Regulatory roles of the GacS/GacA two-component system in
614 plant-associated and other gram-negative bacteria. Mol Plant-Microbe Interact
615 MPMI 14:1351–1363.

616 42. Lapouge K, Schubert M, Allain FH-T, Haas D. 2007. Gac/Rsm signal transduction
617 pathway of γ-proteobacteria: from RNA recognition to regulation of social behaviour:
618 Regulation of RsmA/CsrA binding to RNA. Mol Microbiol 67:241–253.

619 43. Hrabak EM, Willis DK. 1992. The lemA gene required for pathogenicity of
620 Pseudomonas syringae pv. syringae on bean is a member of a family of two-
621 component regulators. J Bacteriol 174:3011–3020.

622 44. Babitzke P, Romeo T. 2007. CsrB sRNA family: sequestration of RNA-binding
623 regulatory proteins. Curr Opin Microbiol 10:156–163.

624 45. Bejerano-Sagie M, Xavier KB. 2007. The role of small RNAs in quorum sensing. Curr
625 Opin Microbiol 10:189–198.

626 46. Chatterjee A, Cui Y, Hasegawa H, Leigh N, Dixit V, Chatterjee AK. 2005. Comparative
627 Analysis of Two Classes of Quorum-Sensing Signaling Systems That Control
628 Production of Extracellular Proteins and Secondary Metabolites in Erwinia carotovora
629 Subspecies. J Bacteriol 187:8026–8038.

630 47. Praillet T, Nasser W, Robert-Baudouy J, Reverchon S. 1996. Purification and
631 functional characterization of PecS, a regulator of virulence-factor synthesis in
632 Erwinia chrysanthemi. Mol Microbiol 20:391–402.

633 48. Liu Y, Cui Y, Mukherjee A, Chatterjee AK. 1998. Characterization of a novel RNA
634 regulator of Erwinia carotovora ssp. carotovora that controls production of
635 extracellular enzymes and secondary metabolites. Mol Microbiol 29:219–234.

636 49. Cui Y, Chatterjee A, Hasegawa H, Dixit V, Leigh N, Chatterjee AK. 2005. ExpR, a LuxR
637 homolog of *Erwinia carotovora subsp. carotovora*, activates transcription of *rsmA*,
638 which specifies a global regulatory RNA-binding protein. J Bacteriol 187:4792–4803.

639 50. Sjöblom S, Brader G, Koch G, Palva ET. 2006. Cooperation of two distinct ExpR
640 regulators controls quorum sensing specificity and virulence in the plant pathogen
641 *Erwinia carotovora*. Mol Microbiol 60:1474–1489.

642 51. Cui Y, Chatterjee A, Liu Y, Dumenyo CK, Chatterjee AK. 1995. Identification of a global
643 repressor gene, rsmA, of Erwinia carotovora subsp. carotovora that controls
644 extracellular enzymes, N-(3-oxohexanoyl)-L-homoserine lactone, and pathogenicity
645 in soft-rotting Erwinia spp. J Bacteriol 177:5108–5115.

646 52. Chatterjee A, Cui Y, Liu Y, Dumenyo CK, Chatterjee AK. 1995. Inactivation of RsmA
647 leads to overproduction of extracellular pectinases, cellulases, and proteases in
648 *Erwinia carotovora subsp. carotovora* in the absence of the starvation/cell density-

649 sensing signal, N-(3-oxohexanoyl)-L-homoserine lactone. Appl Environ Microbiol
650 61:1959–1967.

651 53. Thomson NR, Cox A, Bycroft BW, Stewart GS, Williams P, Salmond GP. 1997. The rap
652 and hor proteins of Erwinia, Serratia and Yersinia: a novel subgroup in a growing
653 superfamily of proteins regulating diverse physiological processes in bacterial
654 pathogens. Mol Microbiol 26:531–544.

655 54. Sjöblom S, Harjunpää H, Brader G, Palva ET. 2008. A novel plant ferredoxin-like
656 protein and the regulator Hor are quorum-sensing targets in the plant pathogen
657 Erwinia carotovora. Mol Plant-Microbe Interact MPMI 21:967–978.

658 55. Valente RS, Xavier KB. 2016. The Trk Potassium Transporter Is Required for RsmB-
659 Mediated Activation of Virulence in the Phytopathogen Pectobacterium wasabiae. J
660 Bacteriol 198:248–255.

661 56. Ludwig A, Tengel C, Bauer S, Bubert A, Benz R, Mollenkopf HJ, Goebel W. 1995. SlyA,
662 a regulatory protein from *Salmonella typhimurium*, induces a haemolytic and pore-
663 forming protein in *Escherichia coli*. Mol Gen Genet MGG 249:474–486.

664 57. Patankar AV, González JE. 2009. Orphan LuxR regulators of quorum sensing. FEMS
665 Microbiol Rev 33:739–756.

666 58. Liang H, Deng X, Ji Q, Sun F, Shen T, He C. 2012. The Pseudomonas aeruginosa Global
667 Regulator VqsR Directly Inhibits QscR To Control Quorum-Sensing and Virulence
668 Gene Expression. J Bacteriol 194:3098–3108.

669 59. Reverchon S, Nasser W, Robert-Baudouy J. 1991. Characterization of *kdgR* , a gene of
670 *Erwinia chrysanthemi* that regulates pectin degradation. Mol Microbiol 5:2203–2216.

671 60. Reverchon S, Expert D, Robert-Baudouy J, Nasser W. 1997. The cyclic AMP receptor
672 protein is the main activator of pectinolysis genes in Erwinia chrysanthemi. J
673 Bacteriol 179:3500–3508.

674 61. Erkosar B, Storelli G, Mitchell M, Bozonnet L, Bozonnet N, Leulier F. 2015. Pathogen
675 Virulence Impedes Mutualist-Mediated Enhancement of Host Juvenile Growth via
676 Inhibition of Protein Digestion. Cell Host Microbe 18:445–455.

677 62. Houtz P, Bonfini A, Bing X, Buchon N. 2019. Recruitment of Adult Precursor Cells
678 Underlies Limited Repair of the Infected Larval Midgut in Drosophila. Cell Host
679 Microbe 26:412–425.e5.

680 63. Murphy KM, Teakle DS, Macrae IC. 1994. Kinetics of Colonization of Adult
681 Queensland Fruit Flies (Bactrocera tryoni) by Dinitrogen-Fixing Alimentary Tract
682 Bacteria. Appl Environ Microbiol 60:2508–2517.

683 64. Morales-Jiménez J, Zúñiga G, Ramírez-Saad HC, Hernández-Rodríguez C. 2012. Gut-
684 Associated Bacteria Throughout the Life Cycle of the Bark Beetle Dendroctonus
685 rhizophagus Thomas and Bright (Curculionidae: Scolytinae) and Their Cellulolytic
686 Activities. Microb Ecol 64:268–278.

687 65. Solà-Ginés M, González-López JJ, Cameron-Veas K, Piedra-Carrasco N, Cerdà-Cuéllar
688 M, Migura-Garcia L. 2015. Houseflies (Musca domestica) as Vectors for Extended-

689 Spectrum β-Lactamase-Producing Escherichia coli on Spanish Broiler Farms. Appl
690 Environ Microbiol 81:3604–3611.

691 66. Park D-S, Oh H-W, Jeong W-J, Kim H, Park H-Y, Bae KS. 2007. A culture-based study of
692 the bacterial communities within the guts of nine longicorn beetle species and their
693 exo-enzyme producing properties for degrading xylan and pectin. J Microbiol Seoul
694 Korea 45:394–401.

695 67. Jang EB, Nishijima KA. 1990. Identification and Attractancy of Bacteria Associated
696 with Dacus dorsalis (Diptera: Tephritidae). Environ Entomol 19:1726–1731.

697 68. Jiang J, Alvarez C, Kukutla P, Yu W, Xu J. 2012. Draft genome sequences of
698 Enterobacter sp. isolate Ag1 from the midgut of the malaria mosquito Anopheles
699 gambiae. J Bacteriol 194:5481.

700 69. Cirimotich CM, Dong Y, Clayton AM, Sandiford SL, Souza-Neto JA, Mulenga M,
701 Dimopoulos G. 2011. Natural Microbe-Mediated Refractoriness to Plasmodium
702 Infection in Anopheles gambiae. Science 332:855–858.

703 70. Quevillon-Cheruel S, Leulliot N, Muniz CA, Vincent M, Gallay J, Argentini M, Cornu D,
704 Boccard F, Lemaître B, van Tilbeurgh H. 2009. Evf, a Virulence Factor Produced by the
705 *Drosophila* Pathogen *Erwinia carotovora* , Is an *S* -Palmitoylated Protein with a New
706 Fold That Binds to Lipid Vesicles. J Biol Chem 284:3552–3562.

707 71. Gibrat J-F, Madej T, Bryant SH. 1996. Surprising similarities in structure comparison.
708 Curr Opin Struct Biol 6:377–385.

709 72. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJE. 2015. The Phyre2 web
710 portal for protein modeling, prediction and analysis. Nat Protoc 10:845–858.

711 73. Ciche TA, Kim K -s., Kaufmann-Daszczuk B, Nguyen KCQ, Hall DH. 2008. Cell Invasion
712 and Matricide during Photorhabdus luminescens Transmission by Heterorhabditis
713 bacteriophora Nematodes. Appl Environ Microbiol 74:2275–2287.

714 74. Waterfield NR, Ciche T, Clarke D. 2009. Photorhabdus and a host of hosts. Annu Rev
715 Microbiol 63:557–574.

716 75. Blackburn null, Golubeva null, Bowen null, Ffrench-Constant null. 1998. A novel
717 insecticidal toxin from *Photorhabdus luminescens*, toxin complex a (Tca), and its
718 histopathological effects on the midgut of *Manduca sexta*. Appl Environ Microbiol
719 64:3036–3041.

720 76. Daborn PJ, Waterfield N, Silva CP, Au CPY, Sharma S, Ffrench-Constant RH. 2002. A
721 single Photorhabdus gene, makes caterpillars floppy (mcf), allows Escherichia coli to
722 persist within and kill insects. Proc Natl Acad Sci U S A 99:10742–10747.

723 77. Dowling AJ, Daborn PJ, Waterfield NR, Wang P, Streuli CH, ffrench-Constant RH.
724 2004. The insecticidal toxin Makes caterpillars floppy (Mcf) promotes apoptosis in
725 mammalian cells. Cell Microbiol 6:345–353.

726 78. Duchaud E, Rusniok C, Frangeul L, Buchrieser C, Givaudan A, Taourit S, Bocs S,
727 Boursaux-Eude C, Chandler M, Charles J-F, Dassa E, Derose R, Derzelle S, Freyssinet
728 G, Gaudriault S, Médigue C, Lanois A, Powell K, Siguier P, Vincent R, Wingate V,

729 Zouine M, Glaser P, Boemare N, Danchin A, Kunst F. 2003. The genome sequence of
730 the entomopathogenic bacterium Photorhabdus luminescens. Nat Biotechnol
731 21:1307–1313.

732 79. Brameyer S, Kresovic D, Bode HB, Heermann R. 2015. Dialkylresorcinols as bacterial
733 signaling molecules. Proc Natl Acad Sci 112:572–577.

734 80. Visick KL, Foster J, Doino J, McFall-Ngai M, Ruby EG. 2000. Vibrio fischeri lux Genes
735 Play an Important Role in Colonization and Development of the Host Light Organ. J
736 Bacteriol 182:4578–4586.

737 81. Sun Y, LaSota ED, Cecere AG, LaPenna KB, Larios-Valencia J, Wollenberg MS,
738 Miyashiro T. 2016. Intraspecific Competition Impacts Vibrio fischeri Strain Diversity
739 during Initial Colonization of the Squid Light Organ. Appl Environ Microbiol 82:3082–
740 3091.

741 82. Cao M, Goodrich-Blair H. 2017. Ready or Not: Microbial Adaptive Responses in
742 Dynamic Symbiosis Environments. J Bacteriol 199.

743 83. Tagkopoulos I, Liu Y-C, Tavazoie S. 2008. Predictive Behavior Within Microbial
744 Genetic Networks. Science 320:1313–1317.

745 84. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in
746 Escherichia coli K-12 using PCR products. Proc Natl Acad Sci 97:6640–6645.

747 85. Yanisch-Perron C, Vieira J, Messing J. 1985. Improved M13 phage cloning vectors and
748 host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene
749 33:103–119.

750 86. Waters CM, Bassler BL. 2006. The Vibrio harveyi quorum-sensing system uses shared
751 regulatory components to discriminate between multiple autoinducers. Genes Dev
752 20:2754–2767.

753 87. Sherwood R. 1966. Pectin lyase and polygalacturonase production by *Rhizoctonia*
754 *solani* and other fungi. Phytopathology 56:279-.

755 88. McMillan GP, Hedley D, Fyffe L, Pérombelon MCM. 1993. Potato resistance to soft-
756 rot erwinias is related to cell wall pectin esterification. Physiol Mol Plant Pathol
757 42:279–289.

758 89. Ryder E, Blows F, Ashburner M, Bautista-Llacer R, Coulson D, Drummond J, Webster
759 J, Gubb D, Gunton N, Johnson G, O'Kane CJ, Huen D, Sharma P, Asztalos Z, Baisch H,
760 Schulze J, Kube M, Kittlaus K, Reuter G, Maroy P, Szidonya J, Rasmuson-Lestander A,
761 Ekström K, Dickson B, Hugentobler C, Stocker H, Hafen E, Lepesant JA, Pflugfelder G,
762 Heisenberg M, Mechler B, Serras F, Corominas M, Schneuwly S, Preat T, Roote J,
763 Russell S. 2004. The DrosDel collection: a set of P-element insertions for generating
764 custom chromosomal aberrations in Drosophila melanogaster. Genetics 167:797–
765 813.

766 90. Chrostek E, Marialva MSP, Esteves SS, Weinert LA, Martinez J, Jiggins FM, Teixeira L.
767 2013. Wolbachia Variants Induce Differential Protection to Viruses in Drosophila
768 melanogaster: A Phenotypic and Phylogenomic Analysis. PLoS Genet 9:e1003896.

769 91. Team RC . R: A language and environment for statistical computing [Internet]. R
770 Foundation for Statistical Computing. Vienna Austria 2012 Cited 2018 Apr 23
771 Available from: http://www.R-project.org/.

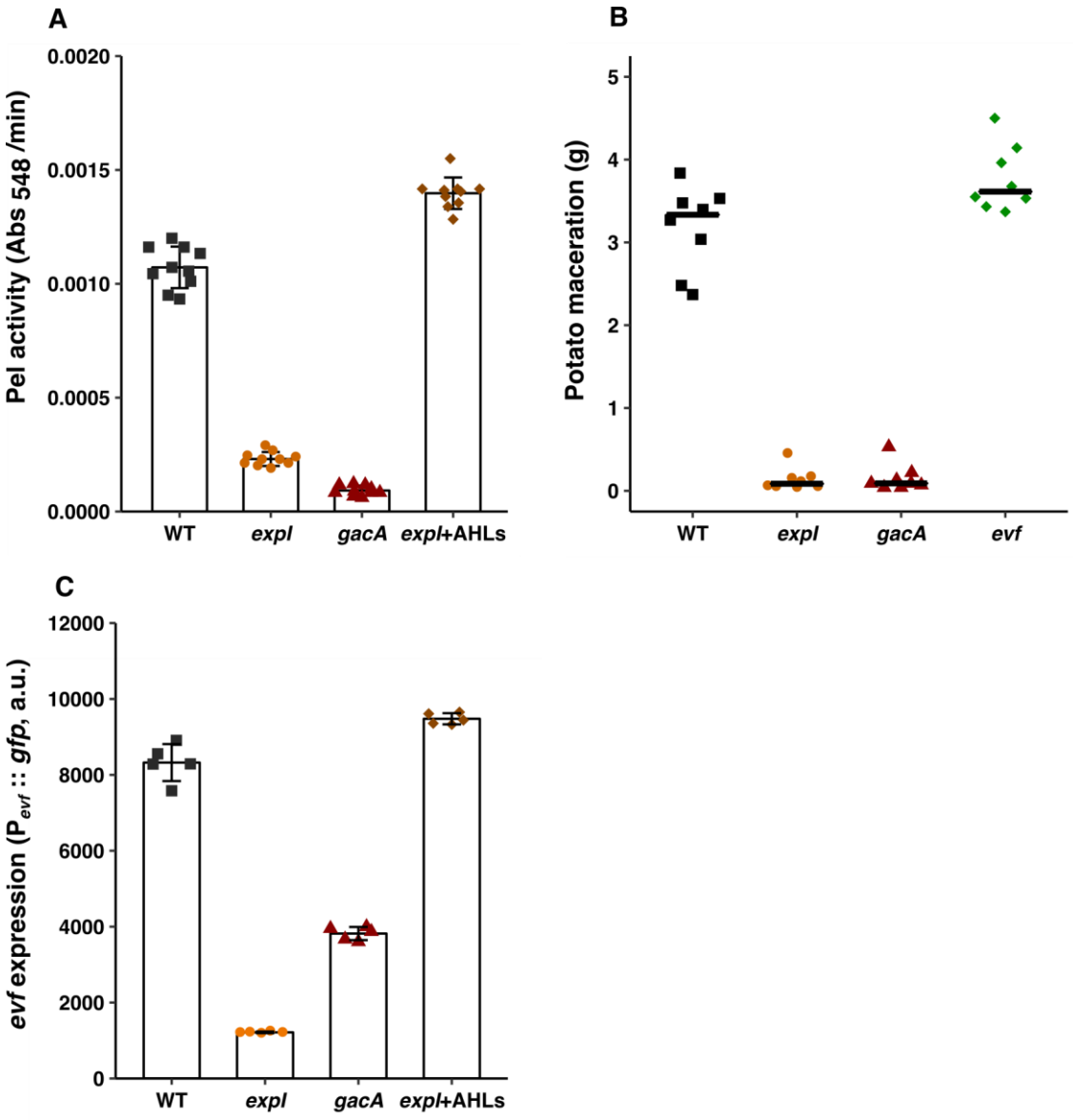
772 92. Wickham H. ggplot2—Elegant Graphics for Data Analysis Aug 2009. 2009: 1–222.

773 93. Bates D, Mächler M, Bolker B, Walker S. 2015. Fitting Linear Mixed-Effects Models
774 Using **lme4**. J Stat Softw 67.

775 94. Kuznetsova A, Brockhoff PB, Christensen RHB. 2017. **lmerTest** Package: Tests in
776 Linear Mixed Effects Models. J Stat Softw 82.

777 95. Hothorn T, Bretz F, Westfall P. 2008. Simultaneous inference in general parametric
778 models. Biom J Biom Z 50:346–363.

779 96. El-Gebali S, Mistry J, Bateman A, Eddy SR, Luciani A, Potter SC, Qureshi M, Richardson
780 LJ, Salazar GA, Smart A, Sonnhammer ELL, Hirsh L, Paladin L, Piovesan D, Tosatto SCE,
781 Finn RD. 2019. The Pfam protein families database in 2019. Nucleic Acids Res
782 47:D427–D432.



783

784 **Fig. 1 Production of pectate lyase and expression of *evf* is dependent on both quorum**
785 **sensing and the GAC system. (A)** Pectate lyase activity in cell-free supernatants of WT
786 *Ecc15*, *expI* and *gacA* mutants at 6 hours of growth in LB + 0.4%PGA. n=10 **(B)** Potato
787 maceration quantification (grams) in potatoes infected with WT *Ecc15*, *expI*, *gacA* and *evf*
788 mutants, 48 hours post-infection. n=8 **(C)** Pevf::*gfp* expression in WT *Ecc15*, *expI* and *gacA*
789 mutants at 6 hours of growth in LB + Spec. n=5

790 Growth curves of the strains used are shown in Fig.S1. Complementation with AHLs was
791 performed with a mixture of 1uM 3-oxo-C6-HSL and 3-oxo-C8-HSL. Error bars represent
792 standard deviation of the mean. For each panel a representative experiment from three
793 independent experiments is shown (other two experiment are shown in Fig. S2). Statistical
794 analysis taking the data of all the three experiments is shown in Fig. S2.
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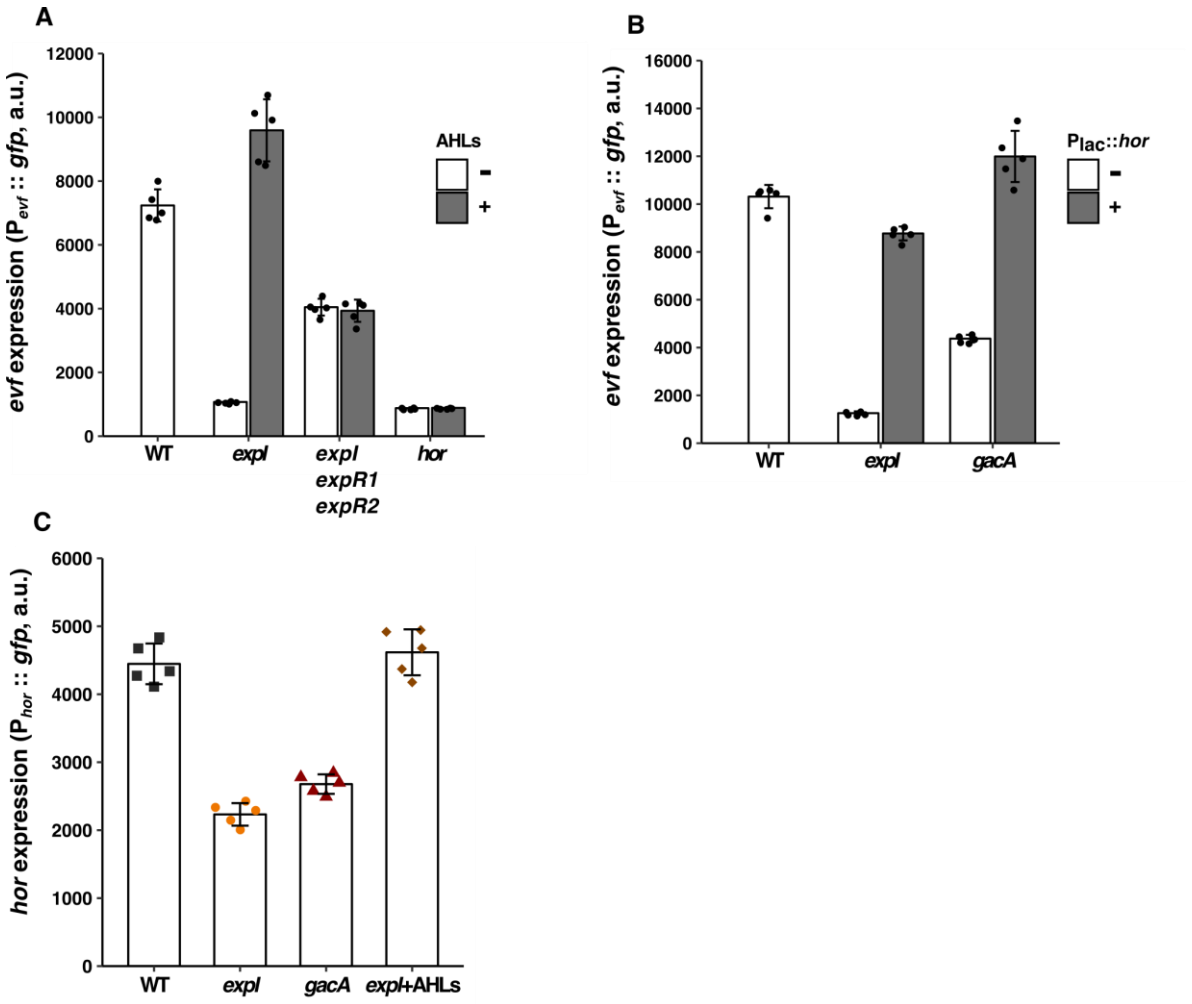

Fig. 2. *evf* regulation by quorum sensing is dependent on ExpR receptors and *hor*. (A) P_{evf}::*gfp* expression without (white bars) or with (grey bars) addition of exogenous AHLs in *Ecc15*, *expI*, *expI expR1 expR2* and *hor* mutants at 6 hours of growth in LB + Spec. n=5 **(B)** P_{evf}**::***gfp* expression in *Ecc15 expI* and *gacA* mutants containing a plasmid with the P_{evf}::*gfp* fusion (white bars)or with both P_{lac}::*hor* and P_{evf}::*gfp* fusions (grey bars) at 6 hours of growth in LB + Spec. n=5 **(C)** P_{hor}**::***gfp* expression in WT *Ecc15*, *expI* and *gacA* mutants at 6 hours of growth in LB + Spec. n=5 Complementation with AHLs was performed with a mixture of 1µM 3-oxo-C6-HSL and 3-oxo-C8-HSL. Error bars represent standard deviation of the mean. For each panel a representative experiment from three independent experiments is shown

806 (other two experiment are shown in Fig. S4). Statistical analysis taking the data of all the

807 three experiments is shown in Fig. S4.

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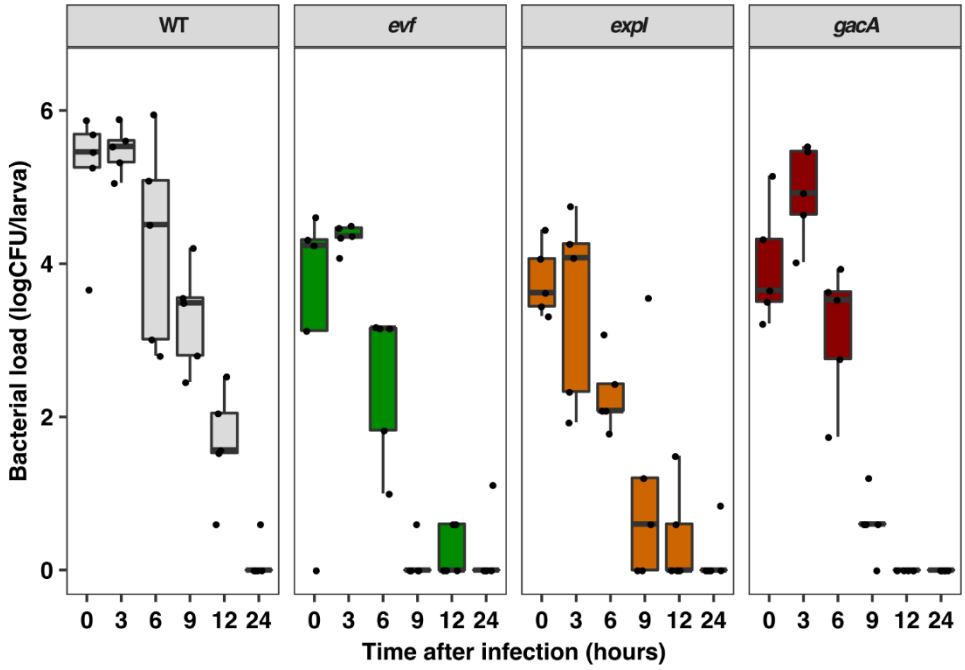
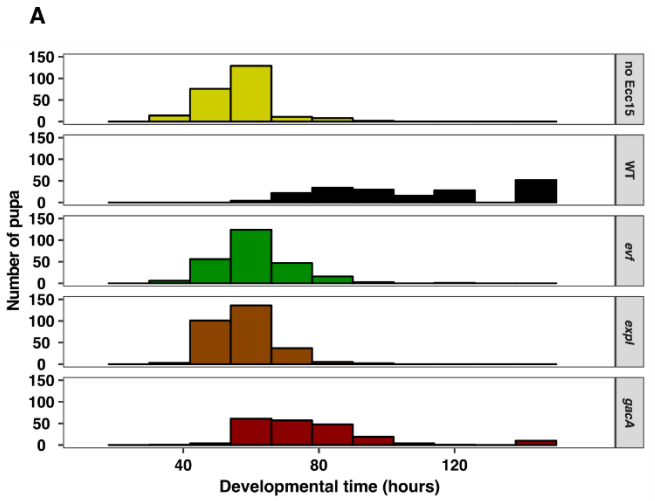


Fig. 3. *Ecc15* loads are higher in *D. melanogaster* larvae orally infected with WT than with mutants impaired in *evf* expression.

D. melanogaster L3 stage larvae were infected with WT *Ecc15*, *evf, expI* and *gacA* mutants for 30 min and then transferred to fresh media. Following the infection period Colony Forming Units (CFUs) of *Ecc15* were measured at the specified time points. Each dot represents CFUs of one single larvae (5 larvae per time point). 0 hours after infection correspond to 30 min of confined exposure to 200µl of an OD_{600}=200. Representative experiment from three independent experiments (other two experiment are shown in Fig. S5). Statistical analysis of the comparison of the entire infection period for each condition tested using the data of all the three experiments is shown in Fig. S5.



B

Bacteria	Developmental Time (Hours)	Statistical group
	Mean ± SD	
No *Ecc15*	54.5 ± 11.1	a
WT	103.8 ± 26.6	e
evf	62.8 ± 12.1	d
expI	58.6 ± 10.9	c
gacA	81.2 ± 18.6	b

D

Bacteria	Developmental Time (Hours)	Statistical group
	Mean ± SD	
No *Ecc15*	52.9 ± 8.6	a
WT	103.8 ± 26.6	c
evf	54.9 ± 8.7	b
p(*evf*+)	NA	NA

Fig. 4. *Ecc15* causes a developmental delay in *D. melanogaster* larvae that is dependent on *evf*, quorum sensing and the GAC system. L3 stage *Drosophila* larvae pupariation time after exposure to **(A)** WT *Ecc15*, *evf*, *expI* and *gacA* mutants or **(C)** WT *Ecc15* overexpressing Evf, compared with non-infected larvae. **(B)** and **(D)** Average developmental time in hours with standard deviation. Representative experiment from three independent experiments (other two experiment are shown in Fig. S6). Statistical groups shown in (B) and (D) were determined using a linear mixed effect model taking in consideration the data from the

831 three experiments. A Tukey HSD test was applied for multiple comparisons using the
832 estimates obtain from the model.
833

834 **SUPPEMENTAL FIGURES**

835

836 **Table S1.** Strains and plasmids used in this study

Strain	Parental strain	Relevant Genotype	Source
E. carotovora			
Ecc15		Wild-type (WT)	(16)
FDV31	*Ecc15*	WT carrying pLIPS	(34)
FDV51	*Ecc15*	*expI::cm*	This study
FDV42	*Ecc15*	*gacA::kan*	This study
FDV163	*Ecc15*	*expIexpR1::cm/expR2::kan*	This study
FDV22	*Ecc15*	*hor::kan*	This study
FDV54	*Ecc15*	WT carrying pFDV54	This study
FDV56	FDV51	*expI::cm* carrying pFDV54	This study
FDV58	FDV42	*gacA::kan* carrying pFDV54	This study
FDV165	FDV163	*expIexpR1::cm/expR2::kan* carrying pFDV54	This study
FDV60	FDV22	*hor::kan* carrying pFDV54	This study
FDV84	*Ecc15*	WT carrying pFDV84	This study
FDV92	FDV51	*expI::cm* carrying pFDV84	This study
FDV86	FDV42	*gacA::kan* carrying pFDV84	This study
FDV104	*Ecc15*	WT carrying pFDV104	This study
FDV114	FDV51	*expI::cm* carrying pFDV104	This study
FDV127	FDV42	*gacA::kan* carrying pFDV104	This study

Plasmids	Relevant genotype	Source
pOM1	Cloning vector, *Spec*^{r}	
pUC18	Cloning vector, *Amp*^{r}	
pLIPS	pOM1 vector containing λ red recombinase system, *Spec*^{r}	(34)
pFDV54	pOM1 vector containing promoter *evf::gfp*, *Spec*^{r}	This study
pFDV104	pOM1 vector containing a promoter lac::*hor* and a promoter *evf::gfp*	This study
pFDV84	pOM1 vector containing a promoter *hor::gfp*, *Spec*^{r}	This study

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843 **Table S2.** Primers used in this study

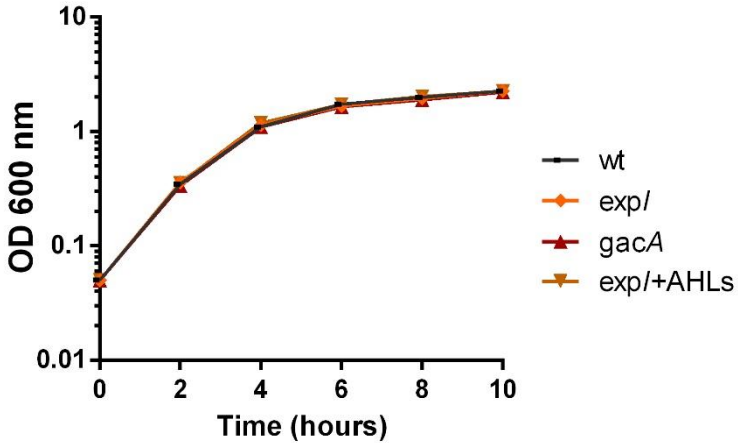
Primer Name	Sequence
1108-Redsystem(pKD46)FWsphI	CCTTACGCATGCCATCGATTTATTATGACAA
1109-Redsystem(pKD46)RVXbaI	CGAGCTTCTAGATACCCATGGATTCTTCGTCT
1127-500Hor500RVSalI	CGAGCTGTCGACGCTAAACAGGTGCAGACCGT
1128-500Hor500FWSalI	CCTTACGTCGACTCAATAAATAGAGTTGTCGCGGG
1130-500gacA500FwSalI	CCTTACGTCGACTATGATGTTCACTATGGACG
1131-500gacA500RvSalI	CGAGCTGTCGACGATATTGCAGGCAGGGGCG
1087-HorDelRVXhoI	CGAGCTCTCGAGCACCTCTCCTTATTGTTAGC
1088-HorDelFWXhoI	CCTTACCTCGAGCTAAATTTGGGTTACGCAGA
1132-DelGacARvXhoI	CGAGCTCTCGAGGAATAATTCTCCAAAAAAGGG
1133-DelGacAFwXhoI	CCTTACCTCGAGGAGTTTCGATGCGTCGGCAT
1134-DelExpIFwXhoI	CCTTACCTCGACTTGCACAGGCTTGATGAGCTGTA
1135-DelExpIRvXhoI	CGAGCTCTCGAGCCTCCATTGAAAAGTTAATAC
1136-500ExpI500FwSalI	CCTTACGTCGACGAATACCGTGTCTGACAACC
1137-500ExpI500RvSalI	CGAGCTGTCGACATCGCCTTTCTCTTGGGAGA
1186-HorDelFw	AATCGTCAGTTATTACAATGGT
1187-HorDelRv	TATGATGAAGCGTTTGCTTGTG
1190-ExpIDelFw	TCAGGCGCTGATGCTGCGTGAT
1191-ExpIDelRv	TCCAGTTATCCCGATGAATGGG
1192-GacADelFw	GGGCGTTACCGCTGACGCGACA
1193-GacADelRV	CAGGCGAACATAGTCAACCTGC
1309-NcoIsiteFW	CCTTACCCATGGTTACGAATTCGAGCT
1310-NcoIsiteRV	CCTTACCCATGGTCATAGCTGTTTCCT
1311-horNcoIFW	CCTTACCCATGGAATTGCCATTAGGAT
1312-horSacIRV	CCTTACGAGCTCCTACGCTTGATTTTCATG
1351-pHor(500bp)_FW	CCTTACAAGCTTTAGAGTTGTCGCAGGAGGTG
1352-pHor(500bp)_RV	CCTTACCTGCAGCACCTCTCCTTATTGTTAGC
1194-pEvfFw	CCTTACAAGCTTTGCTTACAGGAAACCAACAA
1195-pEvf_Rv	CGAGCTGCATGCAATCACTCCTATTGTGGTGG
1411-500evf500FwSalI	CCTTACGTCGACTGCTTACAGGAAACCAACAA
1412-500evf500RvSalI	CGAGCTGTCGACGCATTACTCTACACTTTTCTGAC
1413-EvfDelXhoIFw	CCTTACCTCGAGTTCATAAAATATAGTCAGGG
1414-EvfDelXhoIRv	CGAGCTCTCGAGAATCACTCCTATTGTGGTGG
1415-EvfDelConfFw	CGTTCCCGTTGAAGTCATGG
1416-EvfDelConfRv	CTGGATCGCTGGCTCCAAAC
1235-500-ExpR2-500SalIFw	CCTTACGTCGACGGAGAAGGACGGGAAAGGTA
1236-500-ExpR2-500SalIRv	CGAGCTGTCGACTTGATGATTCGGTGCTGGCG
1237-DelExpR2XhoIFw	CCTTACCTCGAGTGTCATCACGTCTATTTCACT
1238-DelExpR2XhoIRv	CGAGCTCTCGAGGTAACGGCCTCAATAAAAAGCG
1239-ExpR2DelConFw	CTAAAACATTAGCCTCACCGCCG
1240-ExpR2DelConRv	CTAACATGGGCGCGTGTGTATCG
1241-500-ExpR1-500SalIFw	CCTTACGTCGACCACGATTGACGCCAGCTATGA

1242-500-ExpR1-500SalIRv	CGAGCTGTCGACGGCATCAAAGATAACACCGT
1243-DelExpR1XhoIFw	CCTTACCTCGAGAGTTACAGCTCATCAAGCCT
1244-DelExpR1XhoIRv	CGAGCTCTCGAGCCTCAGTCTGAAGAATCAAC
1245-ExpR1DelConFw	CGCCTGGGATCAGGGAGCAA
1246-ExpR1DelConRv	GAAACGAAATCAGAAGAGCT
1353-GFP(noRBS)_FW	CCTTACCTGCAGATGGCTAGCAAAGGAGAAGAACTCT
1354-GFP(noRBS)_RV	CCTTACTCTAGAACCGGATCCTCAGTTGTACAGTTCA
0665-GFP(noRBS)_RV	CCTTACGGATCCTCAGTTGTACAGTTCATCCATGCCA
0576-GFP(noRBS)_FW	CCTTACGCATGCATGGCTAGCAAAGGAGAAGAACTCT
0531_pOM1seq_R	ATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTC
0752-pOM1_seq2_F	CGCCCAATACGCAAACCGCCTCTCCCCGCGCGT
0782- pKD3/4 XhoI Fw	AGTCTCGAGTTGTGTAGGCTGGAGCTGCTTC
0783- pKD3/4 XhoI Rv	GCGCTCGAGCCATATGAATATCCTCCTTAG

Table S3. Orthologues of the Evf protein from *Erwinia carotovora Ecc15* present in the NCBI database (October 2019).

Organism	Sequence ID	Locus Tag	Protein annotated function	% of amino acid query cover	% of amino acid identity	Alignment Template	% of confidence in the predicted structure
***P. carotovorum* Strain 14A**	CP034276.1	EIP93_02725	Hypothetical protein	100	100	c2w3yB	100
***P. carotovorum* Strain Scc1**	CP021894.1	SCC1_3840	virulence factor	100	100	c2w3yB	100
***P. carotovorum* Strain 3-2**	CP024842.1	OA04_05770	virulence factor	100	100	c2w3yB	100
Cedecea neteri	WP_039302011	LH86_RS13095	Hypothetical protein	100	70	c2w3yB	100
Enterobacter sp. AG1	WP_008453376	A936_RS00125	Hypothetical protein	99	69	c2w3yB	100
Rahnella sp. AA	WP_101079538	CWS43_23475	Hypothetical protein	100	68	c2w3yB	100
Klebsiella aerogenes	WP_087858097	B9037_RS05845	Hypothetical protein	100	66	c2w3yB	100
Escherichia coli	WP_113374258	DUL12_RS15125	Hypothetical protein	100	66	c2w3yB	100

The amino acid sequence from *Ecc15* was used as template to identify orthologues. All Proteins are defined as a complete match in the bidirectional best hits. Alignment template stands for the PDB sequence with the highest confidence used by phyre2 to predict orthologs secondary structure, corresponding to *Ecc15* Evf. All sequences were run in both phyre2 (72) and pfam database (96).



856

857 **Fig. S1. Growth curves of WT *Ecc15*, *expI* and *gacA* mutants carrying a P_{evf}::*gfp* reporter**

858 **fusion.**

859

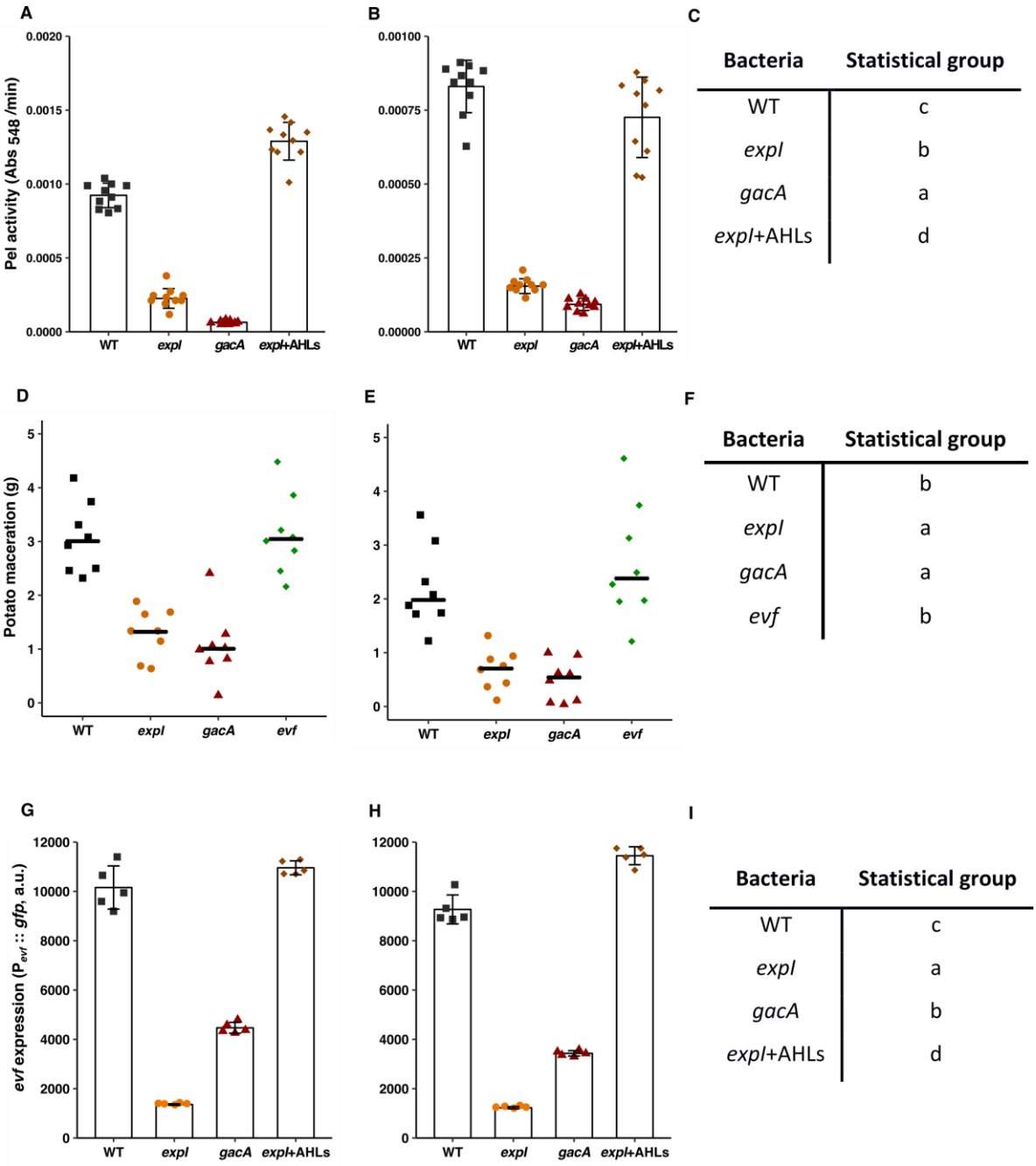
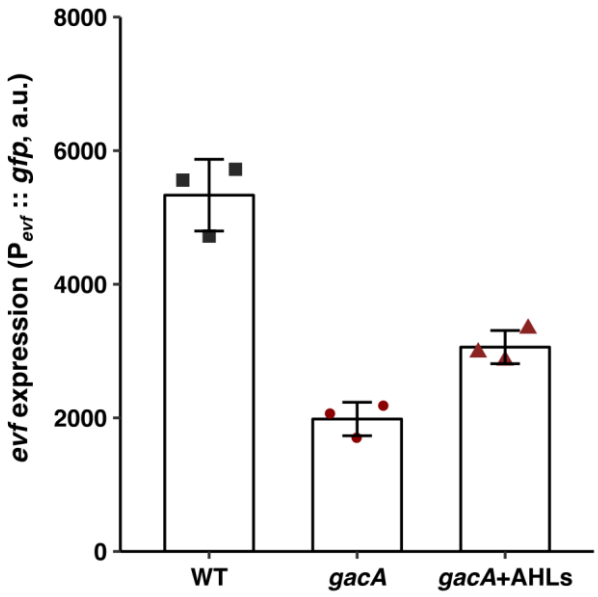


Fig. S2. Independent replicates of the experiments shown in Fig. 1 (Production of pectate lyase and expression of *evf* is dependent on both quorum sensing and the GAC system). **(A, B)** replicates of experiments shown in Fig. 1A, **(C)** Statistical groups of all three experiments from Fig. 1A, **(D, E)** replicates of experiments shown in Fig. 1B, **(F)** Statistical groups of all three experiments from Fig. 1B, **(G, H)** replicates of experiments shown in Fig.

867 1C, **(I)** Statistical groups of all three experiments from Fig. 1C. Statistical analysis was
868 performed using a linear mixed effect model. A Tukey HSD test was applied for multiple
869 comparisons using the estimates obtain from the model.
870



871

872 **Fig. S3. AHLs cannot complement intermediate levels of Pevf::gfp expression in a *gacA***
873 **mutant.** P_{evf}::*gfp* expression in WT *Ecc15* and *gacA* mutant at 6 hours of growth in LB + Spec.
874 n=3. Complementation with AHLs was performed with a mixture of 1uM 3-oxo-C6-HSL and
875 3-oxo-C8-HSL. Error bars represent standard deviation of the mean.

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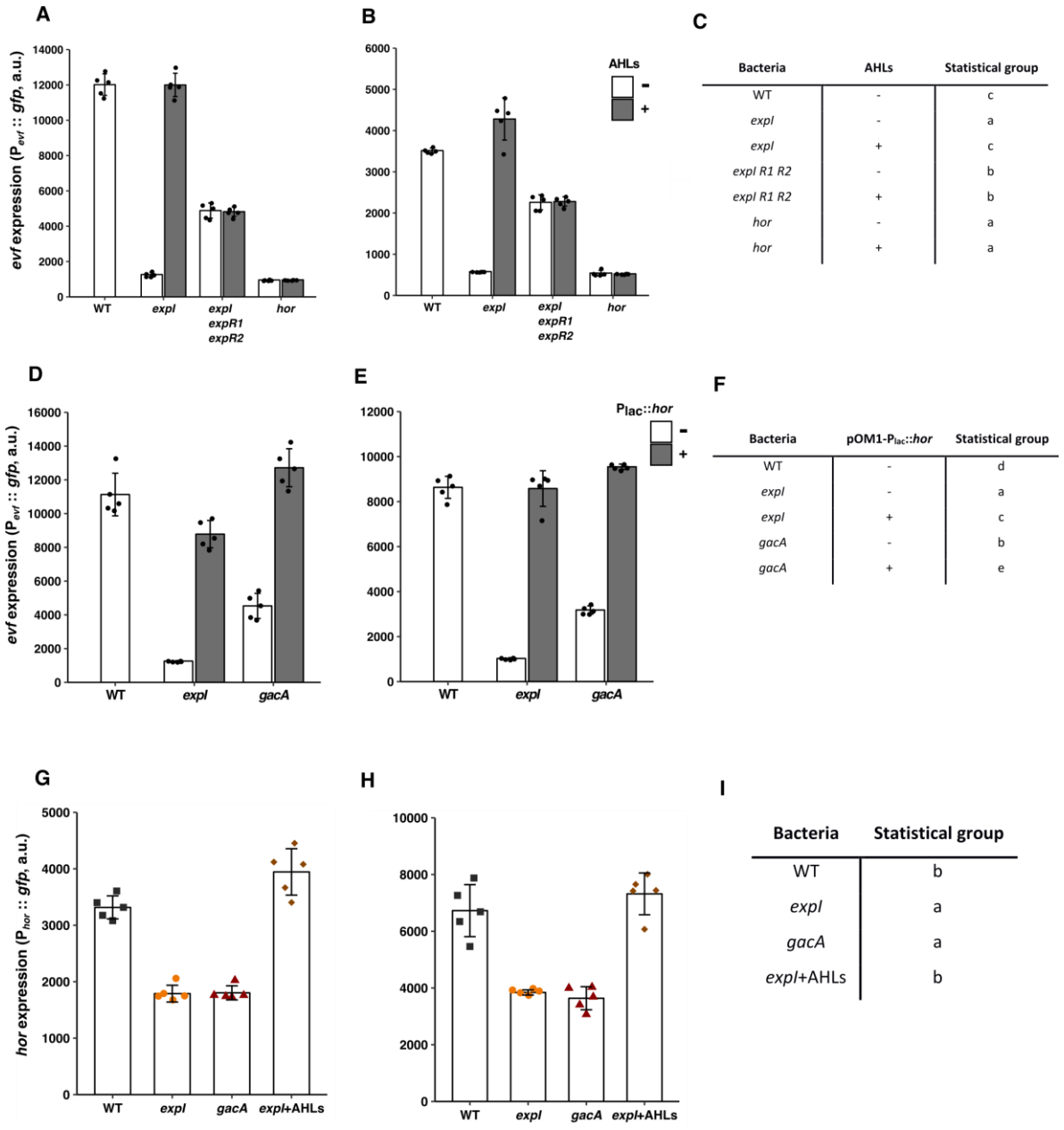
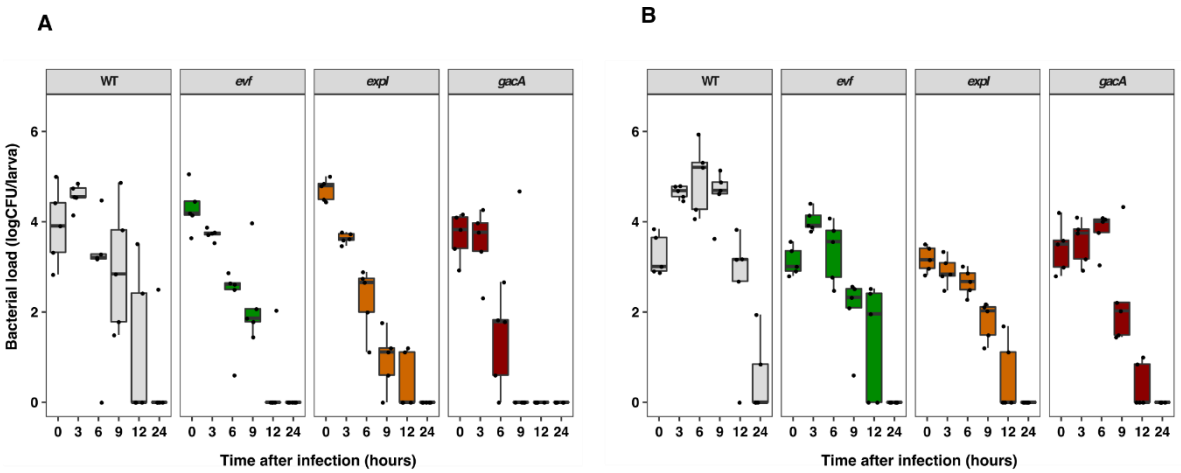


Fig. S4. Independent replicates of the experiment shown in Fig. 2a (*evf* regulation by quorum sensing is dependent on ExpR receptors and *hor*). (A, B) replicates of experiments shown in Fig. 2A, **(C)** Statistical groups of all three experiments from Fig. 2A, **(D, E)** replicates of experiments shown in Fig. 2B, **(F)** Statistical groups of all three experiments from Fig. 2B, **(G, H)** replicates of experiments shown in Fig. 2C, **(I)** Statistical groups of all three experiments from Fig. 2C. Statistical analysis was performed using a linear mixed effect

886 model. A Tukey HSD test was applied for multiple comparisons using the estimates obtain
887 from the model.
888



C

Bacteria	Statistical group
WT	b
expI	a
gacA	a
evf	a

Fig. S5. Independent replicates of the experiment shown in Fig. 3 (*Ecc15* loads are higher in *D. melanogaster* larvae orally infected with WT than with mutants impaired in *evf* expression.) (A, B) replicates of experiments shown in Fig. 3, **(C)** Statistical groups of all three experiments from Fig 3. Statistical analysis was performed using a linear mixed effect model. A Tukey HSD test was applied for multiple comparisons using the estimates obtain from the model.

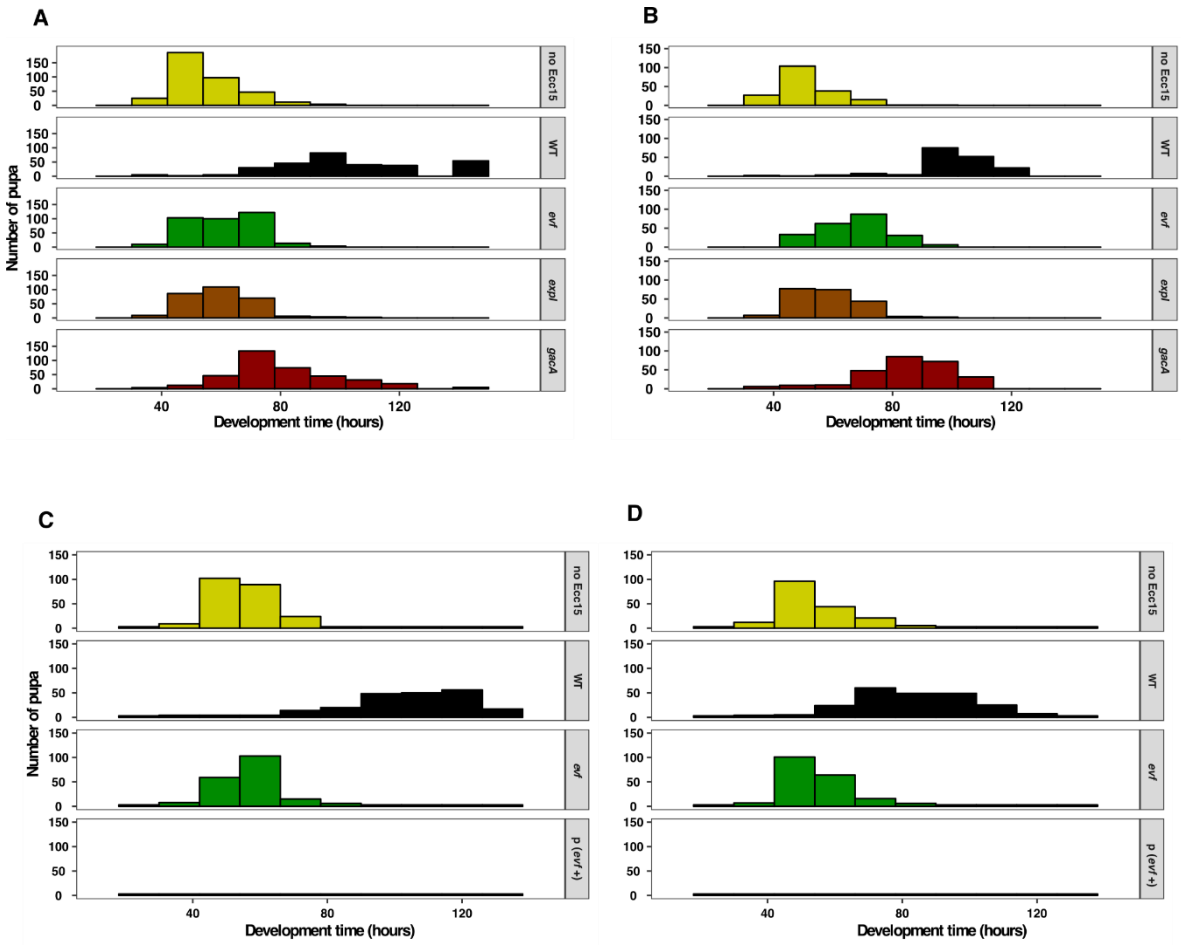


Fig. S6. Independent replicates of the experiment shown in Fig. 4 (*Ecc15* causes a developmental delay in *D. melanogaster* larvae that is dependent on *evf*, quorum sensing and the GAC system). (A, B) replicates of experiments shown in Fig. 4A, **(C, D)** replicates of experiments shown in Fig. 4C.