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Genetic Variation in *Drosophila melanogaster* Resistance to Infection: A Comparison Across Bacteria

Brian P. Lazzaro,*,[1] Timothy B. Sackton† and Andrew G. Clark‡

**Department of Entomology, †Field of Ecology and Evolutionary Biology and ‡Department of Molecular Biology and Genetics, Cornell University, Ithaca, New York 14853*

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ABSTRACT

Insects use a generalized immune response to combat bacterial infection. We have previously noted that natural populations of *D. melanogaster* harbor substantial genetic variation for antibacterial immunocompetence and that much of this variation can be mapped to genes that are known to play direct roles in immunity. It was not known, however, whether the phenotypic effects of variation in these genes are general across the range of potentially infectious bacteria. To address this question, we have reinfected the same set of *D. melanogaster* lines with *Serratia marcescens*, the bacterium used in the previous study, and with three additional bacteria that were isolated from the hemolymph of wild-caught *D. melanogaster.* Two of the new bacteria, *Enterococcus faecalis* and *Lactococcus lactis*, are gram positive. The third, *Providencia burhodogranaria*, is gram negative like *S. marcescens*. Drosophila genotypes vary highly significantly in bacterial load sustained after infection with each of the four bacteria, but mean loads are largely uncorrelated across bacteria. We have tested statistical associations between immunity phenotypes and nucleotide polymorphism in 21 candidate immunity genes. We find that molecular variation in some genes, such as *Tehao*, contributes to phenotypic variation in the suppression of only a subset of the pathogens. Variation in *SR-CII* and *18-wheeler*, however, has effects that are more general. Although markers in *SR-CII* and *18-wheeler* explain >20% of the phenotypic variation in resistance to *L. lactis* and *E. faecalis*, respectively, most of the molecular polymorphisms tested explain <10% of the total variance in bacterial load sustained after infection.

THE stereotypical insect defense against microbial pathogens (reviewed in Leclerc and Reichhart 2004) includes defensive phagocytosis (cellular immunity) and the production of extracellular antibiotic peptides (humoral immunity). Insect immune responses are distinct from those of vertebrates in that insect immune systems lack the adaptive memory of previous infections and high degree of specificity that characterize vertebrate immune systems. Instead, insect antibacterial defenses are generalized and mechanistically simple, with only a small set of genes used to fight against an extremely broad range of bacteria. Despite substantial and increasing understanding of gene function underlying Drosophila antibacterial defense, little is known about the extent and consequences of genetic polymorphism for immune function in natural insect populations. There is evidence that increased immunocompetence in insects can be detrimental to other components of fitness (*e.g.*, Kraaijeveld and Godfray 1997; McKean and Nunney 2001; Kumar *et al.* 2003), potentially allowing selective maintenance of genetic variation in immune function. Additionally, because a comparatively small set of genes is used to combat a broad range of pathogens, it is in principle possible that mutation could increase the quality of response to some bacteria at the expense of the response to others, providing another potential mechanism for the adaptive maintenance of polymorphism. In this study, we evaluate resistance to four different bacteria across a panel of *Drosophila melanogaster* genetic lines to test the degree of concordance in resistance to distinct bacteria and to identify genes harboring natural polymorphism that contributes to phenotypic variation in resistance to infection.

In Drosophila, the humoral immune response to bacteria is initiated when pathogen recognition proteins, such as peptidoglycan recognition proteins (PGRPs) and gram-negative binding proteins (GNBPs), react with conserved components of prokaryotic cell walls. Different PGRP isoforms of the same gene can have different recognition spectra and PGRPs and GNBPs have been shown to interact epistatically (Gobert *et al.* 2003; Werner *et al.* 2003; Pili-Floury *et al.* 2004; Takehana *et al.* 2004; Choe *et al.* 2005; Filipe *et al.* 2005), greatly expanding the breadth of recognition that can be attained through a small number of genes. Once a pathogen has been

[1]*Corresponding author:* Department of Entomology, Cornell University, 4138 Comstock Hall, Ithaca, NY 14853. E-mail: bl89@cornell.edu

recognized, signal is transduced through two primary pathways, named "Toll" and "Imd" after prominent constituent proteins, culminating in a robust transcriptional response, which includes activation of genes encoding secreted antimicrobial peptides. There is some degree of pathogen specificity in the induction of the Drosophila antimicrobial immune response (*e.g.*, Lemaitre *et al.* 1997; Hedengren-Olcott *et al.* 2004), with the Toll pathway primarily responsive to gram-positive bacteria and the Imd pathway primarily responsive to gram negatives. This specificity is not absolute, however, and there is probably concurrent activation of and crosstalk between the two pathways (*e.g.*, Lemaitre *et al.* 1997; Engström 1999; Hedengren-Olcott *et al.* 2004; Stenbak *et al.* 2004). Simultaneous mutational inactivation of both pathways effectively abolishes the Drosophila immune response, making flies susceptible to otherwise innocuous bacteria (*e.g.*, Lemaitre *et al.* 1996) and demonstrating that these two pathways are the primary determinants of Drosophila immunocompetence.

Previous studies have documented naturally occurring molecular variation in Drosophila genes encoding pathogen recognition proteins (Jiggins and Hurst 2003; Schlenke and Begun 2003, 2005; Lazzaro 2005), proteins in the Toll and Imd signaling pathways (Begun and Whitley 2000; Schlenke and Begun 2003), and antibacterial peptides (Clark and Wang 1997; Date *et al.* 1998; Ramos-Onsins and Aguadé 1998; Lazzaro and Clark 2001, 2003). The functional significance of this variation is largely unknown. We previously examined the effects of polymorphism in 21 candidate genes on phenotypic variation in the ability of *D. melanogaster* to suppress infection by a gram-negative entomopathogen, *Serratia marcescens* (Lazzaro *et al.* 2004). In that work, we found that polymorphism in signal transduction and pathogen recognition genes was significantly associated with variability in immunocompetence, but that polymorphism in antibacterial peptide genes did not have a major impact on resistance to infection. In all genes, polymorphisms that were significantly associated with resistance to *S. marcescens* made only small contributions to the overall phenotypic variance in immunocompetence. Most associations explained <15% of the total phenotypic variance (Lazzaro *et al.* 2004). In this study, we reevaluate the same panel of *D. melanogaster* lines for their ability to suppress infection by *S. marcescens* and additionally measure their resistance to three bacteria that were originally isolated from the hemolymph of wild-caught *D. melanogaster* (*Lactococcus lactis*, *Enterococcus faecalis*, and *Providencia burhodogranaria*). With these data, we test the degree to which the lines show correlated abilities to suppress infection by the various bacteria and whether the functional effects of molecular variants in our 21 candidate genes are generalized across pathogens or specific to the microbe used in challenge.

TABLE 1

Number of polymorphic markers typed in each candidate locus

Functional classification, gene name(s)	Cytological position	Locus size (kb)	Markers typed
Pathogen recognition			
PGRP-SC1A, -SCIB, -SC2	44E	10.0	10
SR-CI, SR-CIII	24D	4.3	13
SR-CII	48F	4.4	7
SR-CIV	23F	4.6	7
Signal transduction			
Toll-4	30A	6.8	8
Tehao	34C	7.8	16
18-Wheeler	56F	7.6	6
DIF	36C	20.7	8
cactus	35F	14.8	7
ik2	38D	5.1	5
immune deficiency	55C	4.0	7
Antibacterial peptides			
Attacin AB	51C	4.9	8
Attacin C	50A	3.1	7
Defensin	46D	1.4	5
Diptericin AB	55F	4.7	7
Metchnikowin	52A	1.8	6

MATERIALS AND METHODS

Drosophila and bacterial stocks: Ninety-five lines of *D. melanogaster* were evaluated for resistance to infection by four species of bacteria. These lines are derived from wild flies captured in State College, Pennsylvania, in 1998 and 1999. Each line in the panel is homozygous for an independent second chromosome isolated from the natural population and substituted into a common genetic background. These *D. melanogaster* lines have previously been used to measure natural genetic variation in resistance to *S. marcescens* infection (Lazzaro *et al.* 2004), and their generation is described in detail in the supplemental information to Lazzaro *et al.* (2004). The lines have been genotyped for 127 insertion/deletion and restriction site polymorphisms distributed among 21 genes known or thought to be involved in *D. melanogaster* immunity. Markers were genotyped in coding sequence, introns, and flanking regions of genes encoding peptidoglycan recognition proteins (*PGRP-SC1A*, *-SC1B*, and *-SC2*), class C scavenger receptors (*SR-C I, II, III,* and *IV*), *Toll*-like receptors (*18-wheeler, Tehao,* and *Toll-4*), intracellular signaling proteins (*cactus, DIF, ik2,* and *imd*), and antimicrobial peptides (*Attacins A, B,* and *C*; *Diptericins A* and *B*; *Defensin*; and *Metchnikowin*). A summary of the distribution of markers among candidate genes is found in Table 1. Line genotypes at each marker are presented in supplemental Figure 1 at http://www.genetics.org/supplemental/, and linkage disequilibrium relationships among genotyped markers are presented in supplemental Figure 2 at http://www.genetics.org/supplemental/. Ancestral states of genotyped *D. melanogaster* polymorphisms were determined by comparison to the genome sequences of *D. simulans, D. yakuba,* and *D. erecta* (http://rana.lbl.gov/drosophila/), assuming mutational parsimony. Genotyped markers are identified by their unique nucleotide positions in Release 3.1 of the complete *D. melanogaster* genome assembly.

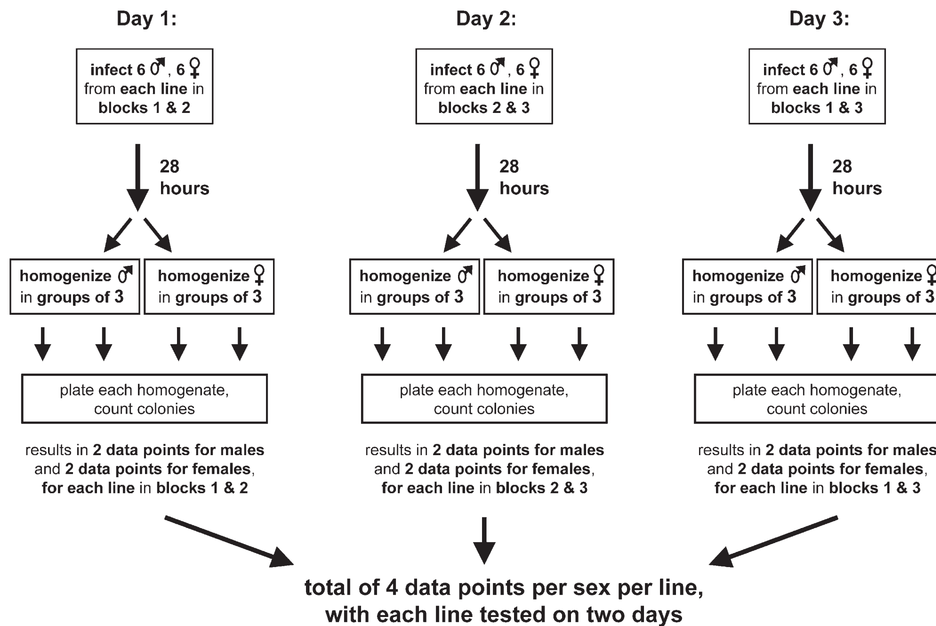

Figure 1.—The design of the phenotypic tests of immune competence. Infections were done in a split-block design, such that all *D. melanogaster* lines were infected on 2 separate days and as many as eight data points were collected for each line. In the diagrammed structure, block 1 contains lines 1–32, block 2 contains lines 33–64, and block 3 contains lines 65–95. The entire structure was conducted independently for each of the four bacteria used in challenge, with lines randomly assigned to blocks separately for each pathogen.

Four bacteria were used to challenge the *D. melanogaster* lines in this study. One of these is the strain of gram-negative bacterium, *S. marcescens*, that we employed in the previous study (Lazzaro *et al.* 2004). This strain is derived from ATCC strain 13880, which was incorrectly identified as ATCC 13315 in our previous publication. The other three bacterial strains were cultured from the hemolymph and thoracic muscle of *D. melanogaster* captured from the same population that gave rise to the test lines in this study (Lazzaro 2002). Two of the strains employed here are gram positive. These have been identified as *E. faecalis* and *L. lactis* on the basis of their sequences at the 16S rDNA locus and the results of API 20Strep (Enterococcus) and API 50CH (Lactococcus) substrate utilization tests (BioMérieux, Marcy-l'Etoile, France). The third strain is gram negative. DNA sequence and metabolic analyses led to the identification of this isolate as a previously undescribed species of Providencia, which was named *P. burhodogranaria* strain B (B. P. Lazzaro and P. Juneja, unpublished results). These three bacteria were chosen for inclusion in this study because they establish sustained infections in *D. melanogaster* that result in high systemic bacterial loads but low fly mortality within the 28-hr experimental period. It should be noted, however, that infection with higher doses of *E. faecalis* than those delivered in this study may elicit marked Drosophila mortality (Lazzaro 2002). We have also obtained other, more virulent, Providencia isolates from wild-caught *D. melanogaster* that induce greater Drosophila mortality than does the strain examined here (B. P. Lazzaro and P. Juneja, unpublished results). All four bacteria in this study are referred to as "pathogens" in this report, although none of them are obligate pathogens of *D. melanogaster* and all are probably opportunistic infectors.

Experimental design: The basic structure of the experiment is diagrammed schematically in Figure 1. The 95 *D. melanogaster* lines were infected with each bacterium in 3-day split block design, with approximately two-thirds of the lines infected on any given day, and each line infected on 2 distinct days. This block structure was repeated independently for each of the four bacteria used in challenge, varying the lines assigned to each replication block between bacteria. Briefly, flies were infected with septic pinprick, and the number of viable bacteria recovered 28 hr after infection was used as a measure of infection severity. Typically, 6–8 replicate data points (representing 18–24 individual flies) were obtained from each *D. melanogaster* genotype after each of the four bacterial challenges, resulting in 2469 data points obtained for the entire experiment.

Bacterial infections were delivered as previously described (Lazzaro *et al.* 2004). The thoraces of individual *D. melanogaster* aged 3–5 days posteclosion were pierced with a 0.1-mm dissecting pin (Fine Science Tools, Foster City, CA) coated in liquid culture ($OD_{600} = 1.0 \pm 0.2$) of the bacterium of interest. This procedure delivers an average of 4×10^3 bacteria to each fly (not shown). All flies were infected between 2 and 5 hr after "dawn" from the flies' perspective. Drosophila were maintained at 22°–24° on a rich dextrose medium for the duration of the experiment. Same-sex trios of flies from each line were homogenized 28 ($\pm$0.5) hr postinfection in 500 μl of sterile LB and then quantitatively plated on standard LB agar plates using an Autoplate 4000 spiral plater (Spiral Biotech, Bethesda, MD). The plates were incubated overnight at 37°, and the concentration of viable bacteria in each homogenate was estimated using the Q-count colony counting system associated with the Autoplate 4000. Because of anticipated high bacterial loads, homogenates of flies infected with *L. lactis* were diluted 100-fold in sterile LB prior to plating. Where possible, two homogenates were obtained from each sex for each genotype on each day. Flies sham infected with sterile needles always failed to yield bacteria within the assay period, and plates were visually checked to make sure that resultant colonies exhibited colony morphology and color consistent with that of the experimental bacteria.

Statistical analysis: Bacterial densities estimated from the Drosophila homogenates ranged from 4.9×10^1 to 3.75×10^5 colony-forming units (CFU) per milliliter, which is equivalent to between 0.8×10^0 and 6.3×10^4 bacteria per fly. Homogenates with densities $>4.0 \times 10^5$ CFU/ml could not be resolved on the counting system and were arbitrarily declared to take a value of 4.5×10^5, undoubtedly an underestimate in many cases. There were 369 such plates, of 2469 plates in the entire experiment. Exclusion of these plates did not substantially change our results (not shown) so we opted to retain them in the analysis. Most of the statistical tests employed here are analyses of variance, which assume that data are normally

TABLE 2

Factors tested in linear models used to evaluate systemic bacterial load (see MATERIALS AND METHODS)

Factor name	Effect type	Effect measured
Line_i ($i = 1, 95$)	Fixed	Overall genetic contribution to phenotype
Sex_j ($j = 1, 2$)	Fixed	Sex of the infected flies
Day_k ($k = 1, 3$)	Random	Day-to-day variability in delivery of infection
Marker_l ($l = 1, 2$)	Fixed	Allelic state at a focal marker
Pathogen_p ($p = 1, 4$)	Fixed	Bacterium used in challenge
$(\text{Line} \times \text{Sex})_{ij}$	Random	Variation among sexes in the effect of background genetic variation
$(\text{Sex} \times \text{Day})_{jk}$	Random	Variation among sexes in day-to-day variability in resistance to infection
$(\text{Sex} \times \text{Marker})_{jl}$	Fixed	Variation among sexes in the effect of a focal marker
$(\text{Sex} \times \text{Pathogen})_{jp}$	Fixed	Variation among sexes in resistance to infection by one or more bacteria
$(\text{Marker} \times \text{Pathogen})_{lp}$	Fixed	Variation among pathogens in the effect of a focal marker
$(\text{Sex} \times \text{Marker} \times \text{Pathogen})_{jlp}$	Fixed	Variation among sexes in pathogen-specific effects of a focal marker
$(\text{Marker} \times \text{Marker})_{lm}$	Fixed	Nonadditive (epistatic) interactions between two markers
$(\text{Sex} \times \text{Marker} \times \text{Marker})_{jlm}$	Fixed	Variation among sexes in nonadditive (epistatic) interactions between two markers
Line_i (Marker_l)	Random	Background genetic variation segregating within genotypes of a focal marker
Line_i ($\text{Marker}_l \times \text{Marker}_m$)	Random	Background genetic variation segregating within a two-locus genotype
Day_k (Pathogen_p)	Random	Day-to-day variability in infection with a given pathogen

distributed, but our data are nonnormal due partially to truncation on the high end of the phenotypic distribution. Log_e-transformation of the raw data provided a fit to normality that was adequate for analysis of variance (NETER *et al.* 1990). Critical values for test statistics were determined by permutation analysis (CHURCHILL and DOERGE 1994) instead of comparison to a parametric distribution, further insulating our conclusions from the effects of nonnormality.

Statistical analyses were conducted using SAS Stat v. 9.1 (SAS Institute, Cary, NC). The factors in all linear models and the components of variation that they describe are listed in Table 2. Unless otherwise indicated, all models were run independently on the data from each of the four bacterial challenges.

Least-squares mean bacterial loads were calculated for each *D. melanogaster* genetic line using PROC MIXED, employing the model

$$Y_{ijkn} = \mu + \text{Line}_i + \text{Sex}_j + \text{Day}_k + (\text{Line} \times \text{Sex})_{ij} + (\text{Sex} \times \text{Day})_{jk} + \epsilon_{ijk}, \quad (1)$$

where Line_i ($i = 1, 95$) and Sex_j ($j = 1, 2$) are considered fixed effects and terms incorporating Day_k ($k = 1, 3$) are considered random effects. Statistical associations between phenotypic value and allelic state at each of the 127 markers were evaluated with a likelihood-ratio test that compared a model that included Marker as a fixed effect to a null model that did not. The mixed models were evaluated independently for each pathogen at each marker using PROC MIXED, method ML, in SAS Stat. The null model takes the form

$$Y_{ijkln} = \mu + \text{Sex}_j + \text{Day}_k + \text{Line}_i(\text{Marker}_l) + (\text{Line} \times \text{Sex})_{ij} + (\text{Sex} \times \text{Day})_{jk} + \epsilon_{ijkl}, \quad (2)$$

where all of the factors except for Sex are random effects, and Marker has just two levels for the two alternative homozygous classes. Because each marker genotype is represented in more than one line, the term $\text{Line}_i(\text{Marker}_l)$ refers to the ith line nested in the lth marker and is used to estimate background genetic effects. The alternative model added fixed main effects of Marker and a Sex × Marker interaction, taking the form

$$Y_{ijkln} = \mu + \text{Sex}_j + \text{Day}_k + \text{Marker}_l + (\text{Sex} \times \text{Marker})_{jl} + \text{Line}_i(\text{Marker}_l) + (\text{Line} \times \text{Sex})_{ij} + (\text{Sex} \times \text{Day})_{jk} + \epsilon_{ijkl}. \quad (3)$$

The strength of association between marker and phenotype was measured as twice the difference between the negative log likelihoods of the test and null models. Critical values were obtained from an empirical null distribution for each marker, generated from 1000 permutations of genotype and phenotype (CHURCHILL and DOERGE 1994). With both true and permuted data sets, genotype–phenotype associations were tested using the full log_e-transformed raw data (as opposed to using mean values for each *D. melanogaster* line). Nominal comparisonwise *P*-values were not corrected for multiple tests across sites or pathogens because it is not clear what experimentwise statistical correction would be appropriate. The nonindependence of sites within loci (due to linkage disequilibrium), the nonindependence of testing the same genotypes in response to different pathogens, the variability in power among sites due to differences in site frequency, and the variation in power across pathogens due to heterogeneity in the phenotypic distributions all serve to make any experimentwise *P*-value correction depend on a set of complex and untenable assumptions. We used the method of STOREY (2002) and STOREY and TIBSHIRANI (2003) to calculate false discovery rates on genotype–phenotype associations when significance is declared at the nominal 5 and 1% levels. After infection with *S. marcescens*, we estimate that 57.2% of the associations detected with $P < 0.05$, and 34.3% of the associations detected with $P < 0.01$, are false positives. The phenotypic resolution was poor after infection with *P. burhodogranaria*, resulting in no associations detected with nominal $P < 0.01$ (see RESULTS) and an estimated false discovery rate of 92.5% on associations declared significant with nominal $P < 0.05$. The false discovery rates after infection with *E. faecalis* are 59.6% ($P < 0.05$) and 57.2% ($P < 0.01$) and after infection with *L. lactis* are 72.4% ($P < 0.05$) and 16.9% ($P < 0.01$). We cannot know which of the detected associations are false positives and which are real, so individual site associations should be interpreted with caution. We can place qualitatively

greater confidence in associations that are repeatedly detected across experiments or challenges with different bacteria, however, so repeatability is used as an informal measure of validation.

Variance components were estimated in SAS Stat using the restricted maximum-likelihood method implemented in PROC VARCOMP. The proportion of the phenotypic variance explained by the *D. melanogaster* genetic line was estimated as the variance attributable to Line in the model

$$Y_{ijkn} = \mu + \text{Line}_i + \text{Sex}_j + \text{Day}_k + \epsilon_{ijk}, \tag{4}$$

where all effects are random, divided by the total phenotypic variance observed. Phenotypic variance attributable to specific polymorphic markers was estimated in an analogous way, after determining the variance attributable to each Marker in the model

$$Y_{ijkln} = \mu + \text{Sex}_j + \text{Day}_k + \text{Marker}_l + \text{Line}_i(\text{Marker}_l) + \epsilon_{ijkl}, \tag{5}$$

where Day and Line(Marker) are random effects. It is important to note that the proportions of the variance attributable to each marker are not expected to sum to the total genetic variance because of nonindependence among sites (linkage disequilibrium) and epistatic interactions among loci. The allelic effects of each marker were defined as the difference in the least-squares mean bacterial loads estimated for each allele. Marker effects were calculated by subtracting the least-squares mean of the allele with the derived marker state from the least-squares mean of the allele with the ancestral marker state. (Note that the ancestral state is determined only for genotyped markers; no inference is made regarding the ancestral state of the phenotypically causal mutations, which are assumed not to be the genotyped markers.) Least-squares means for each allele and the standard error of the estimated difference between alleles were recovered using PROC MIXED to evaluate the model described by Equation 3.

The pathogen specificity of marker contributions to variance was measured as a marker × pathogen interaction. This is the only analysis where data from different bacterial challenges were pooled. To first correct for gross differences in bacterial load achieved by different pathogens, residuals were obtained from the model

$$Y_{kpn} = \mu + \text{Pathogen}_p + \text{Day}_k(\text{Pathogen}_p) + \epsilon_{kp}, \tag{6}$$

where Pathogen_p ($p = 1, 4$) is considered a fixed effect but Day is random. To estimate the significance of any pathogen × marker interaction, the residuals from model (6) above were used as the response variable in the model

$$\begin{aligned} Y_{ijklpn} = {} & \mu + \text{Sex}_j + \text{Day}_k + \text{Marker}_l + \text{Pathogen}_p \\ & + \text{Line}_i(\text{Marker}_l) + (\text{Line} \times \text{Sex})_{ij} + (\text{Sex} \times \text{Day})_{jk} \\ & + (\text{Sex} \times \text{Marker})_{jl} + (\text{Sex} \times \text{Pathogen})_{jp} \\ & + (\text{Marker} \times \text{Pathogen})_{lp} \\ & + (\text{Sex} \times \text{Marker} \times \text{Pathogen})_{jlp} + \epsilon_{ijklp}, \end{aligned} \tag{7}$$

where Pathogen, Marker, and Sex are fixed effects and Day and Line are random effects. The *F*-ratio of the Marker × Pathogen term was retained for each marker and compared to an empirical null distribution generated by running the above two-step analysis on 1000 data sets, where the identity of the pathogen used in infection was randomly permuted within the residuals at the second step.

All possible site pairs were tested for nonadditive interactivity in a general search for epistasis. The significance of the interaction between all marker pairs was evaluated in the mixed model,

$$\begin{aligned} Y_{ijklmn} = {} & \mu + \text{Sex}_j + \text{Day}_k + \text{Marker}_l + \text{Marker}_m \\ & + \text{Line}_i(\text{Marker}_l \times \text{Marker}_m) + (\text{Line} \times \text{Sex})_{ij} \\ & + (\text{Sex} \times \text{Day})_{jk} + (\text{Sex} \times \text{Marker})_{jl} \\ & + (\text{Sex} \times \text{Marker})_{jm} + (\text{Marker} \times \text{Marker})_{lm} \\ & + (\text{Sex} \times \text{Marker} \times \text{Marker})_{jlm} + \epsilon_{ijklm}, \end{aligned} \tag{8}$$

where Sex and the two Marker states ($l = 1, 2$ and $m = 1, 2$) have fixed effects but Day and background genetic Line are random effects. Because of the large number of tests required for the two-site interaction tests, it was not computationally possible to determine critical values for epistatic terms through permutation analysis. Therefore, the *F*-distribution *P*-value of the marker × marker interaction was retained from the analysis of variance as an indicator of significance of the effect.

RESULTS

Genetic variation in immunocompetence: Ninety-five *D. melanogaster* chromosome 2 substitution lines were examined for the ability to suppress systemic growth of four bacteria: *S. marcescens*, *P. burhodogranaria*, *E. faecalis*, and *L. lactis*. Analysis of variance showed that chromosome 2 genotype made a highly significant contribution to phenotypic variation in resistance to infection in all cases [*S. marcescens*, $F_{(85)} = 1.93$, $P < 0.001$; *P. burhodogranaria*, $F_{(86)} = 1.76$, $P = 0.002$; *E. faecalis*, $F_{(93)} = 1.93$, $P < 0.001$; *L. lactis*, $F_{(91)} = 2.02$, $P < 0.001$]. Line genotype explained 70.6% of the nonerror phenotypic variance (10.6% of the overall variance) in resistance to *S. marcescens*, 26.9% (9.7%) to *P. burhodogranaria*, 47.6% (10.1%) to *E. faecalis*, and 57.5% (13.1%) to *L. lactis*. Extreme Drosophila lines differed in pathogen load by a minimum of 4.56 phenotypic standard errors (after infection with *P. burhodogranaria*) to a maximum of 8.32 phenotypic standard errors (after infection with *L. lactis*). Ranked line means and errors are illustrated in Figure 2, and mean values of postinfection load for each *D. melanogaster* genotype are given in supplemental Figure 1 at http://www.genetics.org/supplemental/.

The widest phenotypic distribution in this study was generated after infection with *P. burhodogranaria*, with mean bacterial loads at 28 hr postinfection ranging from 3.02×10^2 to 1.31×10^5 bacteria/fly. The observed variance within lines was also largest with Providencia (Figure 2B). It appears that stochasticity in the early stages of infection by *P. burhodogranaria* has larger effects on the growth dynamics of the bacteria within the fly than is observed with other bacteria (B. P. Lazzaro and P. Juneja, unpublished results), which probably contributes to the higher within-line variances observed with this bacterium. The distribution of mean bacterial loads across Drosophila lines was flattest after challenge

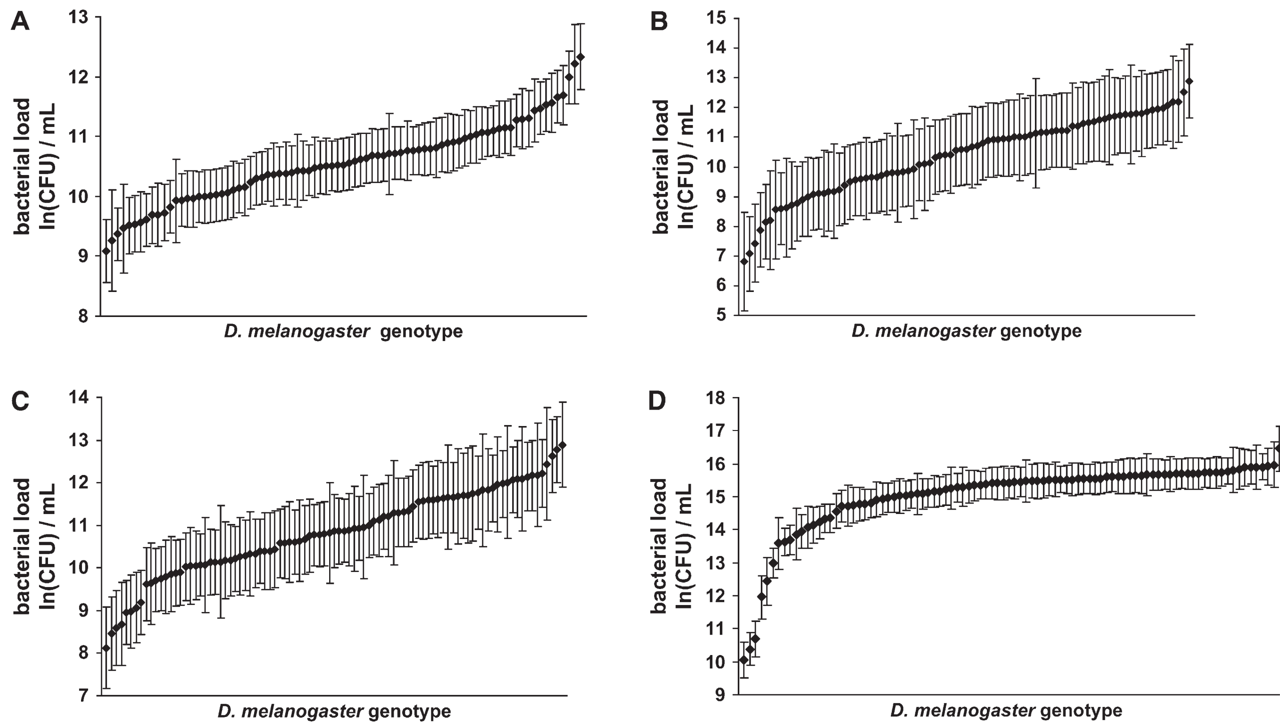

Figure 2.—Mean bacterial loads (±1 SE) sustained by each *D. melanogaster* genetic line measured 28 (±0.5) hr after infection with (A) *Serratia marcescens*, (B) *Providencia burhodogranaria*, (C) *Enterococcus faecalis*, and (D) *Lactococcus lactis*. *D. melanogaster* lines are plotted in rank order within each bacterium and so are ordered differently in each part. Bacterial load is measured as the natural log of the number of colony-forming units per milliliter (CFU/ml) of homogenate from three flies, corrected for the sex of the flies. Genetic line makes a highly significant contribution to phenotypic variance in all cases (ANOVA, $P \leq 0.002$).

with *L. lactis*, with the majority of the line means pushed up to the upper resolution threshold of the plating system (Figure 2D). Of the 536 homogenates derived from *L. lactis*-infected flies, 403 had densities of viable bacteria estimated at $>8 \times 10^4$/ml even after 100-fold dilution of the homogenates. It is therefore likely that the evenness of the mean *L. lactis* loads estimated for the Drosophila lines is the result of inadequate phenotypic resolution and not from a lack of genetic variation. We note that there are several lines distinctively in the low tail of the phenotypic distribution (Figure 2D).

Mean pathogen loads sustained by each Drosophila line were almost universally positively correlated across the bacteria tested, but the correlations were weak (Table 3). Only the correlation between *E. faecalis* and *L. lactis* loads was significant at a nominal $\alpha < 0.05$, and this significance does not survive Bonferroni correction for multiple tests. The weakness of the correlations in resistance to diverse bacteria, in spite of the highly significant contribution of Drosophila genotype to phenotypic variation in resistance to each individual bacterium, suggests that the variability we observe does not simply result from among-line variation in inbreeding depression ("general vigor" effects). Rather, this finding probably reflects biologically heterogeneous aspects of the host–pathogen interaction.

Genotype–phenotype associations: Genotyped polymorphisms in 21 genes known or suspected to be involved in immune response were tested for statistical association with phenotypic variation in bacterial load. The results are summarized in Table 4. Twenty of the 127 genotyped markers were significantly associated

TABLE 3

Slope (top right) and r^2 (bottom left) of the correlation across bacteria in mean load sustained by each *D. melanogaster* line

Bacterium	*S. marcescens*	*P. burhodogranaria*	*E. faecalis*	*L. lactis*
S. marcescens		+0.278	+0.064	+0.192
P. burhodogranaria	0.019		+0.160	−0.079
E. faecalis	0.002	0.016		+0.287*
L. lactis	0.028	0.021	0.045*	

* Correlation significant at $P < 0.05$.

with variability in resistance to one or more of the bacteria tested at a nominal *P*-value of 0.05. At a significance level of $P < 0.05$, 5 markers were associated with resistance to *S. marcescens*, 3 with resistance to *P. burhodogranaria*, 7 to *E. faecalis*, and 8 to *L. lactis*. Seven of those associations are significant at $P < 0.01$: 2 affecting resistance to *S. marcescens*, 2 affecting resistance to *E. faecalis*, and 3 affecting resistance to *L. lactis*. All of the markers associated with resistance to any of the four bacteria are in loci involved in pathogen recognition or signal transduction. None of the 33 markers typed in antibacterial peptide genes were associated with resistance to any of the four bacteria with $P < 0.05$.

Most of the markers associated with variation in intensity of infection are implicated only in resistance to one of the four bacteria, and many of these associations are weak (Table 4). One exception is a complex of polymorphisms within and immediately flanking *SR-CII* intron 2 that seems to be generally associated with resistance to infection. DNA sequence polymorphism flanking this intron is arranged into tight haplotype structure (Figure 3). Linkage disequilibrium rapidly decays outside of the intron (data not shown). Two markers were genotyped in this region: a polymorphic deletion that eliminates 28 bp of the 110-bp intron (marker 7274899) and a silent C/G polymorphism 3 bp from the intron 2 boundary (codon 252, marker 7274975). When the two markers are considered independently, allelic state at the latter marker is a significant predictor of bacterial load sustained after infection with *L. lactis* ($P = 0.002$) and *S. marcescens* ($P = 0.015$) and is suggestive with respect to *E. faecalis* ($P = 0.075$) but not with *P. burhodogranaria* ($P = 0.488$). The deletion state of marker 7274899 is also associated with increased resistance to *L. lactis* ($P = 0.001$), but not to any of the other bacteria. Marker 7274899 was weakly associated with resistance to *S. marcescens* in our previous study ($P = 0.030$; Lazzaro *et al.* 2004), with allelic effects in the same direction as in this study. These two markers can be considered jointly to estimate the effects of the haplotypes illustrated in Figure 3. The two-site genotype indicative of haplotype H3 is highly significantly associated with resistance to *L. lactis* ($P < 0.001$), but the other haplotypes are phenotypically indistinguishable after *L. lactis* infection. After infection with *S. marcescens*, flies with the *iso-1* two-site genotype sustain bacterial loads that are significantly smaller than those sustained by any other genotype ($P = 0.012$). None of the haplotypes were significantly associated with resistance to *P. burhodogranaria* or *E. faecalis*.

Several markers in the *Toll*-like receptor genes *18-wheeler* and *Tehao* were also repeatedly associated with resistance to bacteria used in this study. Five markers in *Tehao* were significantly associated with the suppression of *E. faecalis*, *L. lactis*, or both. No *Tehao* sites were significantly associated with resistance to either of the gram-negative bacteria (Table 4). The five polymorphisms associated with resistance to the gram-positive bacteria are in partial disequilibrium with each other (supplemental Figure 2 at http://www.genetics.org/supplemental/) and so may be correlated with a single phenotypically relevant mutation or haplotype in the *Tehao* gene. These markers are distributed over >2.5 kb of the *Tehao* gene and its promoter, however, making it difficult to pinpoint the physical site of the phenotypically causal polymorphism.

Four of the six markers typed in *18-wheeler* are also associated with variable suppression of the bacteria tested, with at least one marker associated with resistance to each bacterium. A 10-bp insertion/deletion 1.5 kb upstream of the *18w* start (marker 15174292) was significantly associated with variable resistance to *S. marcescens* ($P = 0.006$), but to no other bacterium. A second 12-bp insertion/deletion spanning codons 1361–1364 (marker 15179676) was significantly associated with resistance to *E. faecalis* ($P = 0.001$). This indel is in partial disequilibrium with a synonymous mutation in codon 1212 (marker 15179232) that was weakly associated with variability in suppression of *E. faecalis* ($P = 0.022$) and *L. lactis* ($P = 0.044$) and with a distinct synonymous mutation in codon 1210 (marker 15179526) that was weakly associated with resistance to *P. burhodogranaria* ($P = 0.035$). Given the spatial distribution and incomplete disequilibrium associations among these markers, it is unclear whether there are independent mutations in *18-wheeler* causing variable resistance to each of the four bacteria tested or whether all of the significant associations reflect a smaller number of sites or haplotypes with universal effects on resistance.

Interactions among site pairs: In a general test for epistasis, all pairs of sites were tested for nonadditive interactive effects on variation in resistance to the four bacteria. Multiple site pairs exhibited interactions with nominally significant *P*-values, but these interactions were no more common than might be expected by chance. Following infection with each of the four bacteria, ~5% of the site pairs tested showed interactions with nominal significance $P < 0.05$ and 1% of the site pair interactions tested significant with $P < 0.01$. The absolute number of interacting sites may not be an informative quantity, and sites within a locus are not independent of each other, so it may be of greater interest to consider the significance of the strongest interaction between any two sites in a pair of loci. Even when the data are examined this way, however, there are few strongly discernable patterns (Figure 4). The most significant two-site interactions were detected in response to *L. lactis*, where markers in 17 of the 136 gene pairs (12.5%) exhibited interactions significant at $P < 0.001$. These included interactions within the *PGRP* locus and between the *PGRP*s and seven other genes. Markers in the *PGRP* locus also interacted significantly

TABLE 4

Significant associations between marker genotypes and bacterial load sustained 28 hr after infection with each of four bacteria

	Serratia marcescens	*Providencia burhodogranaria*	*Enterococcus faecalis*	*Lactococcus lactis*
PGRP-SC1A				
Marker 3771856				
A/G, 800 bp 3′ of stop	0.002**	0.370	0.266	0.452
A (56.6%)[a]	10.375 (0.155)	10.226 (0.823)	10.836 (0.424)	11.316 (0.218)
G (43.4%)[a]	10.799 (0.161)	10.566 (0.828)	10.841 (0.430)	11.284 (0.223)
% variance explained	26.9 (4.6)	0.8 (0.3)	0 (0)	0 (0)
PGRP-SC2				
Marker 3777783				
Val/Ile G/A, codon 24	0.607	0.661	0.267	0.044*
G (26.4%)	10.521 (0.179)	10.334 (0.844)	10.465 (0.455)	10.954 (0.251)
A (73.6%)	10.563 (0.129)	10.389 (0.813)	10.908 (0.419)	11.385 (0.220)
% variance explained	0 (0)	0 (0)	5.2 (1.2)	18.9 (4.8)
SR-CI				
Marker 4115944				
Silent G/T, codon 475	0.367	0.046*	0.331	0.420
G (53.3%)	10.483 (0.136)	10.077 (0.829)	10.796 (0.462)	11.263 (0.227)
T (46.7%)	10.631 (0.133)	10.614 (0.826)	10.832 (0.458)	11.314 (0.224)
% variance explained	0.6 (0.1)	3.7 (1.3)	0 (0)	0 (0)
SR-CII				
Marker 7274975				
Silent C/G, codon 252	0.015*	0.488	0.075	0.002**
G (74.5%)	10.666 (0.145)	10.348 (0.905)	10.876 (0.426)	11.433 (0.257)
C (25.5%)	10.211 (0.177)	10.462 (0.933)	10.684 (0.457)	10.830 (0.280)
% variance explained	28.7 (5.3)	0 (0)	0 (0)	33.8 (9.3)
Marker 7274899				
28-bp indel, intron 2	0.678	0.666	0.146	0.001**
Insertion (86.2%)	10.565 (0.134)	10.412 (0.824)	10.876 (0.450)	11.399 (0.219)
Deletion (13.8%)	10.428 (0.211)	10.100 (0.895)	10.478 (0.514)	10.460 (0.283)
% variance explained	0 (0)	28.7 (5.3)	2.5 (0.6)	61.3 (22.7)
Marker 7276155				
60-bp indel, 313 bp 5′ of start	0.013*	0.603	0.197	0.344
Insertion (93.9%)[a]	10.622 (0.135)	10.338 (0.803)	10.803 (0.420)	11.297 (0.272)
Deletion (6.1%)[a]	9.992 (0.281)	10.576 (0.962)	11.024 (0.574)	11.341 (0.375)
% variance explained	43.8 (9.6)	0 (0)	0 (0)	0 (0)
SR-CIII				
Marker 4113576				
Silent A/G, codon 195	0.398	0.031*	0.906	0.196
A (92.0%)	10.597 (0.127)	10.453 (0.815)	10.844 (0.417)	11.311 (0.209)
G (8.0%)	10.314 (0.251)	9.750 (0.935)	10.785 (0.571)	11.226 (0.316)
% variance explained	4.8 (0.7)	5.3 (1.9)	0 (0)	0 (0)
Marker 4114081				
GT/AG, 70–71 bp 3′ of stop	0.049*	0.105	0.305	0.446
GT (18.9%)	10.613 (0.209)	9.860 (0.920)	10.469 (0.485)	11.454 (0.272)
AG (81.1%)	10.536 (0.141)	10.445 (0.870)	10.888 (0.417)	11.267 (0.218)
% variance explained	0 (0)	3.9 (1.5)	3.1 (0.7)	0 (0)
SR-CIV				
Marker 3515646				
Presence/absence of intron 2	0.589	0.925	0.269	0.004**
Presence (67.3%)	10.584 (0.165)	10.420 (0.867)	10.937 (0.437)	11.425 (0.226)
Absence (32.7%)	10.527 (0.189)	10.260 (0.885)	10.668 (0.453)	11.046 (0.242)
% variance explained	0 (0)	0 (0)	1.6 (0.4)	16.1 (3.9)
Tehao				
Marker 13423404				
Noncoding G/A, 1231 bp 5′ of start	0.841	0.837	0.942	0.047*
G (27.8%)	10.498 (0.194)	10.418 (0.791)	10.876 (0.477)	11.172 (0.230)
A (72.2%)	10.586 (0.154)	10.321 (0.754)	10.865 (0.438)	11.345 (0.198)
% variance explained	0 (0)	0 (0)	0 (0)	0 (0)

(*continued*)

TABLE 4

(Continued)

	Serratia marcescens	*Providencia burhodogranaria*	*Enterococcus faecalis*	*Lactococcus lactis*
Marker 13423843				
T/A, 778 bp 5′ of start	0.194	0.597	0.048*	0.014*
T (42.6%)	10.431 (0.158)	10.194 (0.829)	11.196 (0.440)	11.548 (0.208)
A (57.4%)	10.688 (0.145)	10.471 (0.823)	10.645 (0.431)	11.139 (0.201)
% variance explained	8.9 (1.4)	0 (0)	10.2 (2.5)	20.5 (4.8)
Marker 13424088				
G/A, 533 bp 5′ of start	0.251	0.714	0.018*	0.396
G (65.3%)	10.493 (0.138)	10.351 (0.815)	11.076 (0.436)	11.316 (0.206)
A (34.7%)	10.730 (0.161)	10.370 (0.833)	10.463 (0.453)	11.311 (0.222)
% variance explained	6.4 (1.0)	0 (0)	12.2 (3.1)	0 (0)
Marker 13426961				
Cys/Tyr G/A, codon 406	0.117	0.349	0.045*	0.814
G (75.3%)	10.607 (0.123)	10.445 (0.843)	10.751 (0.423)	11.312 (0.211)
A (24.7%)	10.312 (0.171)	10.111 (0.878)	11.087 (0.459)	11.268 (0.244)
% variance explained	12.0 (1.9)	1.4 (0.6)	1.1 (0.3)	0 (0)
Marker 13427284				
Synonymous G/A, codon 512	0.576	1.000	0.441	0.022*
G (35.7%)	10.516 (0.149)	10.401 (0.802)	10.932 (0.429)	11.402 (0.209)
A (65.3%)	10.657 (0.165)	10.405 (0.815)	10.724 (0.444)	11.128 (0.222)
% variance explained	0 (0)	0 (0)	0 (0)	0 (0.6)
18-wheeler				
Marker 15174292				
10-bp indel, 1.5 kb 5′ of start	0.006**	0.303	0.092	0.521
Insertion (91.2%)	10.607 (0.152)	10.423 (0.731)	10.829 (0.430)	11.303 (0.237)
Deletion (8.8%)	10.065 (0.318)	10.386 (0.911)	10.522 (0.548)	11.032 (0.319)
% variance explained	2.7 (0.6)	0 (0)	0 (0)	2.7 (0.6)
Marker 15179526				
Silent T/C, codon 1210	0.257	0.035*	0.201	0.482
C (38.6%)	10.508 (0.163)	10.254 (0.807)	10.834 (0.401)	11.226 (0.236)
T (61.4%)	10.762 (0.174)	10.818 (0.821)	10.916 (0.415)	11.350 (0.247)
% variance explained	6.5 (1.2)	3.7 (1.3)	3.7 (1.3)	0 (0)
Marker 15179232				
Silent G/A, codon 1212	0.495	0.613	0.022*	0.044*
G (82.7%)	10.583 (0.148)	10.495 (0.807)	10.991 (0.489)	11.275 (0.221)
A (13.3%)	10.846 (0.259)	10.240 (0.896)	10.066 (0.557)	11.211 (0.289)
% variance explained	2.8 (0.5)	0 (0)	0 (0)	0 (0)
Marker 15179676				
12-bp indel, codons 1361–1364	0.222	0.842	0.001**	0.357
Insertion (5.1%)	10.010 (0.450)	10.549 (1.042)	9.472 (0.715)	10.790 (0.497)
Deletion (94.9%)	10.504 (0.154)	10.252 (0.830)	10.984 (0.537)	11.291 (0.337)
% variance explained	12.4 (3.1)	2.8 (0.5)	65.8 (22.3)	11.7 (3.1)
DIF				
Marker 17392491				
10-bp indel, intron 6	0.837	0.163	0.044*	0.497
Deletion (73.1%)	10.449 (0.174)	10.656 (0.835)	11.265 (0.469)	11.434 (0.261)
Insertion (26.9%)	10.546 (0.128)	10.229 (0.799)	10.646 (0.428)	11.218 (0.228)
% variance explained	0 (0)	0 (0)	0 (0)	2.6 (0.6)
ik2				
Marker 20644684				
6-bp indel, 1.3 kb 5′ of start	0.788	0.240	0.009**	0.954
Deletion (4.9%)	10.774 (0.359)	9.507 (1.096)	9.583 (0.590)	11.339 (0.434)
Insertion (95.1%)	10.527 (0.160)	10.437 (0.915)	10.883 (0.419)	11.291 (0.217)
% variance explained	0 (0)	6.0 (2.6)	43.8 (13.8)	0 (0)

Markers that are nominally significant at $P < 0.05$ against at least one bacterium are listed. Markers are identified by their position in Release 3.1 of the *D. melanogaster* complete genome assembly, as well as by their position in the relevant candidate gene. The ancestral state for each polymorphism is listed first, with the frequency of each allelic state in parentheses. Each marker/bacterium entry contains the nominal *P*-value of the association, the least-squares mean (SE) load sustained by each genotype, and the percentage of the observed nonerror (total) phenotypic variance explained by the marker. *L. lactis* loads reported are those obtained after 100-fold dilution of the Drosophila homogenates and therefore are smaller than the true loads sustained (see MATERIALS AND METHODS). $^{*}P < 0.05$, $^{**}P < 0.01$.

[a] The ancestral states could not be reliably determined for the two sites in *SR-CII* and *PGRP-SC1A*.

Figure 3.—Haplotype structure surrounding the second intron of SR-CII. Direct sequence obtained from 10 chromosomes collected in Pennsylvania (Lazzaro and Clark 2001) yielded four instances of haplotype H1 (*D. melanogaster* lines 2CPA 1, 105, 118, 122), three instances of haplotype H2 (lines 2CPA 7, 14, 16) and three instances of haplotype H3 (lines 2CPA 12, 51, 103). The *iso-1* haplotype is that of the *D. melanogaster* strain whose whole genome was sequenced (Adams *et al.* 2000). Genotyping of the *D. melanogaster* lines in this study for the C/G polymorphism at marker 7274975 and the deletion defining marker 7274899 suggests that the frequencies of haplotypes H3, *iso-1*, and H1/H2 are 12.4, 14.6, and 73.0% in our sample (supplemental Figure 1 at http://www.genetics.org/supplemental/). Haplotype H3 is associated with resistance to *L. lactis* and haplotype *iso-1* is associated with resistance to *S. marcescens* (see results).

with markers in other genes after infection with the other three bacteria, but to a lesser degree than was seen after infection with *L. lactis* (Figure 4). In general, it appears that a preponderance of the strong interactions involves pathogen recognition loci. It is also apparent that antibacterial peptide loci tend not to interact epistatically with other peptide genes. Of the proteins represented in our study, only DIF and Cactus are known to physically interact. DIF also binds to promoter elements upstream of antibacterial peptide genes. These physical interactions, however, do not appear to result in an increased likelihood of statistical epistasis (Figure 4).

Marker × pathogen interactions: Each marker was tested for heterogeneity in effects across the four bacteria used in this experiment. Six of the 127 markers showed a significant ($P < 0.01$) marker × pathogen interaction. Five of these markers are in *Tehao* (all with $P < 0.005$). As previously mentioned, these sites are in partial linkage disequilibrium with each other, making it impossible to identify the specific mutation(s) driving the interaction. It is clear, however, that the phenotypic effect of genetic variation in *Tehao* depends on the pathogen used in challenge, with effects of larger magnitude detected after infection with gram-positive bacteria. Figure 5 shows the reaction norm of one marker, an A/T polymorphism 778 bp upstream of the *Tehao* start codon (marker 13423843), associated with significantly heterogeneous phenotypic effects across pathogens ($P < 0.001$). The reaction norm for this marker is typical of the significant *Tehao* markers, with the allele conferring greater resistance to gram-positive bacteria resulting in greater susceptibility to gram-negative infection, but with larger allelic effects after gram-positive infection.

The other marker exhibiting a significant ($P = 0.007$) marker × pathogen interaction is an insertion/deletion polymorphism 1.8 kbp upstream of the *SR-CII* start site. The effects of this allele reverse direction between infections with *S. marcescens* and the other three bacteria. Interestingly, this marker did not have a significant marginal effect on resistance to any of the four bacteria, although it is nearly significantly associated with resistance to *S. marcescens* ($P = 0.052$).

Replication of the previously published study: We previously used this same set of *D. melanogaster* lines in a larger-scale analysis of genetic variability in resistance to *S. marcescens* (Lazzaro *et al.* 2004). Those data can be compared to the data from *S. marcescens* infections in this experiment. The phenotypic distribution is narrower in this experiment than in the previous one and is shifted toward higher loads (compare Figure 2A in this study to Figure 1 in Lazzaro *et al.* 2004). In this study, the mean *S. marcescens* load sustained by extreme *D. melanogaster* lines differs by ∼6 phenotypic standard errors, considerably less than the phenotypic spread of 10 standard errors that we previously observed. This may be partially due to the ∼10-fold smaller sample size

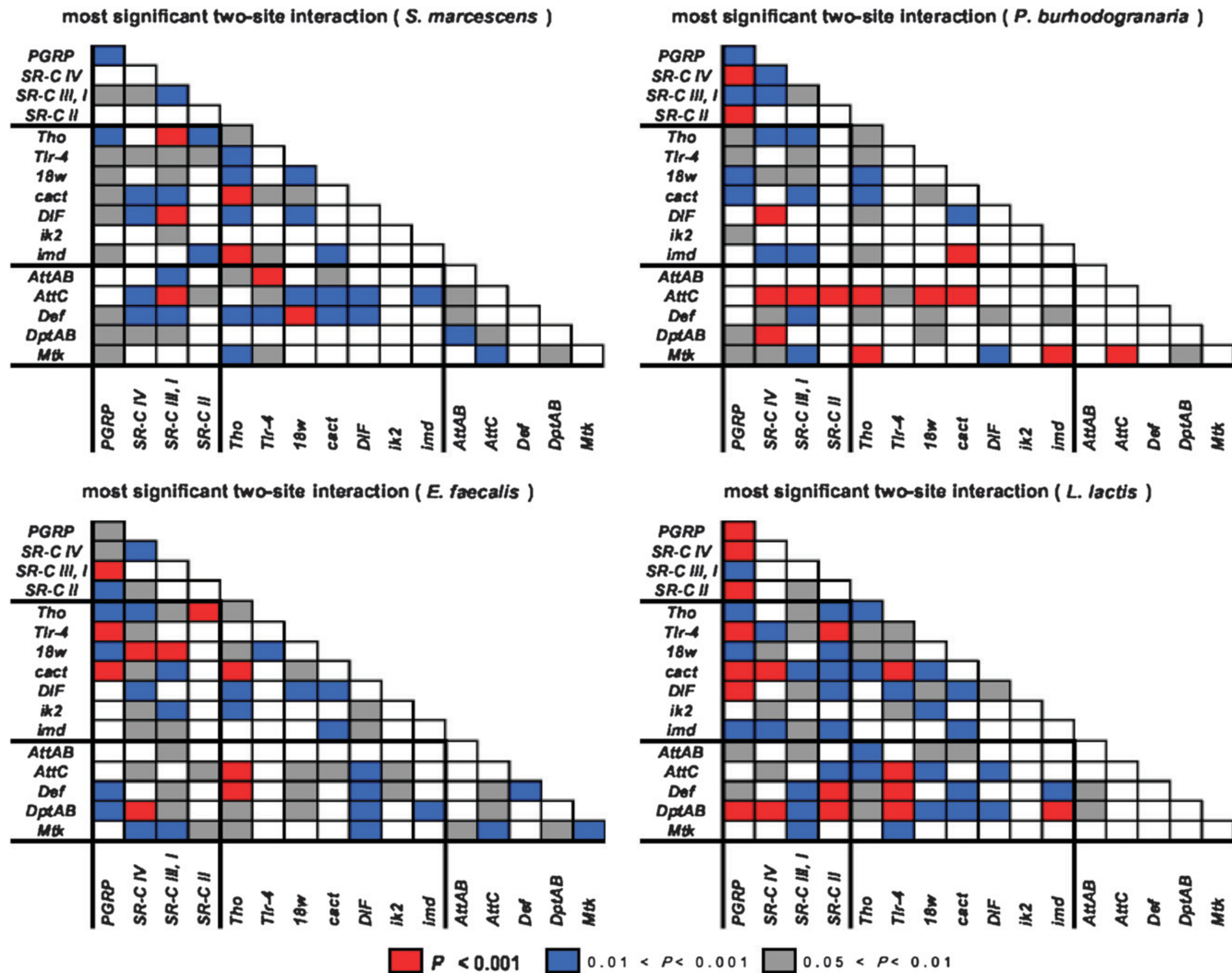

Figure 4.—Matrices of epistatic interactions among loci after challenge with the four bacteria. The most significant interaction term between any two markers in a locus is reported for all pairs of loci (see materials and methods). Gray boxes indicate $0.05 \geq P > 0.01$, blue boxes are $0.01 > P. \geq 0.001$, and red boxes indicate $P < 0.001$.

in the current experiment, where an average of 6.9 observations were made for each *D. melanogaster* line compared to an average of 68.5 observations per line in the previous experiment.

There are some weakly repeated genotype–phenotype associations between the two studies. A 6-bp insertion 1.3 kb upstream of the *ik2* transcriptional start site was associated with resistance to *S. marcescens* in the previous study ($P < 0.001$) and is associated with resistance to *E. faecalis* in this study ($P = 0.009$). The deletion state of the polymorphism leads to higher bacterial loads in both significant cases. A more robustly repeated result is that markers in haplotypes encompassing intron 2 of the scavenger receptor gene *SR-CII* (Figure 3) are implicated in variable suppression of infection by most of the bacteria tested in this study (*L. lactis*, $P < 0.001$; *S. marcescens*, $P = 0.012$; *E. faecalis*, $P = 0.217$; *P. burhodogranaria*, $P = 0.244$) and were associated with resistance to *S. marcescens* in the previous study ($P = 0.030$). Other examples of replication are that a noncoding marker 3′ of *SR-CIII* that is slightly associated with resistance to *S. marcescens* in this study ($P = 0.049$) was more strongly associated with resistance to *S. marcescens* in the previous study ($P = 0.005$) and that a silent substitution in codon 475 of *SR-CI* weakly associated with resistance to *P. burhodogranaria* ($P = 0.044$) was also weakly implicated in suppression of *S. marcescens* in the previous study ($P = 0.050$ in males infected in the morning, $P = 0.128$ overall). A 10-bp deletion 1.5 kb upstream of the start codon of the *Toll*-family receptor gene *18-wheeler* conferred significant resistance to S. *marcescens* in the previous study ($P = 0.023$) and the current one ($P = 0.006$), with the deletion state conferring resistance in both cases.

Notably, however, this study fails to recover as significant some of the strongest site associations seen in the previous study. For instance, a theme in the previous study was that the intracellular signaling genes examined (*DIF*, *imd*, *cactus*, and *ik2*) harbored the majority of the functional variation for resistance to *S. marcescens*

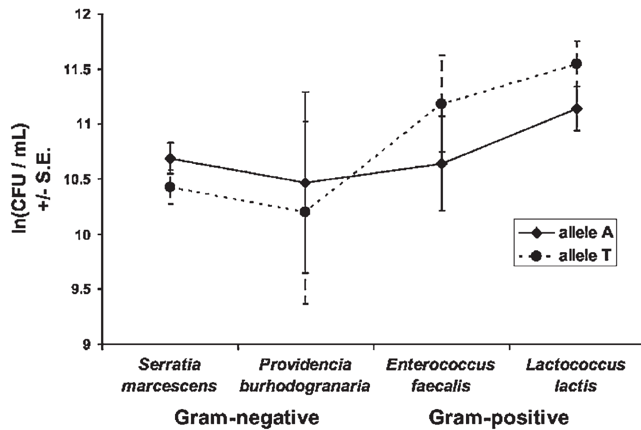

Figure 5.—Plotted norm of reaction for one representative marker in *Tehao*, an A/T polymorphism 778 bp upstream of the start codon (marker 13423843). This marker is one of a complex of markers that are in strong linkage disequilibrium and span several kilobases of the *Tehao* gene and promoter.

infection (Lazzaro *et al.* 2004). None of the markers in these genes are significantly associated with resistance to *S. marcescens* in this study. Furthermore, we noted in the previous study a high incidence of epistatic interactions among intracellular signaling genes and between genes encoding signaling proteins and antibacterial peptides. These interactions were not recapitulated in this study. The differences between the two studies may result either from experimental or from analytical differences, possibilities that are explored in turn.

Genotype–phenotype associations were tested in the previous study with a simple linear model, wherein the response variable was the mean phenotype for each line and the strength of association was determined by the magnitude of the *F*-ratio at each marker (variance attributable to each marker divided by error variance in the model; Lazzaro *et al.* 2004). A relative-likelihood framework is applied to the present data (see materials and methods). To determine whether differences in genotype–phenotype associations detected between the two studies result from differences in analysis of the two data sets, we have reanalyzed the previously published data under the likelihood framework applied to the current data. This new analysis of the old data robustly recovers the published results (not shown), leading us to conclude that differing results between the new and old studies are experimental in nature and not derived from differences in the statistical models employed.

One major experimental difference between the two studies is that this study relies on a substantially smaller number of phenotypic observations than does the previous one. The present failure to recover previously significant site associations may therefore result from decreased statistical power in the smaller study. We estimated allelic effects on resistance attributable to each marker, separately using data from this study (data collected at 28 hr postinfection) and previously published data (data collected at 26 hr postinfection). We can then compare the allelic effects across studies. The estimated marker effect sizes are significantly correlated across the two studies, even when sites whose effects are nonsignificant in either study are included in the comparison ($r^2 = 0.042$, $P = 0.024$; Figure 6A). When the comparison is restricted to sites whose effects were significant in the previously published study, the correlation in effect sizes across experiments becomes much stronger ($r^2 = 0.284$, $P = 0.003$; Figure 6B). The point in Figure 6B is that the largest effect in the previous experiment is in *ik2* (markers 20644684; effect sizes of -0.75 ln(CFU)/ml). This marker was not a significant predictor of resistance to *S. marcescens* in this experiment, but it did significantly predict *E. faecalis* load ($P = 0.009$). The overall correlation in effect sizes across the two experiments suggests that allelic effects are generally repeatable across the two studies and supports the interpretation that reduced statistical power in the second study at least partially explains the differences between the two experiments in the recovery of significant genotype–phenotype associations.

DISCUSSION

We have evaluated the quantitative genetic basis for natural variation in resistance to infection by four different bacteria in *D. melanogaster*. The *D. melanogaster* examined are chromosome 2 substitution lines that were isolated from a natural population in the northeastern United States. The bacteria used in this study, with the exception of *S. marcescens*, were isolated from the hemolymph of *D. melanogaster* collected from that same population, increasing the potential that these are infectious agents of ecological relevance to the experimental Drosophila. The four bacterial strains were specifically chosen because they establish stable infections of moderate intensity with little host mortality. Even so, the bacteria clearly differ in the speed with which they grow in the fly following infection (not shown) and in the ultimate systemic loads achieved (Figure 2).

The *D. melanogaster* genetic line was a highly significant determinant of bacterial load sustained (resistance) after all bacterial challenges ($P \leq 0.002$ in all cases), but the mean bacterial loads sustained by each line were largely uncorrelated. The correlations measured are based strictly on line means and do not account for within-line variances, making it inappropriate to conclude that the lack of significant correlation derives from extreme specificity in the host response. The poor correlation does suggest, however, that the highly significant effects of genetic line do not result from simple differences in vigor (inbreeding depression) among lines. More detailed conclusions from the line means are complicated because the phenotypic resolution varies with the bacterium used in challenge. While some of this difference in phenotypic spread

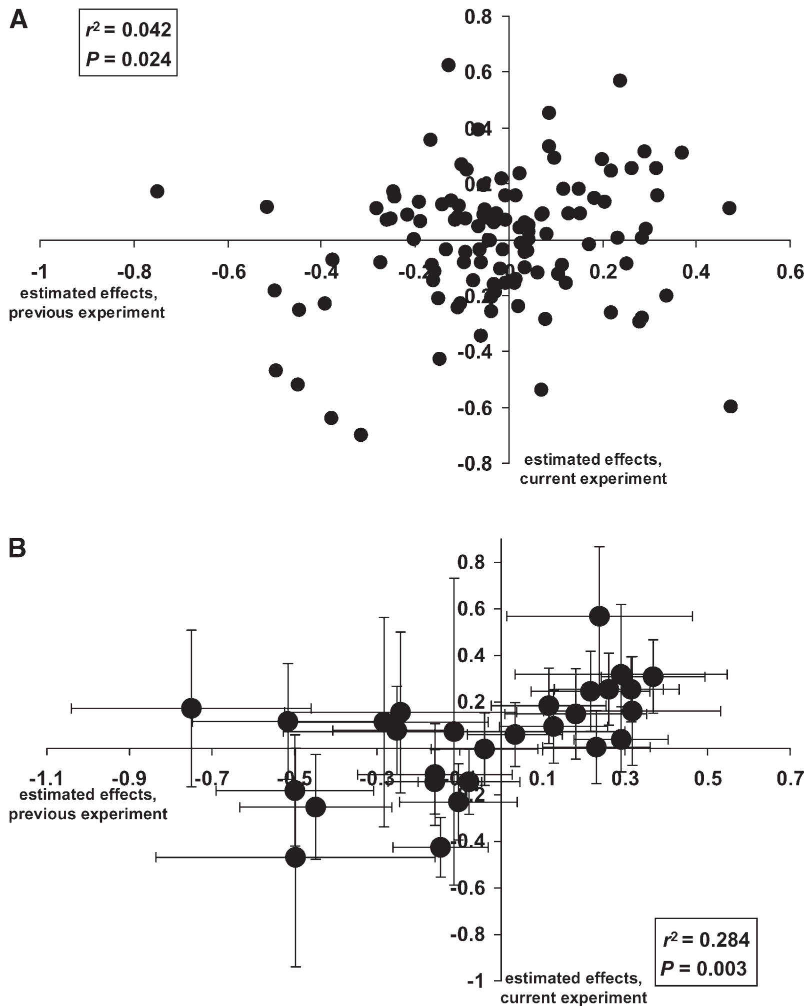

Figure 6.—The correlation between this experiment and a previously published study (Lazzaro *et al.* 2004) in allelic effects on resistance to *S. marcescens* (A) for all 127 markers in the study and (B) for markers that were determined to be significantly associated with resistance in the previously published study.

certainly is caused by biological differences in the interaction between host and pathogen, some of it is probably technical in origin, as exemplified by the *L. lactis* data, where the majority of the flies carried bacterial densities that pushed the upper limit of resolution in our plating system.

One hundred twenty-seven polymorphic markers were genotyped in 21 candidate genes known or thought to be involved in the *D. melanogaster* antibacterial immune response. Genotype at each of these markers was tested for statistical association with bacterial load sustained after infection. Twenty markers in 10 genes were significantly associated with variability in resistance to one or more of the bacteria tested at $P < 0.05$. Seven markers in 5 genes were associated with resistance to infection at $P < 0.01$. Many of the associations between marker genotype and variation in resistance to infection were weak or were significant after infection with only one of the four bacteria (Table 4), although comparison across experiments is complicated by the differences in precision and spread of phenotypes observed after infection with the different bacteria. These differences in the phenotypic distributions translate into variability in statistical power to detect genotype–phenotype associations and make it difficult to interpret associations that are detected after some infection regimes but not others. For instance, the fact that we find fewer genes associated with variation in resistance to *P. burhodogranaria* than to the other bacteria probably does not mean that the genetic basis for resistance to Providencia is simpler, but instead reflects the fact that the observed variance within *D. melanogaster* genetic lines was much larger after *P. burhodogranaria* infection than after infection with other bacteria (the proportion of the non-error phenotypic variance explained by *D. melanogaster* line genotype after *P. burhodogranaria* infection was less than half the variance explained by line after infection with the other bacteria). Despite these complications, there are several consistent observations that bear further discussion.

One is the association of polymorphism in *Tehao* with variable suppression of *E. faecalis* and *L. lactis* infection, although not of infection by *P. burhodogranaria* or *S. marcescens*. Tehao is capable of physical interaction with Toll at the membrane surface and can stimulate immune activation through the Toll signaling pathway,

although the presence of endogenous Tehao activity is not sufficient for immune induction in the absence of Toll (Tauszig *et al.* 2000; Luo *et al.* 2001). The placement of Tehao as a modifier of Toll pathway activity is consistent with our finding that polymorphism in *Tehao* influences that ability to suppress infection by gram-positive, but not by gram-negative, bacteria. The observation that the *Tehao* alleles that are most effective at fighting gram-positive bacteria tend to be less effective against gram-negative bacteria raises the tantalizing prospect that *Tehao* polymorphism may exhibit weak antagonistic pleiotropy in pathogen-specific defense (Figure 5), but additional experimentation is needed to test this hypothesis.

Polymorphic sites in *18-wheeler* and *SR-CII* are associated with variation in resistance to all of the bacteria tested here. These associations may be somewhat unexpected. Despite early reports to the contrary (Williams *et al.* 1997; Hedengren *et al.* 2000), the direct involvement of *18-wheeler* in mounting a systemic induced immune response in adult flies has been called into question (Ligoxygakis *et al.* 2002). *18-wheeler* is, however, required for proper development of the larval fat body and may play a role in inducible larval defenses and hematopoesis (Ligoxygakis *et al.* 2002). There is no direct evidence that *SR-CII* is involved in immune defense, even though *SR-CI*, the closest Drosophila paralog to *SR-CII*, is known to be involved in phagocytosis of bacteria (Rämet *et al.* 2001). *SR-CII* expression is thought to be maximal early in Drosophila development (Rämet *et al.* 2001), and molecular evolutionary analysis reveals *SR-CII* to be on a distinctly more conservative evolutionary trajectory the other three *SR-C*s in Drosophila (Lazzaro 2005). We therefore suggest that the associations we observe between polymorphism in *18-wheeler* and *SR-CII* and variation in resistance to bacterial infection may stem from roles those genes play in physiological processes such as fat body development and cell proliferation, which are essential for organismal immunocompetence but may not be components of the inducible adult immune response *per se.*

One clear negative pattern to emerge both from this study and from our previously published work is that although antimicrobial peptide genes harbor ample molecular variation in *D. melanogaster* (Clark and Wang 1997; Ramos-Onsins and Aguadé 1998; Date *et al.* 1998; Lazzaro and Clark 2001, 2003), polymorphism in these genes does not seem to contribute substantially to whole-organism variation in resistance to infection. We tested 33 markers in seven genes for contribution to phenotypic effect in these two studies, including a null allele of *Attacin A*, large deletions in the promoter of *Attacin B* that affect transcript levels (Lazzaro and Clark 2001; B. P. Lazzaro, unpublished data), and markers that correlate with major haplotype blocks in several antibacterial peptide genes. In neither this study nor a previously published analysis of resistance to *S. marcescens* (Lazzaro *et al.* 2004) did any of these markers associate strongly with resistance to bacterial infection. Given the repeatable absence of genotype–phenotype association across independent experiments and challenge with multiple bacteria, it seems safe to conclude that any whole-organism phenotypic ramifications of polymorphism in antimicrobial peptide genes are too small to be detected in studies such as these. We think that there are two nonexclusive explanations for the failure of peptide variation to explain phenotypic variation. First, Drosophila antimicrobial peptides form a diverse protein group that overlaps in antibiotic activity but that differs in mode of bacterial killing (Imler and Bulet 2005). The antibiotic mechanisms employed by peptides typically are mechanistically simple and the peptides are generally produced in abundance. It therefore may be difficult for bacteria to evolve resistance to even one antimicrobial peptide family, let alone all peptides simultaneously. Minor variations in *cis* transcriptional regulation or antibiotic activity of individual peptides may be effectively neutral with respect to overall host immunocompetence. Second, because peptides are downstream targets of immune signaling and do not provide feedback into the global induction of the immune response, the effects of minor differences in peptide function are not expected to be amplified through the whole of the immune response as effects of functional polymorphism in a transcription factor or signaling protein might.

The replication of a previous association study (Lazzaro *et al.* 2004) as one component of this work provides an unusual opportunity to evaluate the repeatability of quantitative genetic experiments. Statistical power is reduced in the present experiment due to the smaller sample size, but there are a small number of markers whose effects are repeated to varying degrees across experiments (see results). Notably, variability encompassing intron 2 of *SR-CII* was associated with resistance to *S. marcescens* in both studies. There are also, however, some key differences in findings. In the previously published experiment, polymorphism in the intracellular signaling molecules *imd*, *ik2*, *cactus*, and *DIF* was highly significantly associated with variation in the ability to suppress growth of *S. marcescens.* Additionally, there was considerable epistatic interaction among these genes and between these genes and those encoding antibacterial peptides. None of these genes contributed significantly to variation in resistance to *S. marcescens* in this study, however, and the strong epistatic interactions detected in the previous experiment were not recovered in the present one.

Quantitative genetic experiments have often proven difficult to replicate. In Drosophila, for instance, the genetic factors determining the number of neurogenic bristles have been extensively mapped in laboratory settings (reviewed in Mackay and Lyman 2005). The results of several of these laboratory studies failed to be

validated in field settings, despite ample statistical power to do so (Genissel *et al.* 2004; Macdonald and Long 2004; Macdonald *et al.* 2005). Experimental determination of the genetic basis for *D. melanogaster* wing shape has been more replicable, but still imperfect (Palsson *et al.* 2005). Replication of quantitative genetic findings may commonly fail if the original and validation samples differ in their genetic composition (such that the genetic basis for variation in the trait is genuinely different), if environmental conditions are different between studies (influencing the total phenotypic variance or causing substantial differences in genotype × environment interactions), or if statistical power to detect effects is low in either experiment or in both experiments (high type I error). In our study, real biological differences in the physiology of resistance to different bacteria combined with heterogeneity in statistical power may be sufficient to account for the differences we observe across pathogens in genotype–phenotype associations. The differences between the current and previously published experiments on resistance to *S. marcescens* cannot be explained so simply. Because the same *D. melanogaster* lines and the same strain of *S. marcescens* were used in both studies, there is no genetic heterogeneity between the experiments. Both experiments were performed under standardized laboratory conditions, but the two experiments were executed years apart at two different academic institutions, which could introduce environmental differences. One such difference is the medium on which the flies were reared and maintained. The Drosophila medium prepared in the Cornell core facility (this study) is considerably richer that that utilized at Penn State (previous study), a difference that is readily apparent in the developmental time and fecundity of the flies (our unpublished observations). Nutritional state has previously been shown to play a role in the quality of immune response in Drosophila and other insects (*e.g.*, Azambuja *et al.* 1997; Suwanchaichinda and Paskewitz 1998; Vass and Nappi 1998; Koella and Sorense 2002; McKean and Nunney 2005) and may influence the genetic basis for variation in immunocompetence. By assaying the flies in nutrient-rich conditions, we may have inadvertently emphasized genetic differences in resource allocation and development, whereas the comparatively nutrient-poor conditions may have sensitized the previous assay to subtle differences in direct immune function. A variety of other microenvironmental variables may also be involved. Nevertheless, the consistency in allelic effects across the two experiments (Figure 6) suggests that most of the difference in the attainment of statistical significance results from differences in power between the two studies, a probable result of the reduced sample size and shift in the phenotypic distribution toward high loads in this work.

Overall, our data demonstrate that the quantitative genetic basis of *D. melanogaster* antibacterial defense is complex and variable across infecting pathogens. This result, while not surprising, suggests that adaptive evolution in the Drosophila antibacterial immune system may be complicated by genotype × environment interactions and heterogeneity in prevalence of different pathogenic bacteria in time and space. It is clear, however, that substantial and potentially selectable genetic variation exists for antibacterial immune competence in natural populations of *D. melanogaster.* While association studies such as this can implicate genes carrying functional variation in natural populations, the actual mechanistic basis for variation in resistance remains to be determined. It will be of future interest to identify these mechanisms and to explore why variation is allowed to persist in a trait as seemingly critical to fitness as immune capacity.

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