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The *Drosophila melanogaster* Toll Pathway Participates in Resistance to Infection by the Gram-Negative Human Pathogen *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa **is a gram-negative pathogen that infects immunocompromised and cystic fibrosis patients. The molecular basis of the host-*P. aeruginosa* interaction and the effect of specific *P. aeruginosa* virulence factors on various components of the innate immunity pathways are largely unknown. We examine interactions between *P. aeruginosa* virulence factors and components of innate immunity response in the *Drosophila melanogaster* model system to reveal the importance of the Toll signaling pathway in resistance to infection by the *P. aeruginosa* human isolate PA14. Using the two PA14-isogenic mutants *plcS* and *dsbA*, we show that *Drosophila* loss-of-function mutants of Spatzle, the extracellular ligand of Toll, and Dorsal and Dif, two NF-κB-like transcription factors, allow increased *P. aeruginosa* infectivity within fly tissues. In contrast, a constitutively active Toll mutant and a loss-of-function mutant of Cactus, an IκB-like factor that inhibits the Toll signaling, reduce infectivity. Our finding that Dorsal activity is required to restrict *P. aeruginosa* infectivity in *Drosophila* provides direct in vivo evidence for Dorsal function in adult fly immunity. Additionally, our results provide the basis for future studies into interactions between *P. aeruginosa* virulence factors and components of the Toll signaling pathway, which is functionally conserved between flies and humans.**

Microbial pathogens use a variety of complex strategies to subvert host defenses to ensure their multiplication and survival. *Pseudomonas aeruginosa* is a medically important opportunistic human pathogen (10, 42) that, unlike most pathogens, exhibits an extremely broad host range that includes vertebrates (9), insects (3, 5, 7, 15), nematodes (22, 38), and plants (30). The virulence mechanisms used by *P. aeruginosa* to infect these phylogenetically diverse hosts (30) are remarkably conserved (30), suggesting that dissection of the immune strategies used by one model host system to subvert *P. aeruginosa* pathogenesis would provide an understanding of the strategies used by unrelated host organisms.

Drosophila melanogaster defends itself against pathogens via both humoral and cellular immune responses (1, 12). NF-κB-like transcription factors are thought to play critical roles in mediating the immune response of the fly, and at least two signaling pathways appear to trigger their activities. Relish (Rel) functions downstream of the immune deficiency gene *imd* (11), whereas Dorsal and Dif are controlled by the Toll pathway (24, 25, 35). Fungi and gram-positive, but not gram-negative, bacteria predominantly activate the Toll signaling pathway (20, 21, 26, 35) via the extracellular ligand, Spatzle. Toll pathway activation results in the proteolytic degradation of Cactus (27), a homologue of the mammalian I-κB inhibitor of Rel proteins, to mediate the nuclear import of Dorsal (18, 24, 33, 43) and Dif (13, 35, 43). Although both Dorsal and Dif are translocated to the host nuclei following microbial challenge, it is still unknown whether Dorsal participates in the adult fly immune response.

D'Argenio et al. recently reported that the gram-negative pathogen *P. aeruginosa* can infect *D. melanogaster* (5). In light of the striking evolutionary conservation of innate immunity defenses in insects and mammals (1, 12), we have utilized *D. melanogaster* to identify and dissect the disease response pathways involved in *P. aeruginosa* infection. Using virulence factors also required for mammalian pathogenesis, we show here that the *P. aeruginosa* human isolate PA14 establishes a progressive and lethal infection in *Drosophila*. We further show that the Toll signaling pathway, which is highly conserved between flies and mammals, is required to restrict the ability of *P. aeruginosa* to proliferate within fly tissues and be highly lethal. In addition, we present evidence that the *Drosophila* NF-κB-like factor Dorsal plays an important role during disease development and the immune response. These results offer insights into the interactions between *P. aeruginosa* virulence factors and components of the Toll signaling pathway during pathogenesis.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. aeruginosa* strain PA14 and the isogenic PA14 Tn*phoA* mutants were previously described (22, 30, 31, 38). Other

human isolates were obtained from Milton Schroth's UCPP strain collection (University of California at Berkeley). Bacteria were grown at 37°C in Luria-Bertani medium plus 100 μg of rifampin/ml for PA14 or 200 μg of kanamycin/ml for PA14 mutants. None of the *P. aeruginosa* strains used in this study exhibit any in vitro growth defects.

Fly stocks. Fly stocks (obtained from Bloomington Fly Center unless specified) were maintained on standard yeast extract-agar-sucrose-cornmeal medium at 24°C. Tl^{10b} flies carry a constitutively active Toll gene (37). $Tl^{10b/+}$ flies were obtained by mating virgin Oregon-R (OR) females to Tl^{10b} males. Loss-of-function trans-heterozygotes in Toll and Imd pathway mutants were obtained from the crosses TM3/spz^{rm7} × TM1/spz^{197}, CyO/$cact^{A2}$ × CyO/$cact^{IIIG}$, and CyO/dl^{17} × CyO/dl^{1}. F_1 offspring that did not carry the balancer were used for infection assays. $Bc^{1}imd^{1}$ (19) and Rel^{e20} (11) were homozygous for $imd^{-/-}$ and $rel^{-/-}$, respectively. Homozygous recessive Dif^{1} ($dif^{-/-}$) mutants were derived from *ywDD1,cnbw* (35).

Fly infection assays. All experiments used healthy, 4- to 7-day-old adult male flies. Aseptic injuries were produced by pricking flies on the dorsal thorax with a 10-μm-diameter needle (Ernest F. Fullam, Inc., Latham, New York) which had previously been dipped into 10 mM $MgSO_4$. After being dipped into a bacterial suspension containing 5×10^7 CFU/ml from an early stationary phase (optical density at 600 nm, 3.0) bacterial culture, the needle was used to infect flies with 10 to 100 *P. aeruginosa* cells/fly. Fly lethality was determined for groups of ≥25 infected flies for up to 72 h at 24°C. Flies that died within 12 h after infection were excluded. Lethality studies were repeated at least three times. For bacterial growth studies, infected flies were collected at indicated time intervals, ground in 10 mM $MgSO_4$, and plated onto Luria-Bertani agar plates supplemented with appropriate antibiotics, with colony counts scored after 14 to 16 h at 37°C.

Statistical analysis of fly survival kinetics. Statistical analysis was performed using R project software (www.r-project.org/). To test the null hypothesis that the survival kinetics of the mutant is equivalent to that of the wild type, each mutant survival curve was analyzed by two methods: (i) the log-rank test (Mantel-Haenszel) of the Kaplan-Meier estimate of survival (16) and (ii) the Cox proportional hazards regression model (4). The former method is nonparametric, while the latter utilizes a popular parametric model in the field of survival analysis. In each case, a *P* value was calculated from an analysis of the difference of the overall time course, representing the probability of randomly selecting subjects, from those predicted by the null hypothesis, whose survival curves are as different as (or more different than) those actually observed.

Fly cytological studies. Male OR flies infected with PA14 were collected at time intervals, embedded and sectioned in paraffin, stained with hematoxylin and eosin, and examined by light microscopy.

RESULTS

Human isolates of *P. aeruginosa* cause lethal infections in *D. melanogaster*. To determine whether *Drosophila* can serve as a model host for the determination of innate immune responses to *P. aeruginosa* infection, we assessed the viability of male adult OR flies infected with 10 human isolates of *P. aeruginosa* (Table 1). Each fly was infected with 10 to 100 bacterial cells. While control flies, pricked with a sterile needle, quickly recovered from injury (Fig. 1A), human isolates of *P. aeruginosa* caused differing degrees of lethality. In Table 1, these isolates are grouped into three lethality classes (high, medium, and low), members of which showed 93 to 100% lethality (strains PA14, PA37, and PA8); 73 to 84% lethality (strains PA12, PA15, PA38, PA46, PA4, and PA13); and 47% lethality (strain PA2), respectively. Previous studies have shown that other *P. aeruginosa* strains, including the well-characterized PAO1, cause highly lethal *Drosophila* infections (5, 7).

We chose the highly lethal strain PA14 to study the antagonistic interactions of *P. aeruginosa* with *Drosophila*. PA14 is also highly virulent in other model host systems such as plants (30, 31), nematodes (22, 38), the insect *Galleria mellonella* (15), and mice (30). We assessed the resistance of OR flies to PA14 infection by monitoring percent survival and bacterial proliferation over time. Figure 1A shows that PA14-infected flies began to die at 28 h postinoculation and exhibited 0% survival by 48 h. Figure 1B shows that the degree of proliferation of PA14 within fly tissues correlated with the kinetics of mortality. The number of viable bacteria in PA14-infected flies rapidly proliferated, increasing 5 logs within 24 h postinoculation, and bacterial titers continued to increase over time and reached a maximum of 7 logs by 48 h (data not shown). These results indicate that the fly mortality produced by PA14 correlates with the ability of the bacteria to proliferate and establish a lethal infection.

TABLE 1. Human isolates of *P. aeruginosa* cause varying degrees of lethality in *D. melanogaster*

Lethality group	*P. aeruginosa* human isolate	% Fly mortality[a]
High	PA14	100
	PA37	96
	PA8	93
Moderate	PA12	84
	PA15	83
	PA38	83
	PA46	81
	PA4	76
	PA13	73
Low	PA2	47

[a] Percent mortality was determined in adult *D. melanogaster* OR flies at 72 h postinoculation. A minimum of 25 flies were infected with each *P. aeruginosa* strain. The results reflect data from four independent experiments. Human isolates are classified based on the levels of lethality produced in *Drosophila.*

***P. aeruginosa* strain PA14 invades, proliferates in, and colonizes fly tissues.** Following infection in the dorsal thorax, we evaluated stained sections of the infected flies at consecutive time points (Fig. 2). Between 0 and 12 h postinoculation, the

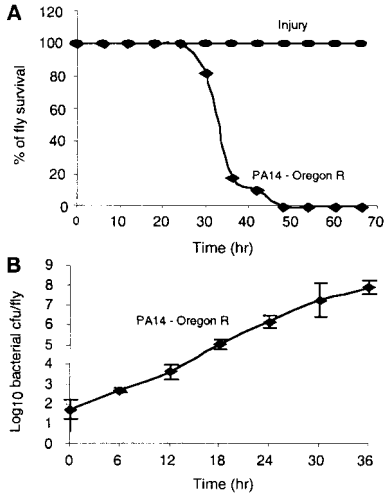

FIG. 1. *P. aeruginosa* proliferates within *D. melanogaster* tissues with a lethal outcome. (A) Percent survival of adult OR flies following injury and infection. Control flies were injured with a needle that had been dipped into 10 mM $MgSO_4$. Infected flies received 10 to 100 bacterial cells/fly. Approximately 40 flies were used for each experiment. The data reflect results from five independent experiments. (B) Proliferation of *P. aeruginosa* strain PA14 in OR flies. Means ± standard deviations (SD) of results for five flies per time point are shown. The data reflect results from four independent experiments.

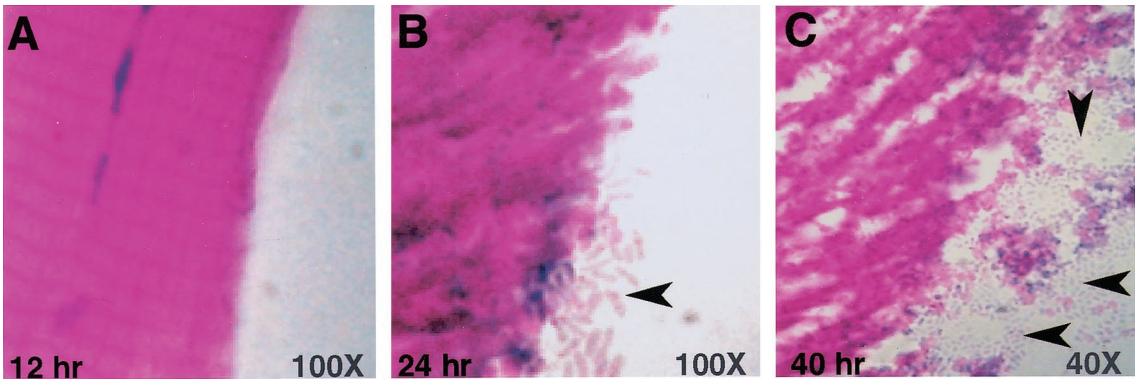

FIG. 2. *P. aeruginosa* invades and degrades *D. melanogaster* tissues. OR flies infected with PA14 were collected and sectioned at 12, 24, and 40 h postinoculation, stained with hematoxylin and eosin, and evaluated by light microscopy. (A) Intact striated flight muscle exhibited no sign of bacterial invasion at 12 h. (B) *P. aeruginosa* cells (arrowhead) associated with flight muscles at 24 h. (C) Flight muscle degradation with large numbers of *P. aeruginosa* cells (arrowheads) at 40 h. Original magnifications: ×100 for panels A and B and ×40 for panel C.

infection was restricted to the focus of inoculation, with the greater portion of the thoracic muscles unaffected (Fig. 2A). By 24 h postinfection and beyond, widespread bacterial invasion was seen and the thoracic muscles began to show muscle fiber disruption (Fig. 2B). By 40 h, significant tissue consumption and complete disruption of striated muscle morphology were observed (Fig. 2C) and bacteria were observed throughout every body organ of the fly (data not shown). These findings demonstrate a progressive invasive process and systemic spread of the pathogen that correlates with PA14 cell proliferation over time and indicate that PA14 is able to invade, colonize, and utilize fly tissues as a nutrient source.

Flies and mammals are susceptible to overlapping subsets of *P. aeruginosa* virulence factors. To determine whether *D. melanogaster* is a suitable model host for the study of specific interactions between *P. aeruginosa* virulence factors and mammalian host responses, we tested 11 PA14 mutants, previously shown to have reduced virulence in mice (23, 32), for their ability to infect flies (Table 2). Virulence genes mutated in these strains encode proteins involved in protein folding, quorum sensing, posttranscriptional control, efflux systems, biosynthesis of redox active compounds, toxins, and proteins of unknown function (23, 32). Some of these genes have previously been shown to be required for virulence in the insect *Galleria mellonella* (15). Table 2 shows that all 11 *P. aeruginosa* virulence-associated genes that are necessary for full mammalian pathogenesis are also essential for maximum pathogenicity in the fly. In addition, all mutants that produced reduced fly lethality also proliferated to lower levels within fly tissues (Table 2). These findings validate *Drosophila* as a host to study interactions between *P. aeruginosa* virulence factors and host innate immunity and suggest that insects and mammals share functionally conserved mechanisms of resistance.

The Toll signaling pathway is required to limit *P. aeruginosa* infection in flies. Since the Toll signaling pathway is thought to mediate pathogen defense in *Drosophila*, we infected fly strains mutant for genetic components of the Toll signaling pathway with the highly virulent strain PA14 and with two PA14-isogenic mutant derivatives, *dsbA* and *plcS*, which display reduced morbidity and mortality in mice (30, 31). The *dsbA* gene encodes a periplasmic thiol-disulfide oxidoreductase that catalyzes proper folding of bacterial proteins, including virulence-

TABLE 2. *P. aeruginosa* infects *D. melanogaster* and mammals using shared subsets of virulence factors

Mutant or gene	% *Drosophila* lethality[a]	% Mouse lethality[b]	Comments	Bacterial replication (log_{10} CFU/fly ± SD)
PA14	100	100	Wild-type strain	8.12 ± 0.32
dsbA	15	60	Periplasmic thiol-disulfide oxidoreductase	3.48 ± 1.20
mtrR	24	53	Transcriptional regulator of multidrug transporter	3.99 ± 2.09
gacA	28	50	Two-component quorum-sensing regulator	4.65 ± 1.95
phzB	28	18	Phenazine biosynthesis	3.46 ± 1.92
33C7	33	0	Unknown[c]	5.40 ± 1.24
plcS	44	40	Phospolipase C, lyses eukaryotic cell	4.00 ± 0.39
toxA	50	40	Exotoxin A, inhibits mammalian protein synthesis	4.67 ± 1.65
44B1	50	56	Unknown[c]	5.43 ± 1.84
pstP	56	0	Transcriptional regulator of RpoN-dependent operons	3.99 ± 2.44
mvfR	56	35	Quorum-sensing regulator	4.52 ± 2.15
pqsB	56	63	Hydroxy-alkylquinoline synthesis–quorum-sensing signaling	4.40 ± 1.77

[a] The lethality rate was measured in adult *D. melanogaster* OR flies. At least 25 flies were inoculated with a needle previously dipped into a 5×10^7/ml bacterial suspension and incubated at 24°C. Similar lethality and proliferation rates were obtained from 3 independent experiments. Bacterial loads were determined 36 hours postinfection.

[b] Mouse lethality rates were obtained using a thermally injured mouse model (30).

[c] Unknown, genes which encode hypothetical proteins of unknown function.

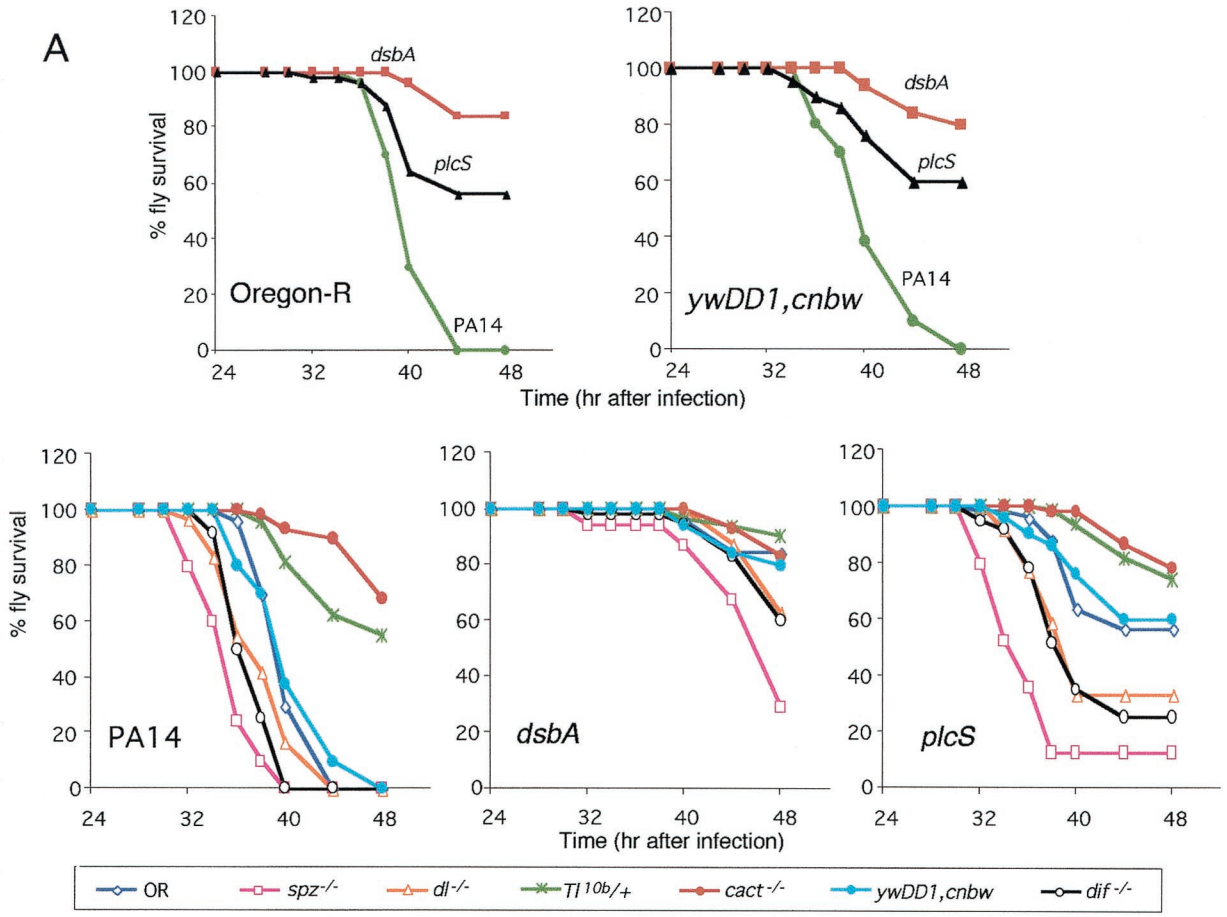

FIG. 3. Mutations in genetic components of Toll signaling alter *P. aeruginosa* lethality and proliferation. (A) Comparative survival analysis of adult flies infected with *P. aeruginosa* strain PA14 or its isogenic mutant derivatives *dsbA* and *plcS*. Survival in wild-type flies (OR and *ywDD1,cnbw*) or Toll component-mutated flies ($spz^{-/-}$, $dl^{-/-}$, $cact^{-/-}$, $dif^{-/-}$, and $Tl^{10b/+}$) was assessed. A total of 100 to 150 flies were infected and monitored for survival at 48 h postinoculation. The results reflect data from three independent experiments. (B) Comparative analysis of bacterial proliferation in adult wild-type flies (OR and *ywDD1,cnbw*) or Toll component-mutated flies ($spz^{-/-}$, $dl^{-/-}$, $cact^{-/-}$, $dif^{-/-}$, and $Tl^{10b/+}$). Flies were infected with *P. aeruginosa* strain PA14 or its isogenic mutants *dsbA* and *plcS*. Five flies per time point were used for CFU determination. Means ± SD of results for five flies per time point are shown. The results reflect data from three independent experiments.

associated proteins, such as extracellular proteases, toxins, and type III secretion apparatus components (32, 44). The *plcS* gene encodes a hemolytic phospholipase capable of degrading eukaryotic cell membrane phospholipids (28) and has been shown to suppress the respiratory burst by neutrophils in mammals (39) and to cause the release of inflammatory mediators from human granulocytes and monocytes in vitro (17).

Figure 3A shows that PA14 caused 100% lethality in wild-type OR and *ywDD1,cnbw* flies while *dsbA* and *plcS* mutant bacteria produced much lower levels of lethality of 20 and 45%, respectively. In contrast, flies that carried loss-of-function mutations in the Toll ligand *spatzle* ($spz^{-/-}$) and the NF-κB-like transcription factors *dorsal* ($dl^{-/-}$) and Dorsal-related immunity factor Dif ($dif^{-/-}$) were more susceptible to infection by *dsbA* and *plcS*, suggesting the importance of the Toll signaling pathway in limiting *P. aeruginosa* lethality (Fig. 3). Specifically, at 48 h postinfection, $spz^{-/-}$, $dl^{-/-}$, and $dif^{-/-}$ flies infected with *dsbA* exhibited 29, 62, and 60% survival, respectively, compared to *dsbA* survival levels for wild-type OR and *ywDD1,cnbw* flies of 85 and 80%, respectively. Similarly, 12, 33, and 25% of $spz^{-/-}$, $dl^{->/-}$, and $dif^{-/-}$ flies, respectively, survived *plcS* infection versus 56 and 60% survival with this strain for the wild-type OR and *ywDD1,cnbw flies*, respectively.

As presented in Table 3, statistical analysis shows that the survival kinetics (Fig. 3A) of the various loss-of-function mutant flies infected with *dsbA* or the *plcS* mutant was indeed affected. Table 3 lists *P* values resulting from comparisons of survival curves from Mantel-Haenszel tests using the Kaplan-Meier estimate of survival (16) and likelihood ratio tests using the Cox proportional hazards regression model (4). As is presented in Table 3, the two tests give similar results. The $spz^{-/-}$ flies began to die at least 5 h earlier than wild-type OR flies when infected by PA14 and *plcS*, indicating increased susceptibility of these mutant flies to the bacterial strains. Although the survival kinetics of $dl^{-/-}$ and $dif^{-/-}$ flies infected with PA14 or *plcS* shows only a slight time difference (1 to 2 h) compared to the OR flies, this difference is statistically significant (Table 3).

Further experiments with both Cactus loss-of-function

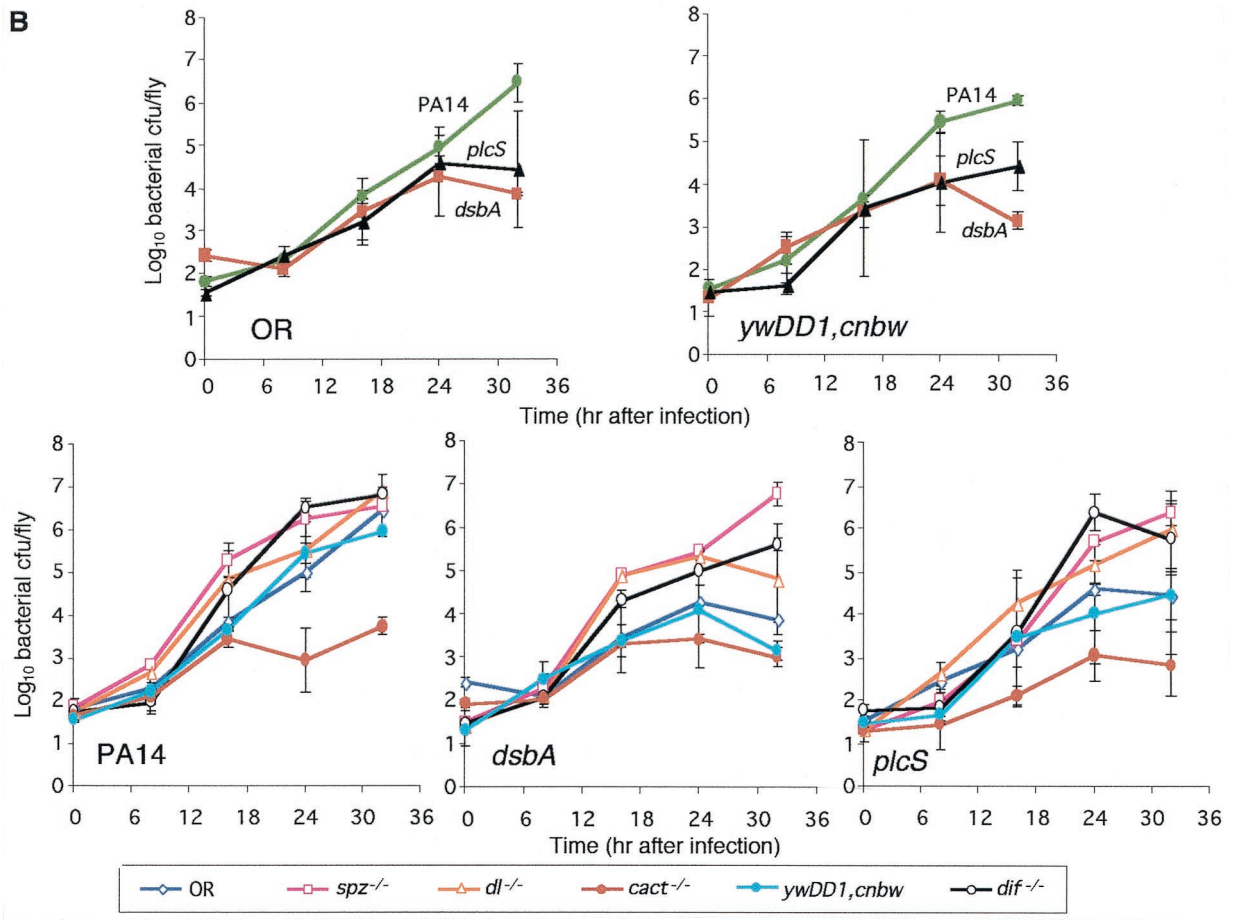

FIG. 3—*Continued.*

(*cact*$^{-/-}$) and Toll gain-of-function (*Tl*$^{10b/+}$) flies provided additional evidence for the role of the Toll pathway in mediating resistance. The Cactus protein is an inhibitor of Toll signaling, and therefore, if the Toll pathway plays a role in mediating resistance, then *cact*$^{-/-}$ mutant flies should exhibit (versus wild-type flies) heightened levels of resistance to *P. aeruginosa* pathogenesis. This result was indeed observed, as shown in Fig. 3A. The *cact*$^{-/-}$ flies were (versus wild-type OR flies) significantly more resistant to infection by the mutant *plcS* and parental strain PA14 (Table 3). Significantly, 68% of the *cact*$^{-/-}$ flies infected with PA14 survived compared to 0% survival in the PA14-infected OR flies. Similarly, gain-of-function *Tl*$^{10b/+}$ flies, which constitutively expressed activated Toll signaling, should have exhibited increased resistance to lethality. As predicted, 55% of infected *Tl*$^{10b/+}$ flies survived PA14 infection compared to the 0% survival of infected OR flies. Furthermore, both *Tl*$^{10b/+}$ and *cact*$^{-/-}$ flies exhibited delayed rates of lethality when infected by either PA14 or the *plcS* mutant strain. Interestingly, a delay in death (versus wild-type flies) was not observed in *Tl*$^{10b/+}$ and *cact*$^{-/-}$ flies or in any other mutant flies when infected with the *dsbA* mutant strain. This result might have been expected, since the *dsbA* mutant strain exhibited highly attenuated virulence in most fly strains (Fig. 3), likely due to the inability of the *dsbA* mutant to properly fold multiple virulence-associated proteins. It is possible that

TABLE 3. Statistical analysis of the survival of wild-type and mutant flies after infection with the wild-type *P. aeruginosa* strain PA14 and isogenic mutants *plcS* and *dsbA* using the Kaplan-Meier (16) and Cox (4) models[a]

Strains	*P* by:	
	Kaplan-Meier	Cox proportional hazards model
PA14-OR vs *dsbA*-OR	0	0
PA14-OR vs *plcS*-OR	4.11*e*−15	2.22*e*−16
PA14-YWDD1 vs *dsbA*-YWDD1	0	0
PA14-YWDD1 vs *plcS*-YWDD1	1.48*e*−14	8.88*e*−16
PA14-OR vs PA14-*spz*	0	0
PA14-OR vs PA14-*dl*	1.03*e*−05	0.000862
PA14-OR vs PA14-*dif*	1.11*e*−16	4.44*e*−16
PA14-OR vs PA14-*Tl10b*	0	0
PA14-OR vs PA14-*cact*	0	0
dsbA-OR vs dsbA-spz	3.84e−13	2.68*e*−14
dsbA-OR vs *dsbA-dl*	0.00328	0.00237
dsbA-OR vs *dsbA-dif*	0.000566	0.000418
dsbA-OR vs *dsbA-Tl10b*	0.21	0.197
dsbA-OR vs *dsbA-cact*	0.985	0.992
plcS-OR vs *plcS-spz*	0	0
plcS-OR vs *plcS-dl*	2.53*e*−05	3.56*e*−05
plcS-OR vs *plcS-dif*	1.23*e*−07	3.01*e*−07
plcS-OR vs *plcS-Tl10b*	0.0013	0.00124
plcS-OR vs *plcS-cact*	0.000156	0.000131

[a] Table lists and compares the *P* values for survival curves from Mantel-Haenszel tests using the Kaplan-Meier estimate of survival (16) and likelihood ratio tests using the Cox proportional hazards regression model (4).

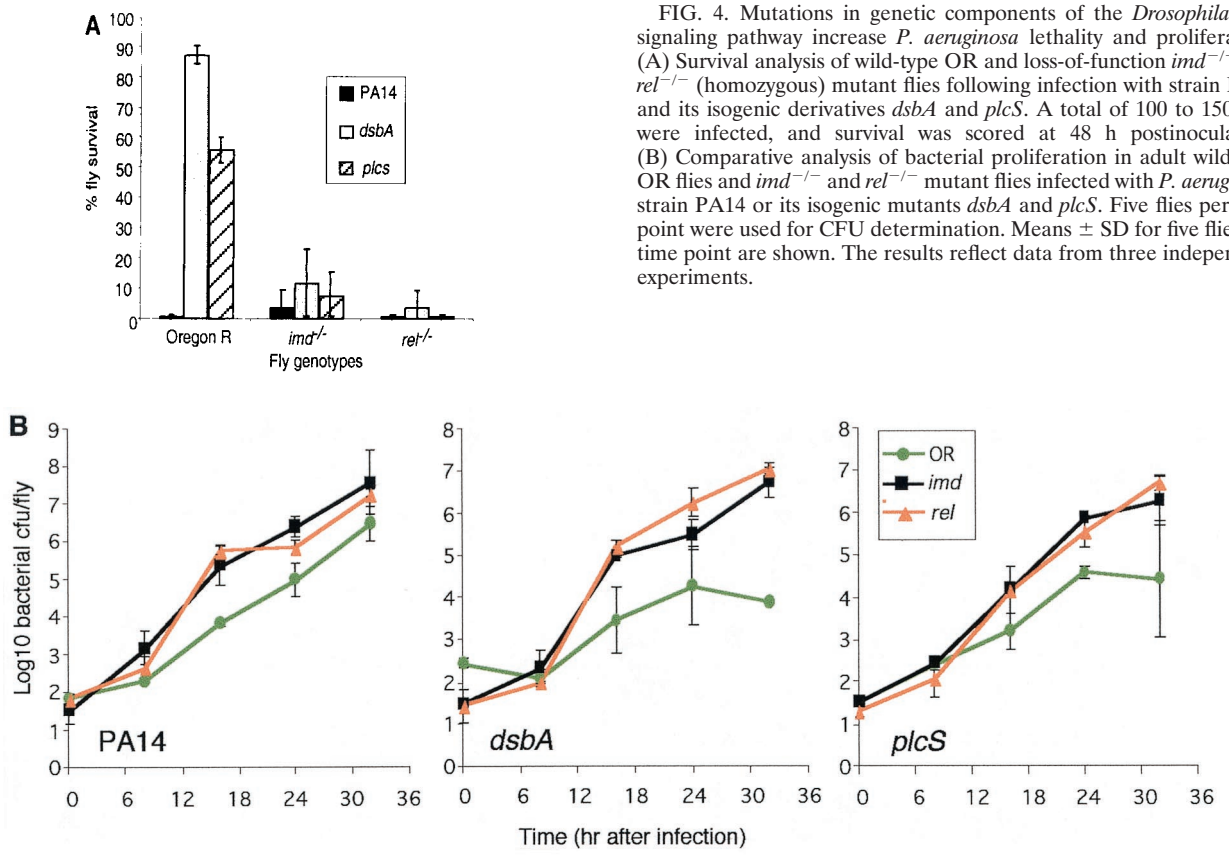

FIG. 4. Mutations in genetic components of the *Drosophila* Imd signaling pathway increase *P. aeruginosa* lethality and proliferation. (A) Survival analysis of wild-type OR and loss-of-function $imd^{-/-}$ and $rel^{-/-}$ (homozygous) mutant flies following infection with strain PA14 and its isogenic derivatives *dsbA* and *plcS*. A total of 100 to 150 flies were infected, and survival was scored at 48 h postinoculation. (B) Comparative analysis of bacterial proliferation in adult wild-type OR flies and $imd^{-/-}$ and $rel^{-/-}$ mutant flies infected with *P. aeruginosa* strain PA14 or its isogenic mutants *dsbA* and *plcS*. Five flies per time point were used for CFU determination. Means ± SD for five flies per time point are shown. The results reflect data from three independent experiments.

the low level of virulence obtained with *dsbA* could be mediated via the gradual accumulation of non-*dsbA*-dependent factors.

Survival of Toll pathway mutant flies correlates with *P. aeruginosa* proliferation. The enhanced susceptibility of $spz^{-/-}$, $dl^{-/-}$, and $dif^{-/-}$ flies and the increased resistance of $cact^{-/-}$ and $Tl^{10b/+}$ flies to the PA14 mutants *plcS* and *dsbA* implicate the Toll signaling pathway in mediating resistance to *P. aeruginosa*. To determine whether the increased fly mortality seen in flies with mutations in components of the Toll and Imd pathways corresponds to an increased ability of *dsbA* and *plcS* bacteria to multiply within these immune-deficient hosts, we profiled the kinetics of bacterial replication. Figure 3B demonstrates that both *dsbA* and *plcS* mutant bacteria displayed decreased growth versus wild-type PA14 bacteria in OR and *ywDD1,cnbw* wild-type flies, correlating with the higher postinfection survival observed with these strains. This is particularly evident at 32 h postinoculation, when the bacterial loads in PA14-infected OR flies were 2.6 and 2.0 logs higher than those seen in OR flies infected with *dsbA* and *plcS*, respectively. In contrast, *dsbA* and *plcS* bacteria replicated to higher loads in $spz^{-/-}$, $dl^{-/-}$, and $dif^{-/-}$ mutant flies (Fig. 3B). Specifically, by 32 h postinoculation, the *dsbA* and *plcS* bacterial loads were 2.9 and 1.9 logs higher in $spz^{-/-}$ than in OR flies, respectively. Similarly, the bacterial loads of *dsbA* and *plcS* in $dl^{-/-}$ flies at 24 and 32 h were 1.1 and 0.9 logs higher and 0.6 and 1.5 logs higher, respectively, than those seen with the wild-type OR flies. Furthermore, by 32 h postinfection, the bacterial loads of *dsbA* and *plcS* in $dif^{-/-}$ flies were 2.5 and 1.3 logs higher than those seen with the wild-type parent *ywDD1,cnbw* flies. These data strengthen the idea of a correspondence between fly survival and decreased bacterial proliferation and suggest that both Dorsal and Dif participate in host immune defense to limit *P. aeruginosa* infection.

Bacterial proliferation was also scored in $cact^{-/-}$ and $Tl^{10b/+}$ flies, which exhibited constitutive Toll pathway activity. As predicted, increased survival of both these flies to bacterial infection correlated with decreased bacterial proliferation over the course of infection. Figure 3B shows that viable PA14 counts for $cact^{-/-}$ flies were approximately 3 logs lower than for OR flies at 32 h, and similar data (data not shown) were obtained for $Tl^{10b/+}$ flies. Furthermore, both *dsbA* and *plcS* strains proliferate to lower bacterial loads throughout the course of infection in $cact^{-/-}$ versus OR flies (Fig. 3B).

***P. aeruginosa* virulence is elevated in *imd* and *rel* mutant flies.** *imd* and *rel* mutations disable the Imd pathway that functions in the *Drosophila* immune response. To ascertain that this pathway also plays a role against *P. aeruginosa* infection, we infected $imd^{-/-}$ and $rel^{-/-}$ flies with PA14, *dsbA*, and *plcS* bacteria. Figure 4A shows that, as seen with Toll pathway mutant flies, *dsbA* and *plcS* lethality was greatly increased in the $imd^{-/-}$ and $rel^{-/-}$ mutants to levels similar to those observed for *dsbA* and *plcS* bacteria in $spz^{-/-}$ flies. These results agree with previous studies showing a requirement for the Imd pathway in fighting gram-negative bacterial infections (6, 19). Figure 4B demonstrates that as seen with the Toll pathway-

deficient flies, PA14, *dsbA*, and *plcS* bacteria replicated to higher densities in the $imd^{-/-}$ and $rel^{-/-}$ versus wild-type OR flies, reaching ≥2 logs higher at 32 h postinfection. These results indicate that both the Toll and Imd pathways are required to confer maximum resistance to *P. aeruginosa* in the adult fly, presumably by generating a fully competent immune system.

DISCUSSION

The results presented in this report provide several significant conclusions with regard to the progression of the host-pathogen interaction between *D. melanogaster* and the human opportunistic pathogen *P. aeruginosa*. These results also provide novel insights into the identity of both pathogen and host genetic factors that enhance or restrict pathogenesis. In addition, these results further establish *Drosophila* as a model system to gain future insights into the mechanisms of *P. aeruginosa* pathogenesis and host resistance.

With regard to the bacterial side of this interaction, we have shown that different *P. aeruginosa* isolates elicit infections in *Drosophila* of differing degrees of lethality and that strain PA14 produces 100% mortality at 36 to 48 h postinfection from an inoculum as low as 10 bacteria. We note that *Drosophila* has previously been reported to be susceptible to other human *P. aeruginosa* isolates (5, 7). We have further shown that the bacteria invade and proliferate in adult fly tissue and that the ability of PA14 and its derivatives to cause maximum lethality correlates with the kinetics of invasion and proliferation. Significantly, PA14 utilizes many of the same virulence factors to infect the fly that it uses to mediate pathogenesis in mammalian hosts and mutations in virulence factors that give decreased mortality exhibit restricted kinetics of proliferation. Additionally, we demonstrated that the *dsbA* and *plcS* virulence gene functions are required for *P. aeruginosa* to produce full disease symptoms (invasion, proliferation, and lethality) in flies.

With regard to the host side of this interaction, we have shown using different fly mutant lines that both the Toll and Imd signaling pathways function to limit the severity of pathogenesis both in the degree of lethality and in the kinetics of bacterial invasion and proliferation. Indeed, the activities of all three *Drosophila* NF-κB-like factors are required to fully restrict infection, as the PA14-isogenic mutants *dsbA* and *plcS* are significantly more virulent in flies that carry mutations in Dorsal, Dif, or Relish. Interestingly, our results on Toll contradict previous reports that the *Drosophila* Toll pathway is not involved in resistance to gram-negative bacterial infections (2, 19, 21, 35, 36). However, the recent microarray analysis by De Gregorio et al. provides evidence that the Toll signaling pathway functions in combating gram-negative infections (6). Here, we functionally demonstrate the importance of the Toll pathway by showing that the two PA14-isogenic mutants *dsbA* and *plcS*, which produce attenuated virulence in wild-type flies, are significantly more virulent in flies that carry mutations in the Toll pathway genes, *spatzle*, *dif*, and *dorsal*.

That the Toll pathway contributes resistance to *P. aeruginosa* infection is further supported by our findings that the gain-of-function Toll pathway mutant $Tl^{10b/+}$, a constitutively active allele, and the loss of function in negative regulator Cactus ($cact^{-/-}$) exhibit heightened resistance to *P. aeruginosa* strain PA14, as manifested by lower fly lethality and in vivo bacterial proliferation. Unchallenged $Tl^{10b/+}$ flies have previously been shown to exhibit strong constitutive expression of the antimicrobial peptides *Drosomycin* and *Metchnikowin*, as well as increased levels (two- to fivefold) of other antibacterial peptides, versus unchallenged wild-type OR flies (14, 20). Because $Tl^{10b/+}$ and $cact^{-/-}$ larvae also exhibit increased numbers of hemocytes (29), we cannot rule out the possibility that the enhanced resistance to *P. aeruginosa* infection observed in $Tl^{10b/+}$ and $cact^{-/-}$ flies results from more phagocytosis and that a collective effort by both humoral (8, 40, 41) and cellular (29, 34) responses underlies the increased survival of $Tl^{10b/+}$ and $cact^{-/-}$ flies to PA14 infections. Future studies using other fly mutants should help to determine the importance of these two classes of responses against *P. aeruginosa*.

Previous studies have indicated that Dorsal, Dif, and Relish play distinct roles in immunity and development (1, 12). To date, no in vivo study has demonstrated the involvement of Dorsal, which controls dorsal-ventral patterning in the developing embryo, in the adult fly immune response. Using genetic data, we provide such evidence, showing that Dorsal is required to mediate a maximal immune response: $dl^{-/-}$ flies infected with *dsbA* and *plcS* exhibit significantly greater susceptibility than do wild-type flies. Interestingly, *dsbA* and *plcS* give decreased levels of lethality and proliferation in $dl^{-/-}$ and $dif^{-/-}$ versus $spz^{-/-}$ flies. One explanation for this result is that Dif and Dorsal functions are partially redundant in the adult fly immune system in a manner similar to that previously suggested to occur for these two activities in *Drosophila* larvae (24).

In conclusion, we show that study of the pathogenic interaction between *P. aeruginosa* and *Drosophila* allows for the dissection of both the pathogenicity of, and the resistance to, this important human pathogen. The *P. aeruginosa-Drosophila* interaction permits the genetic manipulation of both the pathogen and host partners, which is not possible when studying pathogenesis in humans. Significantly, *P. aeruginosa* utilizes many of the same virulence factors to infect insects as it does to infect mammals, including humans, and the *Drosophila* Toll signaling pathway, which is required for full immunity to *P. aeruginosa*, shares striking molecular and functional conservation with the human IL-1/NF-κB signaling pathways. These findings demonstrate the utility of *Drosophila* for probing the mechanistic interactions between *P. aeruginosa* virulence factors and components of the Toll signaling pathway, which should give novel insights into how these components function to provide resistance.

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