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Identification of Drosophila Mutants Altering Defense of and Endurance to *Listeria monocytogenes* Infection

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ABSTRACT

We extended the use of Drosophila beyond being a model for signaling pathways required for pattern recognition immune signaling and show that the fly can be used to identify genes required for pathogenesis and host–pathogen interactions. We performed a forward genetic screen to identify Drosophila mutations altering sensitivity to the intracellular pathogen *Listeria monocytogenes*. We recovered 18 mutants with increased susceptibility to infection, none of which were previously shown to function in a Drosophila immune response. Using secondary screens, we divided these mutants into two groups: In the first group, mutants have reduced endurance to infections but show no change in bacterial growth. This is a new fly immunity phenotype that is not commonly studied. In the second group, mutants have a typical defense defect in which bacterial growth is increased and survival is decreased. By further challenging mutant flies with *L. monocytogenes* mutants, we identified subgroups of fly mutants that affect specific stages of the *L. monocytogenes* life cycle, exit from the vacuole, or actin-based movement. There is no overlap between our genes and the hundreds of genes identified in Drosophila S2 cells fighting *L. monocytogenes* infection, using genomewide RNAi screens *in vitro*. By using a whole-animal model and screening for host survival, we revealed genes involved in physiologies different from those that were found in previous screens, which all had defects in defensive immune signaling.

INTRACELLULAR pathogens are responsible for a large group of infectious diseases; for example, >500 million people worldwide suffer from tuberculosis, AIDS, and malaria each year (http://www.who.int). By residing in a host cell, these pathogens protect themselves from some host immune responses and drug therapies. The ability to enter and survive within a host cell requires a close and intricate interaction between pathogen and host; by manipulating host processes, pathogens can prevent immune responses or subvert host processes to aid in infection.

Listeria monocytogenes is a gram-positive, facultative intracellular bacterium and is the cause of listeriosis, a serious food-borne disease. *L. monocytogenes* has been widely used as a model pathogen to better understand the molecular and cellular aspects of intracellular pathogenesis and mammalian cell-mediated immunity. *In vitro* studies using cultured cells as a model host defined the *L. monocytogenes* intracellular life cycle and virulence factors (Cossart and Lecuit 1998; Portnoy *et al.* 1988; Kreft and Vazquez-Boland 2001; Vazquez-Boland *et al.* 2001) and demonstrated that *L. monocytogenes* can enter professional phagocytes or nonphagocytic cells (Cossart *et al.* 2003). Entry into nonphagocytic cells is dependent on surface proteins called internalins (Cossart and Lecuit 1998; Lecuit *et al.* 1999, 2001). Upon entry, a single-membrane vacuole forms around the bacterium (Cossart and Lecuit 1998). Secretion of a pore-forming cytotoxin, listeriolysin O (LLO), disrupts the phagosome membrane, freeing the bacterium into the cytosol where it can grow and divide (Kuhn *et al.* 1988; Portnoy *et al.* 1988; Gedde *et al.* 2000; O'Riordan and Portnoy 2002). Expression of the actin-nucleating protein ActA facilitates polymerization of host actin at one pole of the bacterium, resulting in directional movement through the cytosol. Bacteria are able to spread to neighboring cells and escape from a second double-membrane phagosome into the cytosol of a new host cell (Tilney and Portnoy 1989; Tilney *et al.* 1990; Domann *et al.* 1992; Kocks *et al.* 1992, 1993).

The development of RNA interference (RNAi) technology provided researchers with a new approach to studying host factors involved in host–pathogen interactions in the fly (Ramet *et al.* 2002; Foley and O'Farrell 2004; Ayres and Schneider 2006). Two recent reports describe the use of RNAi in Drosophila S2 cells (embryo-derived phagocytic cells from Drosophila) to identify host factors involved in *L. monocytogenes* infection; these screens helped clarify the cell biology of the interactions of *L. monocytogenes* with professional phagocytes (Agaisse *et al.* 2005; Cheng *et al.*

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2005). The assays used in these *in vitro* screens measured bacterial loads or qualitative changes in bacterial patterns. This limited the scope of the screens to the identification of fly genes regulating the bacterial life cycle. Surprisingly, these screens did not identify genes affecting the well-characterized pattern recognition pathways, Toll and Imd. Moreover, these *in vitro* systems are obviously limited in their ability to explore how whole-animal physiologies interact with an infecting microbe and how *L. monocytogenes* can enter and survive in a wide variety of cell types.

To overcome these limitations, we performed a forward genetic screen in whole Drosophila to identify host genes required to survive infections. We had three goals. First, we wanted to cast a wider net than has been previously used to measure immunity in Drosophila. Much of what we know about fly immunity has been deciphered using extracellular microbes in immunocompromised flies. By using *L. monocytogenes* in our study we were able to identify host genes important for survival of an intracellular infection. These genes may be specific to a *L. monocytogenes* infection, to intracellular pathogens in general, or to a variety of pathogens. By coupling survival and bacterial proliferation as our output phenotypes, we were able to identify mutants with defense defects as well as lines with pathological defects—those that die from the disease faster without altered bacterial load. We hypothesize that this second class of mutants is less able to endure the stress of an infection. Second, we wanted to determine whether genes required to survive infection in whole flies were the same as those identified in cultured cells by RNAi screens. By doing this, our screen would reveal differences in the host genes involved in the interactions between whole-animal physiology and the microbe, as well as those host genes involved in the interactions between one cell type and the infecting microbe. Third, we wanted to probe the host contributions to the infection because most studies involving host–pathogen interactions with an intracellular pathogen have focused on identifying microbial factors required for infection.

The Drosophila innate immune response has three arms: First, the cellular immune response depends on circulating phagocytic cells that can engulf and clear foreign microbes. Second, the melanization response produces melanin and toxic reactive oxygen species at wounds and sites of infection. The third branch is the humoral immune response, which involves the production of antimicrobial peptides (AMP) by the fat body. These AMPs are produced largely under the control of the Toll and Imd pathways. Both of these pathways are activated by microbial elicitors and disruption of these pathways immunocompromises the fly such that the fly becomes sensitive to normally nonpathogenic bacteria like *Escherichia coli* (Lemaitre *et al.* 1995, 1996, 1997; Kopp and Medzhitov 1999, 2003; Khush *et al.* 2001). Genetic screens in whole flies have primarily concentrated on AMP signaling as an output, strengthening our understanding of signaling through the Toll and Imd pathways (Meng *et al.* 1999; Leulier *et al.* 2000; Rutschmann *et al.* 2000; Wu *et al.* 2001; Khush *et al.* 2002; Gesellchen *et al.* 2005; Yagi and Ip 2005; Kambris *et al.* 2006). In our screen we monitored fly survival as well as bacterial growth in mutant flies. By focusing on phenotypes other than AMP transcription, we anticipated that we would identify host genes affecting a wider array of immunological and pathological functions.

We identified 18 mutants with increased sensitivity to *L. monocytogenes* infection. None of these genes were described previously to have immune or pathogenesis functions. Secondary screens grouped these mutants into phenotypic classes. The flies can be split into classes on the basis of their ability to control the growth of *L. monocytogenes*. We define one group as having defects in endurance and suspect that it has difficulties controlling pathogenesis because mutant flies die faster than wild-type flies even though the bacterial load is the same as in wild-type flies. A second class appears to lack defense functions. We define this group as immunocompromised because it shows increased fly death that is correlated with rapid growth of *L. monocytogenes*. The mutants can also be split into either sensitive or wild-type classes on the basis of their response to *Staphylococcus aureus* or *Salmonella typhimurium*. Secondary screens that used *L. monocytogenes* mutants allowed us to determine that three of our fly mutants may be acting at the vacuole and cytoplasmic stages of the bacteria life cycle. Thus, by screening for survival as an endpoint instead of monitoring the transcriptional output of Toll or imd signaling, we identified host genes involved in immunity and pathogenesis that have not been identified previously.

MATERIALS AND METHODS

Flies, bacterial strains, and media: The Exelixis *Drosophila melanogaster piggyBac* insertion mutant collection was used for the survival screen. To serve as a wild-type control the w1118 line used to generate the isogenic collection was used. All viable lines available at the Bloomington Drosophila Stock Center at the beginning of the screen were obtained (1231 lines in total). Flies were kept in standard fly bottles containing dextrose medium. *L. monocytogenes* strains 10403S, DP-L2161 (Δ*hly*), and DP-L3078 (Δ*actA*) were stored at −80° in brain–heart infusion (BHI) broth containing 15% glycerol. The *L. monocytogenes* mutant strains were derived from the 10403S parent strain and generated in the laboratory of Daniel A. Portnoy (University of California, Berkeley, CA). *S. typhimurium* SL1344 was stored at −80° in LB media containing 15% glycerol. *S. aureus* clinical isolate was from the Clinical Microbiological Laboratory at Stanford University and was stored at −80° in BHI media containing 15% glycerol.

Survival screen: For infection of adult flies, *L. monocytogenes* was grown overnight in BHI medium at 37° without shaking. A total of 10 adult flies, aged 5–7 days old, per mutant line were injected with 50 nl of culture using a picospritzer and pulled glass needle. Flies were injected in the anterior abdomen on the ventrolateral surface with ∼1000 colony-forming units (CFUs). Mutant lines were tested in groups of a minimum of 20 lines, in addition to a wild-type control. Once infected, flies

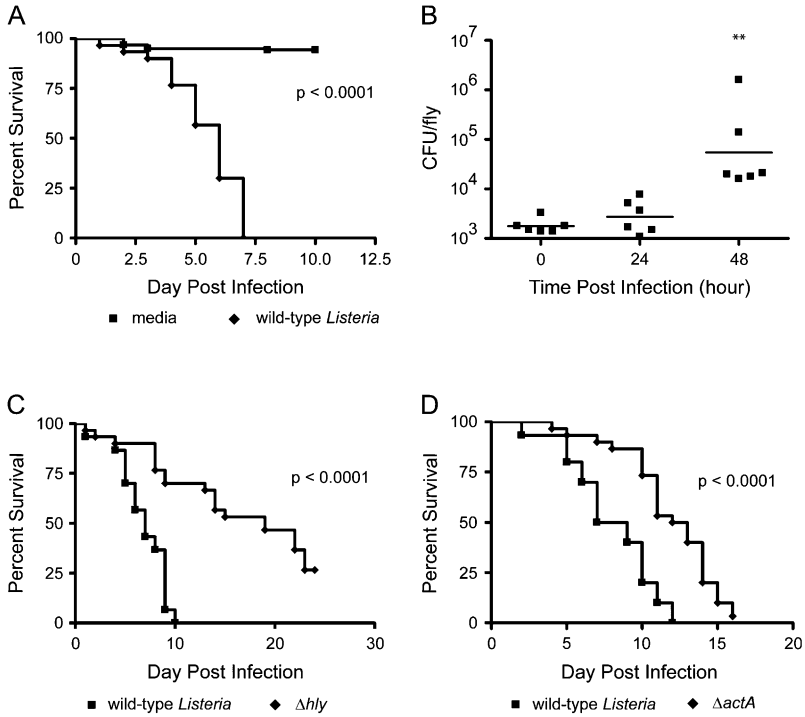

FIGURE 1.—*L. monocytogenes monocytogenes* infection in *Drosophila melanogaster*. Wild-type flies were injected with 10^3 CFUs of either wild-type or mutant *L. monocytogenes*. Survival and growth (growth of wild-type bacteria only) were monitored over the course of the infection. (A) *L. monocytogenes* strain 10403S (wild type) infection. Wild-type flies exhibit a median time to death (MTD) of ~5 days postinfection. (B) Growth of 10403S in flies. Bacteria reach levels $>10^4$ CFUs by 48 hr postinfection. $^{**}P < 0.005$ (*t*-test). (C) Δ*hly* infection in flies. Wild-type flies exhibit a MTD of ~20 days postinfection. (D) Δ*actA* infection in flies. Infected flies exhibit a MTD of ~10 days postinfection. Statistical analysis on survival curves was done using log-rank analysis and lines with $P < 0.05$ were considered statistically significant.

were transferred to vials containing dextrose medium and incubated at 29° and the number of dead flies for each line was counted every 24 hr postinfection until all flies were dead. The median time to death (MTD), the time postinfection when 50% of infected flies have succumbed to the infection, for each line was determined and those exhibiting the most extreme phenotypes were flagged for further testing. Mutant lines that failed to exhibit a phenotype in the initial test were not further tested. Candidate lines were retested in three independent experiments to eliminate any false positives. Thirty-five 7-day-old male flies per line were infected as before and the death rate of the mutant flies for each line was compared directly to the death rate of wild-type control flies, using Graphpad Prism software. Using log-rank analysis, the *P*-value was determined. Lines that exhibited a death rate with a *P*-value <0.05 for all three retests were considered positive mutants.

Secondary screens: *S. aureus and S. typhimurium secondary screens:* For infection of adult flies with *S. aureus*, bacteria were grown overnight in BHI medium at 37° with shaking. Approximately 100 CFUs were injected into each fly. For infection with *S. typhimurium*, bacteria were grown in LB medium at 37° and ~10,000 CFUs were injected into each fly. For all infections, 35 7-day-old male flies per mutant line were infected as mentioned above and the death rate for each line was directly compared to that of wild-type flies and analyzed as previously mentioned.

Quantification of CFUs in flies: Infected flies were homogenized in BHI media supplemented with 1% Triton X-100. Homogenates were serially diluted and plated on BHI agar and incubated overnight at 37°. The number of CFUs per mutant line was compared to that of wild-type flies using Graphpad Prism software for three independent experiments. Using an unpaired *t*-test, the *P*-value was determined. Mutant lines that exhibited a *P*-value <0.05 for all three retests were considered to have significantly different bacterial growth compared to the wild-type control.

***L. monocytogenes* mutants:** For infection of adult flies with both DP-L2161 and DP-L3078, bacteria were grown at 37° overnight without shaking. Thirty-five 7-day-old male flies per mutant line were infected for three independent replicates for each mutant fly line and flies were injected with ~1000 CFUs. The death rate for each line was directly compared to a wild-type control and analyzed using Graphpad Prism software as described above.

Verification of the PiggyBac insertion site: Inverse PCR was done to determine the insertion site for the PiggyBac (PBac) element following a protocol provided by Exelixis. Briefly, Genomic DNA from each mutant fly line was isolated using the QIAGEN (Valencia, CA) DNeasy kit. 5′- and 3′-end digestions were done using *Sau*3AI and *Hin*PI, respectively, and incubated at 37° for 3 hr. Ligations were done using T4 DNA ligase at 4° overnight. PCR reactions were performed using the primer sequences and reaction conditions provided by Exelixis (http://flystocks.bio.indiana.edu).

RESULTS AND DISCUSSION

Screening for host genes important for survival: To search for host genes that are important for surviving an infection, we conducted a forward genetic screen in a mutant population of Drosophila and monitored death rates following challenge with *L. monocytogenes*. *L. monocytogenes* establishes a lethal infection upon injection into Drosophila (Figure 1A) (MANSFIELD *et al.* 2003). Wild-type flies injected with 10^3 CFUs exhibited a MTD of 4–5 days postinfection. The predictable death kinetics allowed us to use survival as an output phenotype for our screen. We hypothesized that screening flies for their ability to survive *L. monocytogenes* infections would allow us to determine immune mechanisms used to fight this microbe, host mechanisms exploited by the pathogen, and physiologies that drive pathogenesis in the fly.

TABLE 1

Distribution of mutant fly lines

Mean time to death (day)	No. of PBac lines
1	85
2	145
3	249
4	510
5	168
6	58
7	16

After an initial test, the MTD for each mutant Drosophila line was determined. The majority of mutant lines exhibit a MTD of 4 days postinfection and the most extreme phenotypes observed were 1 and 7 days postinfection. Mutants exhibiting the most extreme phenotypes were flagged for further testing to eliminate false positives.

TABLE 2

Screening for genes affecting defense and endurance

Retest no.	No. of lines tested	No. of positive lines	No. of negative lines	% positive lines
Initial test	1231	101	—	8.2
1	101	35	66	34.6
2	35	25	10	71.4
3	25	18	7	72

Over 1200 PBac insertion mutant lines were tested for their ability to survive a *L. monocytogenes* infection and the MTD for each line was determined. The mutant lines that exhibited the most extreme phenotypes with MTDs of day 1 and day 7 (~100 mutants) were considered positive hits and tested further to eliminate any false positives. The candidate mutants were tested in three independent experiments, the death curves for each were compared to the wild-type control, and log-rank statistical analysis was done. Lines that exhibited a death rate with a *P*-value <0.05 for all three retests were considered positive mutants. After three rounds of retesting >80% of these mutants were considered false positives and a total of 18 mutants, all with increased sensitivity to infection, were identified as positive mutants that affect host susceptibility to *L. monocytogenes* infection.

For our initial screen we tested 1231 viable mutants from the publicly available collection of Exelixis PBac homozygous transposon insertion lines. We tested only homozygous viable lines and this number represents ~8% coverage of the Drosophila genome. Age-matched male flies from each mutant line were injected with 10^3 CFUs of *L. monocytogenes* and survival was monitored. Approximately half of the mutants tested exhibited a MTD of 4 days postinfection (Table 1). The mutant lines that exhibited the most extreme phenotypes with MTDs of day 1 and day 7 (~100 mutants) were tested further to eliminate any false positives. The candidate mutants were tested in three independent experiments and the death curves for each were compared to the wild-type control and statistical analysis was done. After three rounds of retesting >80% of these mutants were considered false positives and a total of 18 mutants, all with increased sensitivity to infection, were identified as positive mutants that affect host susceptibility to *L. monocytogenes* infection (Table 2). For our study, we define any fly that exhibits a reproducible, significantly different faster death rate compared to wild-type flies as sensitive and any fly with a slower death rate compared to wild type as resistant. We injected the positive mutants with media alone to confirm that the increased mortality was infection dependent (data not shown).

Identification of genes: A benefit of using the Exelixis PBac collection is that the insertion sites of the transposons for the mutants in the collection are publicly available. The Bloomington Drosophila Stock Center (http://flystocks.bio.indiana.edu) has verified the reported insertion sites. For additional verification, we identified the PBac-element insertion site in each of our positive mutants using iPCR and by BLAST sequence analysis of the flanking DNA sequences. Our gene identities agree with those reported by Exelixis (Table 3). We also outcrossed our 18 mutants to the parental strain and challenged the F_4 generation with *L. monocytogenes* to reduce the possibility of background effects on host survival. Using the computed gene (CG) numbers for the genes we determined the gene ontology (GO) terms from FlyBase (http://flybase.bio.indiana.edu), which are listed in Table 3.

Ubiquitination components: From our screen we identified three mutant lines whose candidate genes can be categorized as genes involved in the ubiquitination process. These are *crossbronx*, *CG15120*, and *CG2247*. This was not surprising as previous studies demonstrated the importance of ubiquitin-mediated protein degradation in the regulation of the Toll and Imd pathways. Activation of the Toll pathway requires the ubiquitination and degradation of cactus, the inhibitor of the NF-kb-like transcription factor DIF. Lys-63 polyubiquitination of TRAF6 is necessary for signaling through the Imd pathway (Zhou *et al.* 2005). Additionally, ubiquitination is important for controlling the cytotoxicity of LLO after release of *L. monocytogenes* from the vacuole during infection.

cbx is characterized as a ubiquitin-conjugating E2 enzyme. It has been studied in processes including axon pruning, sperm development, and sperm individualization. In the context of fly immunity, *cbx* has been identified from a genetic screen to possibly have a role in crystal cell development, a type of immune cell in the fly (Milchanowski *et al.* 2004). The identification of this gene may reveal a role for crystal cells in the host defense against bacterial infection.

CG15120 is a homolog of the Parkin coregulated gene protein, PACRG, which is involved in ubiquitination; moreover, polymorphisms in PACRG and a related gene, PARK2, are linked to increased susceptibility to

TABLE 3

Summary of positive Drosophila lines

PBac line	CG no.	Symbol	PBac orientation relative to gene	Gene ontology/molecular function
10036	*CG11489*[a]		Within fifth intron of *CG11489*	ATP binding, receptor signaling, protein serine/threonine kinase activity
10067	*CG10536*[a]	*cbx*	Within first intron of *cbx*	Ubiquitin-conjugating enzyme activity
10269	*CG13564*[a]		Within 5′-UTR of *CG13564*	
10496	*CG14899*[a]		Within 5′-UTR of *CG14899*	
10618	*CG11293*[a]		400 bp from *CG11293*	
10675	*CG15120/CG16926*		Within 3′ end of *CG15120* and *CG16926*	
10743	*CG13788*[a]	*gr28b*	Within first intron of *Gr28b*	Taste receptor activity
10881	*CG32706*[a]		Within 100 bp of 3′ end of *CG32706*	mRNA binding
17872	*CG4857*[a,b]		Within first intron of *CG4857*	
18050	*CG2247*[a]		Within first intron of *CG2247*	
18270	*CG3527*[b]		Within 5′-UTR of *CG3527*	rRNA processing, ribosomal small subunit processing
18398	*CG2152*[a,b]	*pcmt*	Within 200 bp of 5′ end of *pcmt*	Protein-L-isoaspartate (D-aspartate) *O*-methyltransferase activity
18609	*CG12487*[a]	*bobA*	Within coding region of *BobA*	Notch signaling pathway, cell fate specification
18678	*CG2914*[a,b]	*ets21C*	Within first intron of *Ets21C*	DNA binding, transcription factor activity
19181	*CG6128*[b]		Within coding region of *CG6128*	α-L-fucosidase activity, fucose metabolism
19207	*CG2397*[b]	*cyp6a13*	Within coding region of *Cyp6a13*	Electron transporter activity, oxidoreductase activity
19220	*CG3056*[b]		Within 3′ end of *CG3056*	mRNA binding
19305	*CG7408*[b]		Within 5′-UTR of *CG7408*	*N*-acetylgalactosamine-4-sulfatase activity

The insertion sites and closest gene of the PBac element for each mutant line are listed. The insertion sites were determined by Exelixis using iPCR and verified by FlyBase and are publicly available.

[a] We verified the insertion sites of lines by iPCR. The proposed molecular functions of the genes as provided by FlyBase are also listed.

[b] Genes tested in the RNAi screens by Cheng *et al.* (2005).

the intracellular pathogen *Mycobacterium leprae* in humans (Lorenzetti *et al.* 2004; Schurr *et al.* 2006). Polymorphisms in PACRG and PARK2 have been linked to the susceptibility of humans to the pathogens *S. typhi* and *S. paratyphi* (Ali *et al.* 2006). The third ubiquitin-related gene, *CG2247* represented in line 18050, contains an F-box domain that facilitates the interactions between proteins during polyubiquitination. Our identification of these genes may reveal new processes in host defense and pathogenesis in which ubiquitin-mediated protein degradation is involved.

RNA binding/processing components: The second main class of mutants we identified from our screen includes proteins involved in various processes involving RNA. *CG3527* is involved in rRNA processing, specifically processing of the small ribosomal subunit. *CG32706* and *CG3056* are reported to have mRNA-binding activity. Expression and mutation analyses have not been published for these genes.

Additional interesting genes recovered: Two additional genes we recovered that we find of great interest are *ets21C* and *gr28b*. *ets21C* is a DNA-binding protein with transcription factor activity. Previous studies have demonstrated that *ets21C* is immune regulated: Its expression is upregulated in Drosophila S2 cells in response to an LPS challenge and regulation is dependent on activation of JNK signaling via the Imd pathway (Boutros *et al.* 2002; Park *et al.* 2004). No immune function for this gene has been demonstrated.

gr28b is a seven-pass transmembrane gustatory receptor important for taste perception. Expression studies have shown this protein is expressed in the bitter taste cells of the sensilla in the fly taste organs. Identifying a gene involved in taste perception in the context of immunity and pathogenesis was somewhat surprising. Future experiments will reveal if the feeding behavior of the fly is affected, thus affecting the immune response in the fly. Alternatively, *gr28b* may be serving a function other than taste perception in the fly.

Phenotypic characterization of mutants—defense defects *vs.* endurance defects: To distinguish which of our mutants had defects in immunity *vs.* pathogenesis, we performed three secondary screens. First, we measured *L. monocytogenes* growth to determine whether flies had a defect in preventing growth of this microbe. Second, we measured survival when infected with *S. aureus* to determine whether the mutants had a general defect in preventing gram-positive bacterial growth

TABLE 4

Characterization of Drosophila mutants—sensitivity to *S. aureus* and *S. typhimurium* and growth of *L. monocytogenes*

PBac line	CG no.	Symbol	*S. aureus*	*S. typhimurium*	Bacterial load
10067	*CG10536*	*cbx*	+	0	+
10675	*CG15120/CG16926*		+	0	+
10881	*CG32706*		+	0	+
17872	*CG4857*		+	0	+[a]
18050	*CG2247*		+	0	+
19207	*CG2397*	*cyp6a13*	+	0	+[b]
19220	*CG3056*		+	0	+
10036	*CG11489*		+	0	+
18609	*CG12487*	*bobA*	+	0	+[b]
18270	*CG3527*		+	0	0
18398	*CG2152*	*pcmt*	+	0	0
19305	*CG7408*		+	0	0
10496	*CG14899*		0	0	+
19181	*CG6128*		0	0	+
18678	*CG2914*	*ets21C*	0	0	0
10269	*CG13564*		0	0	0
10618	*CG11293*		+	+	0
10743	*CG13788*	*gr28b*	+	−	+

Mutant lines were injected with 10^2 CFUs of *S. aureus* or 10^4 CFUs of *S. typhimurium* and the survival of the flies was monitored over the course of the infection. The growth of *L. monocytogenes* in mutant lines was determined as described in MATERIALS AND METHODS. + indicates increased growth or sensitivity compared to wild-type flies, − indicates resistance or decreased growth compared to wild-type flies. 0 indicates no change in sensitivity or growth compared to wild type. Statistical analysis on survival curves was done using log-rank analysis and lines with $P < 0.05$ were considered statistically significant. Statistical analysis on bacterial growth was done using a *t*-test and lines with $P < 0.05$ were considered statistically significant.

[a] Increased growth at 24 hr postinfection only.

[b] Increased growth at 48 hr postinfection only.

(Toll mutants are very sensitive to both these microbes). Third, we measured survival when infected with *S. typhimurium* to determine whether the mutants were sensitive to gram-negative bacteria (as might be expected for Imd pathway mutants) (Table 4).

To monitor *L. monocytogenes* growth, flies were infected, homogenized at 0, 24, and 48 hr postinfection, and plated to count viable bacteria. Twelve mutants had increased *L. monocytogenes* growth when compared to wild-type flies. We consider the nature of these mutations to be immunocompromising to the fly because they allow increased growth of *L. monocytogenes*. An alternative explanation for some of these mutants could be that the environment of the pathogen in the mutant fly is more favorable to *L. monocytogenes* growth and the mutation affects physiology outside of what would normally be considered the immune system. The remaining mutants exhibited no significant difference in *L. monocytogenes* levels compared with wild type. This is a new class of mutant phenotype in the sense that it is not commonly studied in the field of fly immunity. Since microbial proliferation is unchanged in this group, these mutants should not be regarded as immunocompromised; instead, we suggest that these flies have an altered physiology that makes them more sensitive to the pathology induced by the *L. monocytogenes* infection. These flies are less able to endure the infection than wild-type flies even though bacterial levels are similar.

Often, studies examining the Drosophila immune response to gram-positive bacteria involve challenges with nonpathogens and have used specific molecular readouts such as AMP production to assess immune activity (MICHEL *et al.* 2001; DEGREGORIO *et al.* 2002; RUTSCHMANN *et al.* 2002; TAUSZIG-DELAMASURE *et al.* 2002). Defects in signaling resulted in an inability to mount an immune response to all tested gram-positive microbes. On the basis of our examination of our mutants, it is clear that the Drosophila response to gram-positive bacteria is more complex than has been previously appreciated. Not all of our mutants are sensitive to a second gram-positive pathogen, *S. aureus*. The presence of the two mutant lines that are clearly immunocompromised with respect to *L. monocytogenes* but show no change in *S. aureus* susceptibility indicates that there are multiple mechanisms and pathways used to fight gram-positive pathogenic bacteria.

To determine whether mutations causing increased sensitivity to *L. monocytogenes* infection could also affect infections with gram-negative bacteria, we challenged flies with *S. typhimurium*. Only one gene, *CG11293*, shows increased sensitivity to *S. typhimurium*. This mutant exhibits increased sensitivity to *L. monocytogenes* and *S. aureus*

TABLE 5

Characterization of *L. monocytogenes* mutants in Drosophila lines

PBac line	CG no.	Symbol	Δ*hly*	Δ*actA*
18270	*CG3527*		−	−
10067	*CG10536*	*cbx*	−	−
10269	*CG13564*		−	−
10496	*CG14899*		−	−
10618	*CG11293*		−	−
10675	*CG15120/CG16926*		−	−
18050	*CG2247*		−	−
19181	*CG6128*		−	−
19207	*CG2397*	*cyp6a13*	−	−
19220	*CG3056*		−	−
19305	*CG7408*		−	−
18678	*CG2914*	*ets21C*	−	−
18609	*CG12487*	*bobA*	−	−
10036	*CG11489*		−	0
17872	*CG4857*		−	0
10881	*CG32706*		0	−
18398	*CG2152*	*pcmt*	−	−
10743	*CG13788*	*gr28b*	0	0

Fly lines were infected with Δ*hly* or Δ*actA* mutant lines as described in MATERIALS AND METHODS. − indicates resistance compared to 10403S infection. 0 indicates no change in sensitivity compared to 10403S infection. Statistical analysis on survival curves was done using log-rank analysis and lines with $P < 0.05$ were considered statistically significant.

infection but unaltered *L. monocytogenes* growth. We suggest that these flies have a general pathogenesis defect because they are sensitive to three different pathogenic bacteria even though the bacterial load is comparable to that of wild-type flies. *gr28b* exhibits increased resistance to *S. typhimurium* infection while being more sensitive to *S. aureus*. This increased resistance was an unexpected phenotype; we are not aware of any other fly mutations that cause resistance to gram-negative bacteria but increased sensitivity to gram-positive bacteria.

Characterization of *L. monocytogenes* mutants in Drosophila mutants: We chose *L. monocytogenes* as the pathogen for our screen because the life cycle and virulence factors have been well characterized, and, by using bacterial mutants, we can test for changes in pathogenesis in our mutant flies. After being taken up by a host cell, *L. monocytogenes* secretes LLO to lyse the phagosome membrane and release the bacteria into the cytoplasm. The protein ActA is important for nucleating host actin at one pole of the bacterium. Actin polymerization drives motility of *L. monocytogenes* in the cytoplasm of an infected cell and enables the bacteria to move from one host cell to another without entering the extracellular space, avoiding immune defenses of the host. We found that Δ*hly* (LLO deletion) and Δ*actA* mutants are strongly attenuated in wild-type Drosophila, with a MTD of 18–20 days and 11–12 days, respectively (Figure 1, C and D). We examined susceptibility of our fly mutants to infection with the *L. monocytogenes* mutants Δ*hly* and Δ*actA* to determine if any of our Drosophila mutants are involved in specific steps of the bacterial life cycle. We hypothesized that if any of our fly mutations are involved in specific stages of the life cycle, then the fly susceptibility to mutant bacterial infections will be different compared to that to a wild-type bacterial infection.

In 14 mutant fly lines, we found that the mutant *L. monocytogenes* strains acted as they do in wild-type flies, killing flies more slowly than do wild-type *L. monocytogenes* (Table 5) (individual death curves are provided in the supplemental materials). For 4 of our fly mutants (*CG11489*, *CG4857*, *CG32706*, and *gr28b*) the flies were equally sensitive to at least one *L. monocytogenes* mutant and wild-type *L. monocytogenes*. Two mutants, *CG11489* and *CG4857*, are just as sensitive to infection with Δ*actA* as they are to infection with wild-type bacteria while being much less sensitive to Δ*hly* mutants. In other words, even though the Δ*actA L. monocytogenes* are defective in cell-to-cell spread, these mutant fly lines are just as sensitive as if they had been infected with wild-type bacteria. However, these two mutant fly lines respond like wild-type flies to an infection with *L. monocytogenes* lacking LLO—they are more resistant. On the basis of their RNAi screen results, CHENG *et al.* (2005) suggested that mutants like this might be more sensitive to LLO and that the knocked-down genes were involved in controlling LLO toxicity. This model could also apply to our mutants; however, we find that our mutants are also sensitive to *S. aureus* and presumably sensitivity to *S. aureus* is not caused by the same perturbed process that might result in sensitivity to LLO. Perhaps this mutant affects a more general process involved in sensing bacteria.

CG32706 is sensitive to Δ*hly* mutants but is comparatively insensitive to Δ*actA* infection. That is, even though the Δ*hly* strain is defective in vacuole escape, it kills this mutant fly line with similar kinetics as wild-type *L. monocytogenes*. Yet this fly line is resistant to *L. monocytogenes* that lack ActA and are defective in cell-to-cell spread. Perhaps in these flies LLO is not required for *L. monocytogenes* to be released into the cytosol. Similar phenotypes have been observed in *in vitro* screens in which genes involved in later stages of vesicular trafficking had been knocked down (CHENG *et al.* 2005).

gr28b is sensitive to Δ*hly* and Δ*actA L. monocytogenes*. As shown above, *gr28b* is sensitive to *L. monocytogenes* and *S. aureus*, but has increased resistance to *S. typhimurium*. Because the mutant strains of *L. monocytogenes* are not attenuated in this fly line, we suggest that LLO and ActA are not required for the bacteria to elicit pathogenic effects on this fly line. In other words, *L. monocytogenes* lacking factors required for normal intracellular pathogenesis are not attenuated, indicating that *L. monocytogenes* in the *gr28b* mutant may be an extracellular population.

Members of immunity pathways were not identified: Two known immunity genes were present in the collection of mutant flies we tested (imd and *kenny*).

Because previous studies of fly immunity have primarily focused on the Toll and Imd pathways, we anticipated that these two genes would be identified from our screen. These mutants had median times to death of 2 days, but were not tested further because they were outside of our cutoff of a median time to death of 1 day. As we screened a relatively small number of mutants, it is possible that we did not hit any members of the Toll signaling pathway because of our small sample size. In addition, some Toll pathway mutants would not meet our viability requirement and would be excluded. Regardless, this screen demonstrates that fighting an infection and the interactions that occur between host and pathogen are complex, and many processes in addition to the pathways regulating AMP transcription are involved.

The importance of performing both *in vivo* and *in vitro* screens: We compared our list of mutant genes with the hundreds of genes identified in RNAi screens monitoring *L. monocytogenes* growth in cultured Drosophila cells. Because many mutations in essential functions would have a lethal phenotype in the whole fly, we anticipated that we would not find some genes in the whole fly that were identified *in vitro*. Conversely, we also expected to find genes that were important in the whole animal but not in tissue culture. Nonetheless, we expected significant overlap between the sets of genes isolated in these two screens; instead, we found no overlap. The screen by Agaisse *et al.* (2005) was a genomewide screen and theoretically all of our mutants should have been tested in that screen. The dsRNA library used by Cheng *et al.* (2005) contained eight of our positive mutants, none of which were identified from their *in vitro* screen (see Table 3). Both of these *in vitro* screens selected for bacterial phenotypes, including growth and vacuole escape, and utilized one cell type. The majority of genes identified from these *in vitro* screens are, not surprisingly, involved in vesicular trafficking and phagocytosis. By screening for host survival in a whole-animal model, we were able to identify different host genes involved in a variety of processes during infection, including the immune response and pathogenesis.

Conclusions: In this study we demonstrate the power of combining two genetically tractable organisms, one host and one pathogen, to reveal a previously uncharacterized group of genes involved in immunity and pathogenesis. Previous genetic studies using each organism separately greatly limited the scope of genes and processes that can be identified. The use of Drosophila to study the innate immune response to infection facilitated our understanding of signaling events that lead to the production of AMPs. Previous screens in the fly and in S2 cells have focused on identifying genes involved in these signaling pathways, but they did not look beyond this process. *In vitro* studies using *L. monocytogenes* have expanded our knowledge to better understand pathogenic mechanisms used by intracellular pathogens. Yet these genetic studies have focused more on the bacteria than on the host genes involved in the infection. Attempts to overcome these limitations have been made with RNAi screens in S2 cells infected with *L. monocytogenes*. These screens have revealed important host factors required for internalization and growth of *L. monocytogenes* in host cells but were not capable of identifying immunity- or pathogenesis-related genes. By screening for host survival in a whole-fly model, we could probe the host contributions to infection that extend to processes beyond those found by other screens and uncovered genes that were not discovered by the other methods.

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