[SII-2]

Multi-host Pathogenesis *by Pseudomonas aeruginosa* and Use of *Drosophila melanogaster* as a New Model Host

You-Hee Cho,[1,2] Gee Lau,[2] and Laurence Rahme[2]

Department of Life Science, Sogang University[1],
Department of Surgery, Harvard Medical School, Massachusetts General Hospital
and Shriner's Burns Hospital[2]

Summary

Fruit fly, *Drosophila melanogaster* has developed efficient immune mechanisms to prevent microbial infection, which are consisted of cellular and humoral responses. During the systemic or local infection, two distinct pathways (Toll and Imd) play major roles in antimicrobial peptide synthesis. The Toll pathway is required to defend Gram-positive bacterial and fungal infections, whereas the Imd pathway is important in Gram-negative bacterial infection. We have shown that the infection of the opportunistic Gram-negative bacterium, *Pseudomonas aeruginosa* strain PA14 (PA14) into fly dorsal thorax can kill the flies within 48 h (100% mortality) in our optimized infection condition, suggesting that the PA14 strain can cause disease progress in fly model system. We found that flies carrying a constitutively activated mutant form of the Toll receptor (Tl^{10b}) showed increased resistance to *P. aeruginosa* infection and that flies carrying mutations in the Toll signaling pathway as well as in the Imd signaling pathway was more susceptible to PA14 infection. All these results imply that the Toll pathway might be important in the resistance to this pathogenic Gram-negative bacterial infection.

Introduction

Host-pathogen interactions are complex and multifactorial interactions that involve both the host and the pathogen. The invading pathogen encounters host defenses that restrict its establishment and multiplication. For a pathogen to cause disease, it must possess mechanisms that allow it to establish and proliferate within its specific host by defeating or evading host defenses. Although, there remains much to be learned regarding the role that various bacterial products play during infection, recent studies on pathogenesis have shown how bacterial pathogens interact with eukaryotic host cells, thereby inhibiting or eliciting host responses of importance for the infectious disease process. Based on investigations that have uncovered striking conservation of innate immunity defenses in plant, insects and mammals, which points to a common ancestry of these systems (reviewed in Hoffmann et al., 1999), we have developed the he use of genetically tractable hosts to model *P. aeruginosa* pathogenesis is based on our initial discovery showing a remarkable conservation in the virulence mechanisms used by *P. aeruginosa* to infect hosts that are evolutionarily diverged (Rahme et al., 1995, Rahme et al., 1997, Mahajan-Miklos et al., 1999; Tan et al., 1999, Jander et al., 2000).

There is an extensive body of literature regarding the mechanism by which insects, like their vertebrate counterparts, defend themselves against pathogens by triggering both the humoral and cellular immune defense systems in response to infection. Upon exposure to microbes, the Toll/Dorsal or IL-1R/NF-κB pathways are activated in flies and mammals. In *Drosophila*, the production of antimicrobial peptides, which are the flies' major defense against invading pathogens, are specifically induced by three signaling pathways in response to microbial challenge. The Toll pathway confers resistance to fungal infection but not to *E. coli* (Lemaitre et al., 1996; Rutschmann et al., 2000), whereas a Toll-related receptor known as 18-Wheerer (18W, Toll2) is critical in the antibacterial immune response (Williams et al., 1997). Regulation is achieved by controlling the nuclear translocation of two NF-κB-like proteins, Dorsal, Dif and Relish (Reichhart et al., 1993; Meng et al, 1999; Hedengren et al., 2000; Rutschmann et al., 2000). In the absence of immune challenge, Dif and Dorsal are associated with their inhibitor, Cactus, an IκB-like homolog in the cytoplasm. Upon immune challenge, the receptors relay phosphorylation signals that causes the ubiquitination and proteolytic degradation of Cactus, thus allowing nuclear translocation of Dorsal and Dif. The Toll pathway controls the nuclear localization of both Dorsal and Dif, while 18-Wheeler mediates antimicrobial peptide *Attacin* expression through translocation of Dif only (Williams et al., 1997; Wu and Anderson, 1998). Thus, separate pathways exist to discriminate between Cactus-Dorsal and Cactus-Dif complexes to promote an appropriate response. A third pathway involving the immune-deficiency locus (*imd*) also appears to be important in the response to bacterial infection, as *imd*$^{-/-}$ show a lower survival rate than wild-type flies when challenged by *E. coli*, but not when infected by various entomopathogenic fungi (Lemaitre et al., 1995).

The large body of elegant works on *D. melanogaster* immune responses to bacterial challenge is based on the use of non-pathogenic, yet highly immunogenic microorganisms (Lemaitre et al., 1995; Williams et al., 1997; Wu and Anderson, 1998; Basset et al., 2000). Innate immune studies using microorganisms that are unable to establish an infection in flies do not address host immune responses that occur during disease development. In this presentation, we use *D. melanogaster* to facilitate the dissection of disease responsive pathways to the Gram-negative pathogen, *P. aeruginosa*. We show that *P. aeruginosa* strain PA14 establishes an infection and kills *D. melanogaster*. Some PA14 isogenic mutants and clinical isolates that exhibit decreased virulence in other virulence models, are also less-virulent in *D. melanogaster*, showing the high conservation of multi-host pathogenesis mechasnism of PA14. Furthermore, we demonstrate that the Toll signaling pathway is required for full resistance to *P. aeruginosa* infection.

P. aeruginosa proliferates and kills *D. melanogaster*

The success of a pathogen is measured by its ability to survive and proliferate in an infected host. To use *D. melanogaster* as a model host to study susceptible host-pathogen interactions, we tested for the ability of *P. aeruginosa* to establish an infection in *D. melanogaster*. We compared the ability of *D. melanogaster* Oregon R to resist infection by *E. coli* strain DH5α and two *P. aeruginosa* strains UCBPP-PA14 and PAO1, by monitoring fly mortality and bacterial proliferation.

A

% survival

120
100
80
60
40
20
0

0   20   40   60   80

Time (hr)

B

Log10 of bacterial cfu/fly

10

7.5

5

2.5

0

0   10   20   30   40   50

Time (hr)

—○— Oregon R - Aseptic injury
—▲— Oregon R - E. coli
—◆— Oregon R - PA14
—☐— Tl 10b /+ - PA14
—✕— Oregon R - PAO1

—●— PA14 - Oregon R
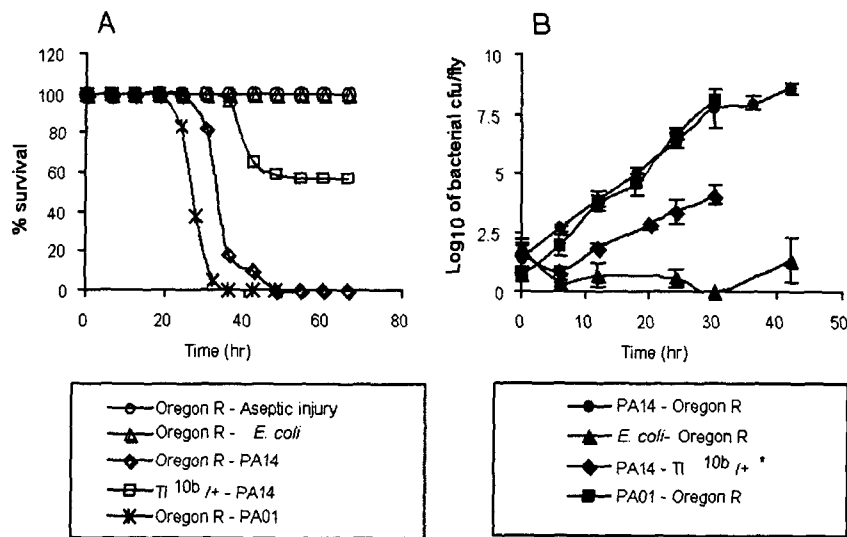—▲— E. coli- Oregon R
—◆— PA14 - Tl 10b /+ *
—■— PAO1 - Oregon R

Figure 1. Survival of Flies and Time Course of Bacterial Proliferation in Infected Flies. A. % survival of flies following aseptic injury (pricking with a 10 μ needle) or infection with an inoculum of 10 to 100 bacterial cells/fly. Fly survival was monitored up to 72 hours post-infection. Three independent experiments provided similar results. Approximately the total of 40 flies were used for each experiment. B. Proliferation of P. aeruginosa strains PA14, PAO1 and E. coli in Oregon-R and Tl10b/+ flies. Means of five flies per time point + SD are shown. Three independent experiments provided similar results. *Bacterial growth in Tl10b/+ flies is plotted up to 30 hours. Beyond that point flies began to die.

Our control experiments showed that pricking flies with a sterile needle does not cause death, and that flies quickly recover from injury (Fig. 1A). Similarly, pricking flies with needles containing DH5α does not cause death (Fig. 1A; Lemaitre et al., 1996). In contrast, both PA14 and PAO1 are lethal to D. melanogaster. PA14-infected flies start dying at about 30 hours post-infection, and reach 100% mortality within approximately 44 hr, while PAO1 causes mortality beginning at an earlier time point, approximately 24 hours post infection (Fig. 1A). Both PAO1 and PA14 were lethal with a LD$_{50}$ of one bacterium (data not shown).

Growth of DH5α, PA14 and PAO1 within infected flies was determined to examine whether the killing of the flies correlated with the ability of the bacteria to proliferate and establish an infection, rather than a result of a toxic compound secreted by P. aeruginosa. As expected, no bacterial multiplication was observed in flies infected with DH5α and almost all the bacteria were cleared shortly after infection (Fig. 1B). In contrast, Oregon R flies were unable to restrict the growth of both P. aeruginosa virulent strains PA14 and PAO1. The growth profile of both strains was essentially identical. The number of viable bacteria in both PAO1 and PA14-infected flies increased by approximately 5 logs within 24 hr post-infection, reaching a maximum bacterial titer of approximately 7 logs at fly death (Fig. 1B).
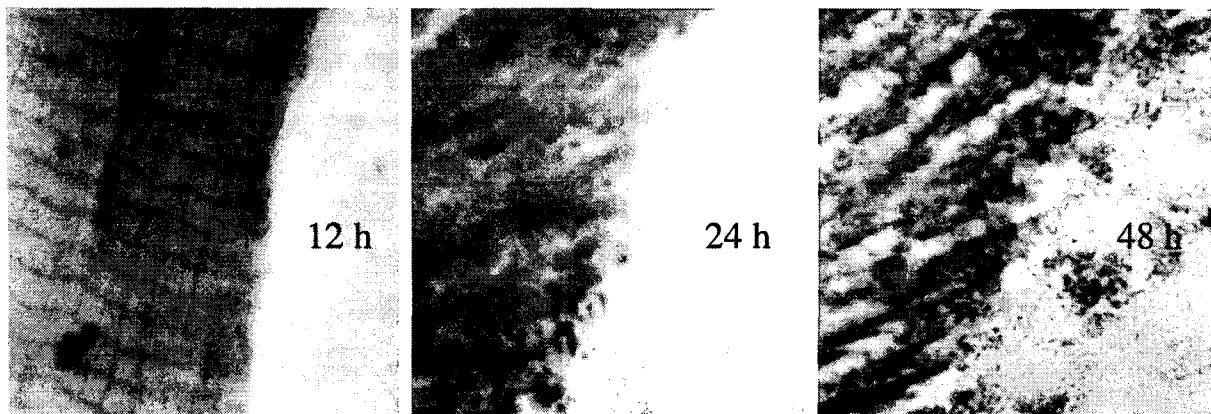
Fig. 2. Cytology of PA14-infected flies. Male Oregon-R flies infected with PA14 were collected at indicated intervals and sectioned. Histologic preparations were stained with Haemotoxylin & Eosin and evaluated by light microscopy. Original magnification (100X).

P. aeruginosa is capable of invading and colonizing *D. melanogaster* tissues.

Following injection with PA14 in the dorsal part of flight thorax, we sectioned and morphologically evaluated the infected flies at consecutive time points. At early time points (0 -12 hr), the infection is localized and restricted to the focus of injection (data not shown), and the remainder of the thorax muscle remained healthy as demonstrated by the intact striated muscle (Fig. 2A). Beyond 24 hr, we observed a more widespread invasion by the bacteria, which are present in the abdominal section of the fly, and the infected dorsal thorax muscle began to show signs of degradation with increasing amounts of PA14 present (Fig. 2B). By 36 and 40 hr, the amount of bacteria is overwhelming and can be observed in almost every organ of the fly. Significant consumption of flight muscles has occurred and the appearance of striated muscle is no longer apparent (Fig. 2C). These findings indicate a progressive invasion process and systemic spread of the pathogen that corr elates with the degree of bacterial proliferation over time. Bacteria are able to invade, colonize, and utilize the tissues of the fly as a source of nutrients. By 40 hours, there is significant tissue consumption, which is incompatible with physiologic functioning of the flies and results in their death.

Identification of PA14 mutants attenuated in *D. melanogaster* infection

To determine whether the *P. aeruginosa-D. melanogaster* model system would be suitable to study some of the universal mechanisms underlying *P. aeruginosa* transposon mutants, previously identified as pathogenic in *Arabidopsis thaliana* or *Caenorhabditis elegans* screens and subsequently shown to be relevant for mammalian pathogenesis (Rahme et al., 1997; Mahajan-Miklos et al., 1999; Tan et al., 1999). As summarized in Tables 1 and 2, PA strains and PA14 isogenic mutants exhibited reduced virulence in flies as indicated by less than 65% fly mortality after infection.

Table 1. Virulence of *P. aeruginosa* clinical isolates in *D. melanogaster* pathogenesis model

	Strain	% Mortality		Strain	% Mortality
Group A	PA14	100	**Group C**	CF27	50.0
Highly	PA37	96.0	Attenuated-in-	PA2	47.5
virulent	CF2	96.0	virulence	CF1	28.0
	PA8	93.3		CF29	27.0
Group B	PA4	75.8	**Group D**	CF4	9.0
Virulent	PA12	83.8	Highly	CF30	4.0
	PA13	72.8	Attenuated-in-	CF5	3.0
	PA15	83.3	virulence	CF3	0
	PA38	83.0			
	PA46	80.8			

Table 2. Virulence of *P. aeruginosa* attenuated mutants in *D. melanogaster*

Mutant & gene	Fly Mortality	Mouse Mortality	Comments
pho15 (dsbA)	4	62	Periplasmic disulfide bond formation
plcS	23	40	Hemolysis, degrades phospholipids of eukaryotic membrane
35A9 (mtrR)	24	53	Multi-drug transporter, decreased surface attachment
12A1 (lasR)	24	50	Quorum sensing regulator
6A6 (phzB)	24, 28	18	Phenazine biosynthesis
33C7	50	0	Methyl transferase motif
50E12	56	0	for accumulation of poly-ß-hydroxybutyrate
toxA	50	40	Exotoxin A inhibiting eukaryotic protein synthesis
34B12 (mvfR)	50	50	Global regulator of exoproducts (i.e. pyocyanin)
115E3	100	0	Pathogenicity islet (not present in PAO1)
33A9	90	0	Reduced motility, increased surface attachment
36A4	78	0	Homologue of *P. syringae hrpM*
25F1	85	0	Homologue of *Chlorobium tepidum orfT*

Toll pathway is required for increased resistance to PA14 Infection

To determine whether the host innate immunity responses of *D. melanogaster* against *P. aeruginosa* involve components of the Toll pathway, we infected Toll-dominant/+ heterozygous flies (Tl^{10b}/+) with strain PA14. As shown in Fig. 3A, Tl^{10b}/+ flies were more resistant to PA14 infection than Oregon R flies. Mortality rates of Tl^{10b}/+ flies are reduced and delayed over time. Approximately 60% of these flies survived infection compared to 0% survival of the infected Oregon R 64 hours post infection (Fig. 1A).

Decreased killing of Tl^{10b}/+ flies correlates with restricted ability of the bacteria to proliferate in Tl^{10b}/+ flies (Fig. 1B). Although bacterial titers increased steadily during the first 30 hours of infection, bacterial titers in Tl^{10b}/+ flies were lower than in the wild-type Oregon R flies by a factor of approximately 100 folds.

We tried to measure the mortality of the fly mutants defective in Toll signaling cassette. Several toll-signaling-related flies (Toll, Spätzle, Dif and Dorsal) are more susceptible to PA14 infection in terms of death kinetics as well as imd and Relish mutant flies (data not shown). Further as shown in Fig. 3, even the virulence-attenuated mutants (*33C7* and *plcS*) and avirulent isolate (PA2) were as virulent as PA14 in Toll-signaling-
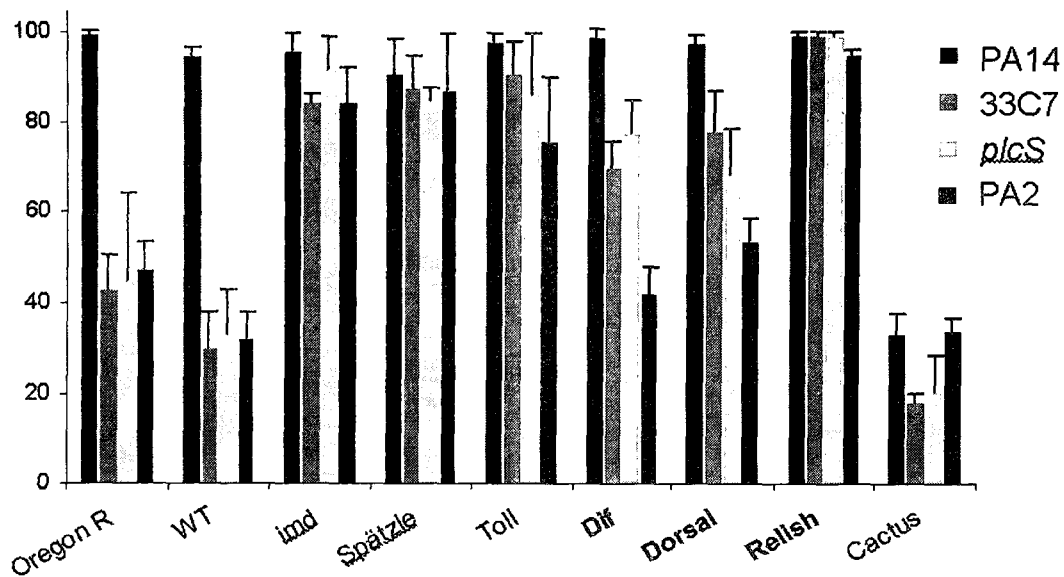
Fig. 3. Mortality of Toll and imd pathway mutants of *D. melanogaster*. Mortality was calculated as the fraction of dead flies at 48 hours post-infection with an inoculum of 10 to 100 bacterial cells of each strain (PA14 and PA2) or attenuated mutant (33C7 and *plcS*). At least five independent experiments were perfomed for standard deviation. Approximately the total of 40 flies per infection were used.

(Toll, Spätzle, Dif and Dorsal). This leads us to the suggestion that the genes involved in each virulence mechanism are important in full virulence in the wild type *D. melanogaster* and that *P. aeruginosa* is able to affect components of the Toll signaling pathway by directly or indirectly limiting and delaying the transient nuclear localization of the transcription factor Dorsal and restraining the degradation of Cactus.

These results further confirm the importance of the components of the Toll pathway during Gram-negative infections and demonstrate that a fully virulent *P. aeruginosa* strain prevents an early and prolonged presence of Dorsal in the nuclei of the fat-body cells.

Discussion

The host-pathogen interaction responses described in this work are based on antagonistic interactions between *D. melanogaster* and the Gram-negative pathogen *P. aeruginosa* strain PA14. We show that the PA14 proliferates to extremely high titers, causes disease and effectively kills *D. melanogaster*. This ability is not unique to one particular *P. aeruginosa* strain, as flies are also susceptible to killing by the well-characterized *P. aeruginosa* strain PAO1. Using *D. melanogaster* as a genetically tractable host that provides tools for the dissection of host innate immunity responses during infection, we demonstrate that *D. melanogaster* immune defense response elicited as a consequence to infection caused by *P. aeruginosa* requires components of the Toll pathway as opposed to *E. coli*.

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Multifactorial nature of *P. aeruginosa* infection in *D. melanogaster*

Mortality studies with the PA14 isogenic mutants reveal that several virulence factors relevant in mammalian pathogenesis also affect the efficacy of bacterial proliferation and killing in *D. melanogaster* (Table 1). Our cytological studies of infected fly tissues, which show massive muscle tissue consumption, suggest the importance of extracellular degradative enzymes such as lipases and proteases during the infection. Indeed, two quorum sensing regulatory mutants, *gacA* (Rahme 1997; Pesci and Iglewski, 1997; Reimmann et al., 1997) and *mvfR* (Rahme et al., 1997; Cao et al., 2001) that show defects in the production of these enzymes cause lower percentage of killing compared to the wild-type strain PA14. It is interesting to note that flies die earlier following infection with strain PAO1 than with strain PA14. This finding is likely mediated by the action of toxins produced specifically by strain PAO1. It has been reported that an uncharacterized toxin regulated by quorum-sensing regulators LasR and RhlR, rapidly and lethally paralyzes *C. elegans* (Darby et al., 1999). This toxin is not present in PA14, and may contribute to the early mortality in our PAO1 infected flies.

The variety of mammalian virulence-associated genes identified as relevant in fly virulence indicates that the use of *D. melanogaster* as a host to model mammalian pathogenesis has few limitations with regard to the categories of *P. aeruginosa* virulence-related functions that can be studied.

Toll pathway is important in resistance of *D. melanogaster* to Infection by *P. aeruginosa*.

One of the most common targets of mammalian immune suppression caused by Gram-negative bacteria such as Yersinae and Salmonellae is the IL-1R/ NF-κB pathway. The Yersinia virulence factors, YopJ, was shown to bind directly to the superfamily of Map Kinase Kinase (MKKs) and I kappa Kinase (IKK,, blocking both phosphorylation and subsequent activation of the MKKs and IKK (Ruskdeschel et al., 1997; Boland and Cornelis, 1998; Orth et al., 1999; Palmer et al., 1999). Previously, Lemaitre et al (1996) showed that the *Drosophila* Toll pathway is required for resistance to fungal infection, but not to the Gram-negative bacterium *E. coli*. A number of previously conducted expression studies of antimicrobial peptides in mutants affecting components of the *Drosophila* Toll pathway, such as *Toll*, *pelle* and *tube* lend some clues to the potential role played by Toll in the antibacterial immune responses (Lemaitre et al., 1996; Lemaitre et al., 1997; Wu and Anderson, 1998). Homozygous recessive mutations in the components of the *Drosophila* Tl pathway, such as *Tl$^{-/-}$*, *pelle$^{-/-}$* and *tube$^{-/-}$* strongly diminish the expression of *Drosomycin* (antifungal) and *Metchnikowin* (antifungal and antibacterial) and to a lesser extent *Cecropins* (antifungal and antibacterial) *Defensin* (antibacterial) in response to fungal and *E. coli* infections, respectively (Lemaitre et al., 1996; Lemaitre et al., 1997). Accordingly, gain-of-function unchallenged Toll (Tl^{10b}/+) mutant flies show strong constitutive expression of *Drosomycin* and *Metchnikowin* and higher levels (2- to 5-fold) of all antimicrobial peptides than the unchallenged wild type Oregon R. However, since *E. coli* does not infect flies, it is not possible to determine the involvement of the Toll pathway responding to bacterial infection.

Using gain-of-function Toll (Tl^{10b}/+) mutant flies and the *P. aeruginosa* strain PA14 we demonstrate the importance of the Toll signaling pathway in resistance to infection by this Gram-negative virulent bacterium.

Our findings show that the $Tl^{10b}/+$ flies are approximately 60% more resistant to PA14 infection, and that PA14 proliferates less vigorously in $Tl^{10b}/+$ flies than in the wild type Oregon R flies. It is possible that the constitutive activation of Toll pathway results in above basal level accumulation of antimicrobial peptides, which then may serve as the first line of D. melanogaster host defense against the P. aeruginosa infection. In support of this notion are our findings that demonstrate that the expression of Drosomycin, known to be uniquely controlled by the Toll pathway (Lemaitre et al., 1996), is substantially reduced at the early stages of the infection in wild-type flies. Additional demonstration of the importance of the Toll pathway and of Drosomycin in the first line of D. melanogaster defense to the Gram-negative pathogen P. aeruginosa comes from our expression studies involving the attenuated in virulence mutants 33C7 and plcS discussed above.

Immune suppression by modulating Toll-mediated immune response

Early modulation of mammalian immune responses has been shown to play a crucial role in the impressive ability of Yersiniae to overcome the defenses of the mammalian host and to overwhelm it with massive bacterial growth. Yersiniae use a type III secretion system to inject several virulence factors (Yops) into target cells, which act in concert at different stages of infection to suppress the immune response. Though not well characterized, P. aeruginosa harbors a type III secretion system that translocates various effector proteins into the host cells to modulate host proinflammatory responses (reviewed in Frank, 1997; Yahr et al, 1997; Yahr et al., 1998; Sawa et al., 1999). It is remarkable that Yersiniae could modulate immune responses immediately upon contact with the host cell. It has been shown by Andersson et al. (1996) that a YopH-dependent dephosphorylation of several host immunity-related proteins occurs within one minute following bacterial host-cell contact. In this respect, it is of further interest that the proteins secreted by Enteropathogenic E. coli (EPEC) also exhibit an immediate effect on host cells. Thus, killing of extracellularly adherent EPEC as early as 5 to 10 min after the initial contact with epithelial cells still allows the bacteria to trigger the full program of A/E lesion formation (Rosenshine et al., 1996). It is possible then, that the inability of PA14-infected flies to fully express early systemic defense responses, not even the expected response as a consequence of the aseptic injury, may be a result of a local immune suppression initiated by bacterial-host cell contact. This pathogenic strategy may lead to the delay of the onset of the systemic defense responses, thus allowing host colonization and disease development.

P. aeruginosa targets components of the Toll pathway in order to cause disease in D. melanogaster.

Our findings show that P. aeruginosa is able to modulate host protective measures by restraining early host defense responses. The immunohistochemistry studies demonstrate that this modulation targets at least one of the components of the Drosophila Toll pathway Dorsal, not previously shown to be affected by Gram-negative bacteria (data not shown). These findings provide us with the opportunity to perform in vivo studies using Drosophila as a model host and elucidate the role of the components of Toll pathway as well as facilitate the identification of bacterial products involved in the modulation of Toll-related responses. Based on the

overall number of immuno-reactive nuclei to the Dorsal antibody, nuclear localization of Dorsal peaks 24 hr post-infection. The delay of the nuclear translocation of Dorsal in Phase I and the transient nuclear presence of Dorsal in Phase II correlates with the timing and the limited expression of the antimicrobial peptides. Despite the correlation between Dorsal translocation and the expression of antimicrobial genes, Cactus degradation is not observed. A very plausible explanation is that Cactus remains in complex with Dorsal having only limited amount of Cactus to be degraded, that is beyond our detection limit. The over-abundance of Cactus protein may physically inhibit the nuclear localization of the transcriptional activator Dorsal leading to the prevention of the early induction of the *Drosophila* immune responses. This interpretation can explain the limited and transient nuclear presence of Dorsal into the fat-body nuclei. Recently, it has been shown by Neish et. al. (2000) that non-pathogenic (commensal) bacteria have also evolved ways to interfere with the NF-κB activation. Inhibition of IKK has been shown in Yersiniae infection by the way of inhibiting Map kinase kinase (Orth et al., 1999). In addition, it is possible that Dorsal may translocate into fat-body nuclei via a Toll-dependent but Cactus-independent manner. This behavior may be similar to Cactus-independent, but ventral signal-dependent regulation of Dorsal nuclear import during dorsal-ventral polarity determination in *Drosophila* embryo (Drier et al., 1999; 2000). Similarly, Cactus degradation is the direct result of ventral signaling from Toll receptor rather than a secondary consequence of its separation of the Dorsal/Cactus complex (Belvin and Anderson, 1996).

The lack or limited Cactus degradation during *P. aeruginosa* infection may be also significant in the context of the cellular response. If Cactus degradation is required for hemocyte differentiation as indicated by the overabundance of hemocytes in flies lacking Cactus protein (Qiu et al., 1998), then a disproportionately high amount of Cactus resulting from the PA14-infection will inhibit Dorsal and prevent hemocyte differentiation. This finding suggests that even though NF-κB-like transcriptional factors are induced, the cellular response is still non-functional. Work is in progress to determine the role of Cactus and the effect of PA14-infection on hemocyte differentiation and proliferation during *P. aeruginosa* infection.

Based on our results, we propose that one of the *in vivo* mechanisms that *P. aeruginosa* uses to block the onset of a systemic defense response is to impose an early and most likely immediate inhibition on the Toll–dependent immune responses at the site of infection. Inhibition of the local immune response by bacterial virulence factors provides an appropriate environment for colonization and establishment of infection.

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