Porphyromonas gingivalis Virulence in a *Drosophila melanogaster* Model[▽]

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Porphyromonas gingivalis has been implicated in the etiology of adult periodontitis. In this study, we examined the viability of *Drosophila melanogaster* as a new model for examining *P. gingivalis*-host interactions. *P. gingivalis* (W83) infection of *Drosophila* resulted in a systemic infection that killed in a dose-dependent manner. Differences in the virulence of several clinically prevalent *P. gingivalis* strains were observed in the *Drosophila* killing model, and the results correlated well with studies in mammalian infection models and human epidemiologic studies. *P. gingivalis* pathobiology in *Drosophila* did not result from uncontrolled growth of the bacterium in the *Drosophila* hemolymph (blood) or overt damage to *Drosophila* tissues. *P. gingivalis* killing of *Drosophila* was multifactorial, involving several bacterial factors that are also involved in virulence in mammals. The results from this study suggest that many aspects of *P. gingivalis* pathogenesis in mammals are conserved in *Drosophila*, and thus the *Drosophila* killing model should be useful for characterizing *P. gingivalis*-host interactions and, potentially, polymicrobe-host interactions.

Porphyromonas gingivalis is a Gram-negative, obligate anaerobe that has been strongly implicated in the etiology of adult (chronic) periodontitis (21, 29), a destructive disease that affects the gums and supporting structures of the teeth. *P. gingivalis*-host interactions have previously been studied using several animal models, the most common of which are murine models (5, 7, 18, 20, 28), including an abscess model (39), a subcutaneous chamber model (24), and a periodontal bone loss model (41). Studies performed using murine models have demonstrated that *P. gingivalis* strains vary in their ability to cause periodontal bone loss (20, 40) and soft tissue destruction and death (28, 43, 56) and that strain W83 is highly virulent relative to many other strains of *P. gingivalis* (28, 43, 56). Murine models have also been used to identify *P. gingivalis* components that are important for pathogenesis (25, 43, 51, 59) and to characterize the host response to *P. gingivalis* infection (6, 11, 31, 35).

The fruit fly, *Drosophila melanogaster*, has been well established as a nonmammalian model for studying host-pathogen interactions (1, 17, 52, 55, 63). *Drosophila* relies solely on an innate immune response to combat invading microbes, and this immune response strongly parallels the mammalian innate immune response (47, 48). Like the mammalian innate immune response, *Drosophila* uses pattern recognition receptors to detect conserved microbial motifs on invading microbes, and the flies activate an immune response that is specific for the type of invading microbe. The absence of an adaptive immune response makes *Drosophila* useful for studying the interactions between microbes and the host innate immune response, in isolation. Numerous tools exist for the genetic manipulation of *Drosophila*, and these have been used to generate thousands of transgenic and mutant lines, including lines useful for identi-

fying host factors that promote or fight infection (9, 52). The *Drosophila* genome has been sequenced, which has facilitated the development of microarray, proteomic, and RNA interference (RNAi) technologies for genome-wide analysis of *Drosophila* processes (14, 23, 65, 66). Additionally, the relative affordability, short generation time (10 to 14 days), and ease of use allow sample sizes that are large enough to permit statistical analysis of the data and make *Drosophila* an attractive complement to mammalian models.

The *Drosophila* killing model has been successfully used to characterize host-microbe interactions of bacterial (9, 13, 15, 17, 22, 52, 55), fungal (1, 3), and parasitic (62) pathogens, and the results of these studies suggest that there is good correlation between pathogenesis in mammals and in nonmammalian animals such as *Drosophila*. In these studies the microbe of interest is either fed to *Drosophila* or introduced directly into the hemocoel (body cavity) through the thorax by using a needle, and the survival of the infected animals is monitored over time.

P. gingivalis grows optimally at 37°C, and in contrast to other bacteria studied using the *Drosophila* model, it (and other oral pathogens) is an obligate anaerobe. It was therefore unclear whether this model would be viable for studying *P. gingivalis*-host interactions. The objective of the current study was to determine the viability of the *Drosophila* killing model for studying *P. gingivalis*-host interactions. We demonstrate that *P. gingivalis* is pathogenic in *Drosophila*, killing the animals in a dose-dependent manner. *P. gingivalis* killing of *Drosophila* was not due to uncontrolled bacterial growth or overt damage to *Drosophila* tissues. Heat-killed and live *P. gingivalis* microorganisms were equally pathogenic in *Drosophila*, which suggests that *P. gingivalis* cell surface components and *Drosophila* immune responses play important roles in causing pathology in this model. Differences in the virulence of several clinically prevalent *P. gingivalis* strains were observed using the *Drosophila* killing model and correlated well with studies in mammalian infection models (24, 28, 43, 56) and human epidemiologic studies (30). Additionally, multiple *P. gingivalis* components

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TABLE 1. Bacterial and *Drosophila* strains used in this study

Strain or stock	Description or genotype	Source
P. gingivalis strains		
W83	Lab strain, originally isolated from an oral infection	Margaret Duncan
ATCC 49417	Lab strain, originally isolated from an oral infection	Joseph Zambon
381	Lab strain, originally isolated from an oral infection	Joseph Zambon
ATCC 33277	Lab strain, originally isolated from an oral infection	Joseph Zambon
A7A1	Lab strain, originally isolated from an oral infection	Joseph Zambon
23A4	Lab strain, originally isolated from an oral infection	Mike Curtis
W50	Lab strain; renamed W50UK for these studies; wt for E8 and GPC	Mike Curtis
E8	*rgpA rgpB* double mutant (arginine-specific protease)	Mike Curtis
GPC	Capsule mutant	Mike Curtis
W83 2741	Lab strain, renamed W83VA for these studies; wt for V2577	Janina Lewis
V2577	*kgp* mutant (lysine-specific protease)	Janina Lewis
ATCC 33277	Lab strain, renamed 33277FL for these studies; wt for YPF1 and SMF1	Richard Lamont
YPF1	*fimA* mutant (major fimbriae)	Richard Lamont
SMF1	*mfa1* mutant (minor fimbriae)	Richard Lamont
W83	Lab strain, renamed W83JP for these studies; wt for M1217	Koji Nakayama
M1217	*mgl* mutant (L-methionine-α-deamino-γ-mercaptomethane-lyase)	Koji Nakayama
P. aeruginosa PA01	Lab strain	Neil Baker
E. coli DH5α		Invitrogen
D. melanogaster Canton S	Wild type	Amanda Simcox

that are involved in virulence in mammals were found to be involved in *Drosophila* killing.

MATERIALS AND METHODS

Bacterial and *Drosophila* strains and growth conditions. Bacterial and *Drosophila* strains used in this study are described in Table 1. *P. gingivalis* strains were grown on brucella blood agar (BBA; Anaerobe Systems), at 37°C in an anaerobic chamber (85% N_2, 10% H_2, 5% CO_2). *Drosophila* stocks were maintained and propagated at 26°C in standard culture vials containing corn flour-molasses medium. Only 3- to 5-day-old female flies were used in experiments.

Infection of adult female *Drosophila*. Bacterial strains were grown in 40 ml of Trypticase soy broth (TSB) for 24 h at 37°C. *Pseudomonas aeruginosa* and *Escherichia coli* were grown aerobically with shaking. *P. gingivalis* was supplemented with hemin (5 μg/ml) and vitamin K (1 μg/ml) and incubated anaerobically. The bacteria were harvested by centrifugation at 4,000 rpm for 8 min and diluted in TSB to the following optical densities at 600 nm (OD_{600}; unless otherwise noted): *P. aeruginosa*, 0.7; *E. coli* DH5α, 2.0 (1×10^{11} CFU ml^{-1}); *P. gingivalis*, 2.0 (1.09×10^{11} CFU ml^{-1}). For heat killing, *P. gingivalis* was incubated at 60°C in a water bath for 1 h, and an aliquot of the preparation was plated on BBA to confirm loss of viability. The bacteria were introduced into the hemocoel (body cavity) of CO_2-anesthetized *Drosophila* through the thorax, using a 30-gauge needle dipped into 500 μl of bacterial culture, or sterile TSB was used for mock infections (vector controls [VC]). *Drosophila* flies were returned to culture vials, and the number of surviving animals at time point 0 h was recorded. Infected *Drosophila* flies were incubated at 30°C, and the number of dead flies was recorded every 12 h for 7 days (unless otherwise noted). All experiments were repeated.

FITC labeling of *P. gingivalis* and microscopy. *P. gingivalis* strain W83 was grown in TSB and harvested as described above. The bacteria were washed once in phosphate-buffered saline (PBS, pH 7.4) and diluted in PBS to an OD of 2.0. A 0.2-ml volume of fluorescein isothiocyanate (FITC) solution (2 mg/ml) was added to 0.4 ml of the resuspended bacteria, and the mixture was incubated at room temperature in the dark with occasional vortexing for 30 min. After incubation with FITC, the bacteria were pelleted, washed two times with PBS to remove unbound FITC, and resuspended in 0.4 ml of PBS. Adult female *Drosophila* flies were infected as described above. Infected animals were killed 2 h postinfection (exposed to −20°C for 5 min) and immediately visualized using a Nikon Eclipse E600 microscope equipped with a FITC/Texas Red filter set. Bright-field and fluorescent images were taken of representative animals by using a Nikon DXM 1200 digital camera.

P. gingivalis growth within infected *Drosophila* animals. Thirty-nine *Drosophila* flies per group were infected (as described above) with the following *P. gingivalis* strains: W83, ATCC 49417, 381, ATCC 33277, A7A1, and 23A4. Three animals per group were ground in 80 μl of PBS with a Teflon pestle at the following time

points (in hours) postinfection: 0, 4, 8, 12, 24, 36, 48, 60, 72, 84, 96, 108, and 120. Viable *P. gingivalis* cell counts were determined on BBA plates containing gentamicin (8 μg/ml), amphotericin B (3 μg/ml), and vancomycin (1 μg/ml). The experiment was repeated, and the results were averaged.

Statistical methods. (i) Sample size. Power calculations based on pilot data estimated that a sample size of 136 *Drosophila* flies per group would be sufficient to detect a relative risk of mortality (RR) of at least 2.0 at an α-level of 0.05 with 90% power when comparing different infections. A sample size of 150 animals per group was used unless otherwise noted. Depending on the number of experimental groups involved, each experiment was divided into three, four, or five parts for feasibility.

(ii) Data analysis. Survival data were analyzed using the SAS statistical software package (SAS Institute, Cary, NC). A Cox proportional hazards (P-H) model was fitted to the survival data. Likelihood ratio tests were performed, and RR values were obtained from the fitted Cox P-H model and adjusted for the individual "experiments" and "parts." RR values with *P* values of <0.05 were considered significant.

RESULTS

P. gingivalis pathogenesis in the *Drosophila* killing model. To determine whether *P. gingivalis* is pathogenic in *Drosophila*, groups of adult female *Drosophila* flies were infected with *P. gingivalis* strain W83, a *P. aeruginosa* strain (PA01) that was previously shown to be highly pathogenic (19), an *E. coli* strain (DH5α) that was previously shown to be nonpathogenic (15), or mock infected. The survival curves of infected and mock-infected *Drosophila* are shown in Fig. 1. *Drosophila* animals infected with *P. gingivalis* strain W83 were significantly more likely to die than mock-infected animals (RR for W83 versus mock, 3.58; *P* = 0.0038) and *E. coli* DH5α-infected animals (RR for W83 versus DH5α, 2.78; *P* = 0.01) but significantly less likely to die than *P. aeruginosa*-infected animals (RR for W83 versus PA01, 0.39; *P* = 0.0012).

To determine whether *P. gingivalis* kills *Drosophila* in a dose-dependent manner, groups of *Drosophila* flies were infected with increasing concentrations of *P. gingivalis* strain W83. The survival curves of *Drosophila* flies infected with different amounts of strain W83 are shown in Fig. 2. An increase in the amount of *P. gingivalis* that was inoculated into *Drosophila*
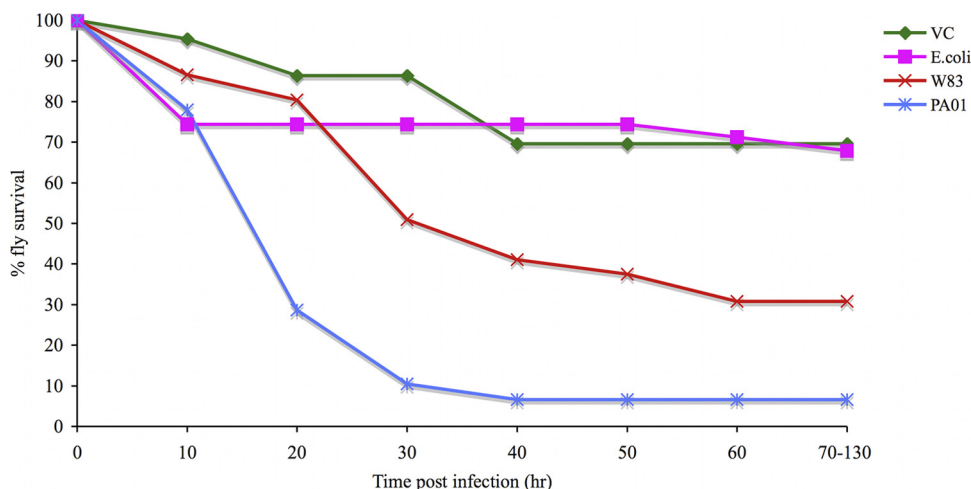
FIG. 1. Survival curves of adult, female *Drosophila* flies infected with *P. gingivalis* and other bacterial species. Eighteen *Drosophila* flies per group were infected with *P. gingivalis* (W83), *P. aeruginosa* (PAO1), or *E. coli* DH5α or mock infected, and the number of dead animals was recorded every 10 h for a period of 130 h. The experiment was repeated for a total sample size of 36 animals per group.

resulted in an increase in the number of animals that were killed during the time course of the experiment.

To determine whether *P. gingivalis* spreads systemically once introduced into *Drosophila*, animals were infected with FITC-labeled strain W83 or mock infected and examined using fluorescence microscopy 2 h postinfection. As there were no fluorescent *P. gingivalis* reference strains, FITC labeling was used. Representative bright-field and FITC images of *P. gingivalis*- and mock-infected animals are shown in Fig. 3. By 2 h postinfection strong FITC fluorescence was observed at the site of inoculation (white arrows), and the fluorescent bacteria were detected throughout the abdomen of the animals (Fig. 3B and D, gray arrows), as has been observed with other bacterial infections in *Drosophila* (22, 55). This was not surprising given the open circulatory system of *Drosophila* and the direct inoculation of the bacteria into the hemocoel. FITC fluorescence was not observed in mock-infected animals (Fig. 3F and H).

Virulence of *P. gingivalis* strains in the *Drosophila* killing model. To compare the virulence of several clinically prevalent *P. gingivalis* strains, *Drosophila* animals were infected with strains W83, ATCC 49417, ATCC 33277, 381, A7A1, or 23A4 or mock infected. The *P. gingivalis* strains that were used in this experiment are commonly used lab strains that were originally isolated from individuals with oral infections, and they were selected for this study because they were identified in previous epidemiologic studies as being prevalent in human populations (30, 49). The survival curves for animals infected with each of the *P. gingivalis* test strains are shown in Fig. 4. The RR values for pairwise comparisons of the *P. gingivalis* strain infections are shown in Table 2. Based on the RR values, *Drosophila* animals were significantly more likely to die from infection with any of the *P. gingivalis* test strains than from a mock infection. *Drosophila* animals were significantly more likely to die from a W83 infection than from an infection with any of the
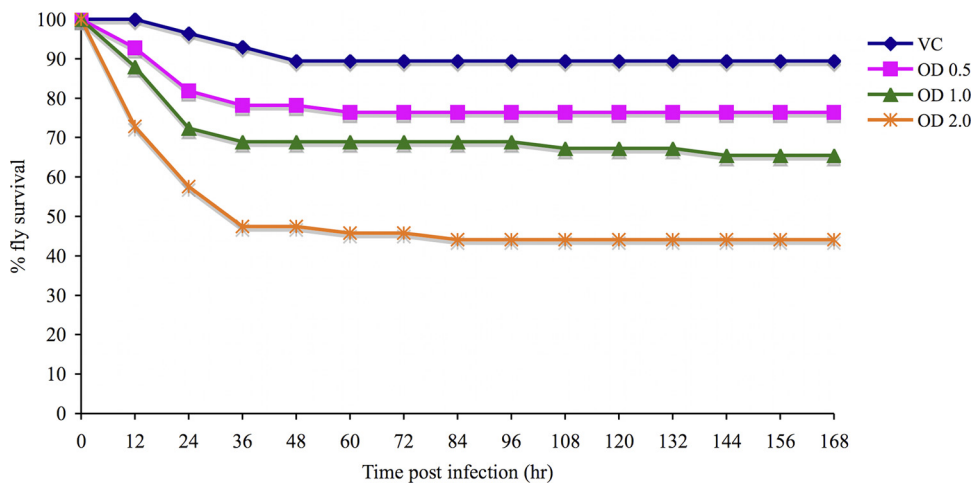

FIG. 2. Survival curves of adult female *Drosophila* flies infected with different amounts of *P. gingivalis* (W83). Thirty *Drosophila* flies per group were infected with strain W83 at an OD_{600} of 0.5, 1.0, or 2.0 or were mock infected. The experiment was repeated for a total sample size of 60 animals per group.
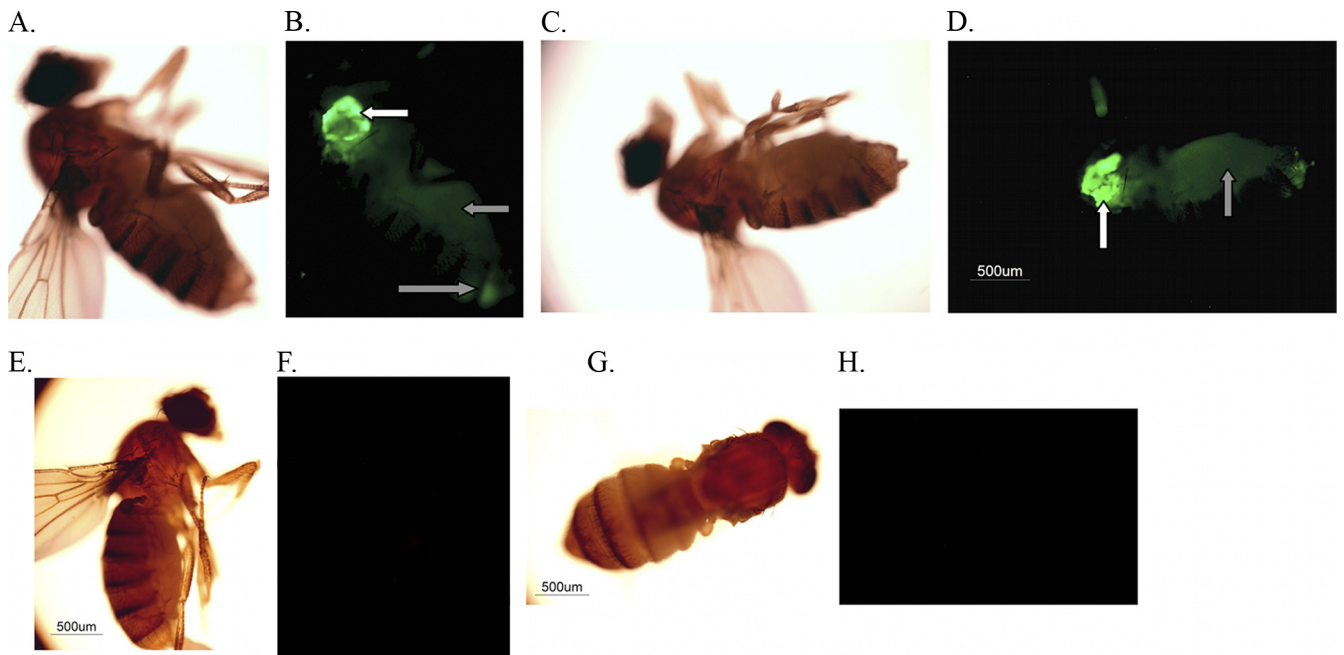
FIG. 3. Location of FITC-labeled *P. gingivalis* within infected *Drosophila* animals. *Drosophila* animals were infected with FITC-labeled *P. gingivalis* strain W83 or mock infected and observed 2 h postinfection by using bright-field and fluorescence microscopy. (A to D) *P. gingivalis*-infected animals; (E to H) mock-infected flies. White arrows indicate sites of septic injury in the thorax. Gray arrows indicate FITC-labeled *P. gingivalis* within the abdomens of the infected animals.

other *P. gingivalis* test strains, and they were also more likely to die from an A7A1 infection than from a 23A4 infection.

To examine the growth of *P. gingivalis* strains within infected *Drosophila*, colony counts were used to determine the level of viable *P. gingivalis* present in infected animals at specific time points postinfection. *P. gingivalis* colony-forming units that were recovered per animal at several time points are shown in Fig. 5. Based on the number of bacteria recovered immediately after infection (time point zero), between 5.10×10^3 CFU and 3.17×10^4 CFU of *P. gingivalis* were inoculated into the ani-

mals. Although small increases in the amount of *P. gingivalis* recovered per *Drosophila* were sometimes observed, there was an overall downward trend in the amount of bacteria recovered from the animals over time. A similar trend was observed when real-time PCR was used to quantitate *P. gingivalis* DNA within the animals (data not shown). Significant numbers of viable *P. gingivalis* could persist in the animals postinfection, which was not surprising, as the bacterium exhibits a high degree of aerotolerance and shows no loss of viability after exposure to air for at least 5 h (54). Viable *P. gingivalis* could be recovered
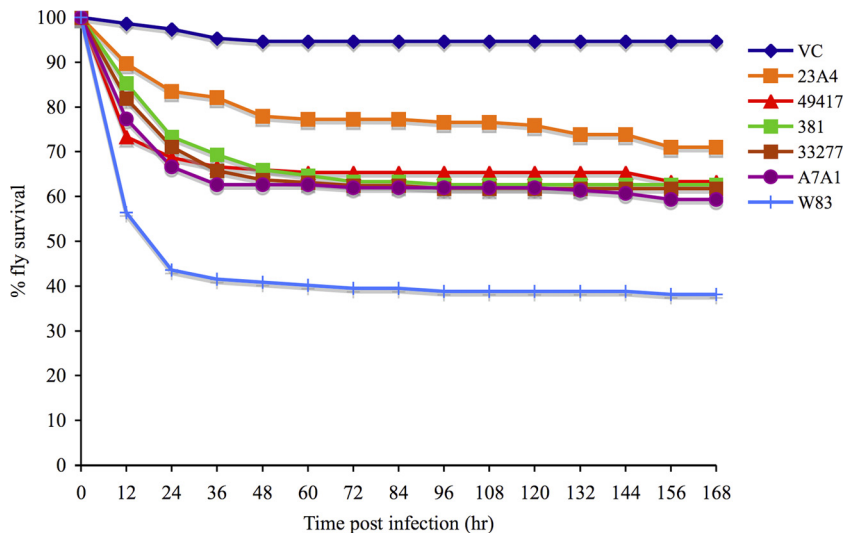

FIG. 4. Survival curves of adult female *Drosophila* flies infected with several *P. gingivalis* strains. *Drosophila* animals were infected with *P. gingivalis* strain W83, ATCC 49417, ATCC 33277, 381, A7A1, or 23A4 or were mock infected.

TABLE 2. Relative risk of mortality for pairwise comparisons of *P. gingivalis* infections in *Drosophila*[a]

Infection	RR of mortality (*P* value) for pairwise comparison					
	W83	A7A1	ATCC 33277	ATCC 49417	381	23A4
A7A1	**1.7 (0.0014)**					
ATCC 33277	**1.86 (0.0002)**	1.10 (0.621)				
ATCC 49417	**1.93 (0.0001)**	1.13 (0.503)	1.03 (0.859)			
381	**1.93 (0.0001)**	1.13 (0.498)	1.04 (0.855)	1.00 (0.996)		
23A4	**2.62 (<0.0001)**	**1.54 (0.031)**	1.41 (0.093)	1.36 (0.133)	1.36 (0.133)	
VC[b]	**15.81 (<0.0001)**	**9.30 (<0.0001)**	**8.49 (<0.0001)**	**8.21 (<0.0001)**	**8.21 (<0.0001)**	**6.04 (<0.0001)**

[a] Values are for pairwise comparisons of infections with groups listed horizontally versus those listed vertically. Results are shown in bold when *P* was <0.05.
[b] VC, mock-infected animals.

from the *Drosophila* up to 24 h postinfection for strain 23A4, up to 48 h postinfection for strain ATCC 49417, and up to 60 h postinfection for strains W83, 381, ATCC 33277, and A7A1.

To investigate the mechanism of *P. gingivalis* killing of *Drosophila*, the effect of *P. gingivalis* infection on the viability of *Drosophila* tissues was examined, and the survival of animals infected with live versus heat-killed bacteria was compared. Animals infected with strain W83 were dissected, and the viability of their tissues was assessed using the vital dye trypan blue and a LIVE/DEAD cytotoxicity assay. No gross differences in tissue integrity were observed between *P. gingivalis*- and mock-infected animals (data not shown). W83 was heat killed at 60°C for 1 h, which also inactivates the proteases (gingipains) (64). When the survival rates of *Drosophila* animals infected with live and heat-killed W83 were compared (Fig. 6), no differences were observed in the survival of the two groups of animals.

Effects of *P. gingivalis* putative virulence factor gene knockouts on *Drosophila* survival. To determine whether similar mechanisms are involved in *P. gingivalis* virulence in mammals and in *Drosophila* and whether multiple *P. gingivalis* factors are

involved in *Drosophila* killing, the virulence levels of the following *P. gingivalis* mutants were compared to their wild-type (wt) parental strains using the *Drosophila* model: *rgpA rgpB* deficient (arginine-specific proteases), *kgp* deficient (lysine-specific protease), *fimA* deficient (fimbrillin, major fimbriae), *mfa1* deficient (minor fimbriae), capsule deficient, and *mgl*-deficient (L-methione-α-deamino-γ-mercaptomethane-lyase) (Table 1). Groups of wt *Drosophila* were infected with the *P. gingivalis* mutant strains, the corresponding wt strains, or mock infected, and the survival rates of the infected animals were compared.

Survival curves for wt *Drosophila* infected with the wt or mutant *P. gingivalis* strains are shown in Fig. 7. The RR values for pairwise comparisons of infections with wt versus mutant strains of *P. gingivalis* are shown in Table 3. An RR value greater than 1 indicates that *Drosophila* animals were more likely to die from infection with the wt *P. gingivalis* strain than from an infection with the mutant strain. *Drosophila* animals infected with *P. gingivalis rgpA rgpB*, *kgp*, *fimA*, *mfa1*, and capsule mutants were less likely to die than *Drosophila* animals infected with the corresponding wt strains (Fig. 7A to E and
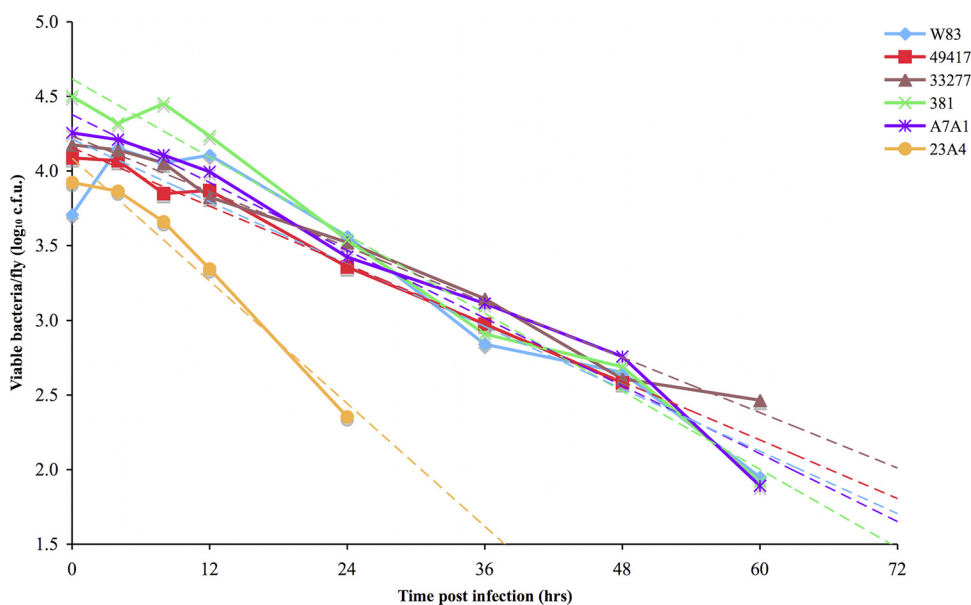

FIG. 5. Growth of *P. gingivalis* in adult female *Drosophila* flies. *Drosophila* flies were infected with *P. gingivalis* strain W83, ATCC 49417, ATCC 33277, 381, A7A1, or 23A4, and viable cell counts were determined on BBA. Each data point represents the $\log_{10}$ value of the average CFU per animal recovered at a specific time point postinfection. Trend lines are shown as dashed lines.
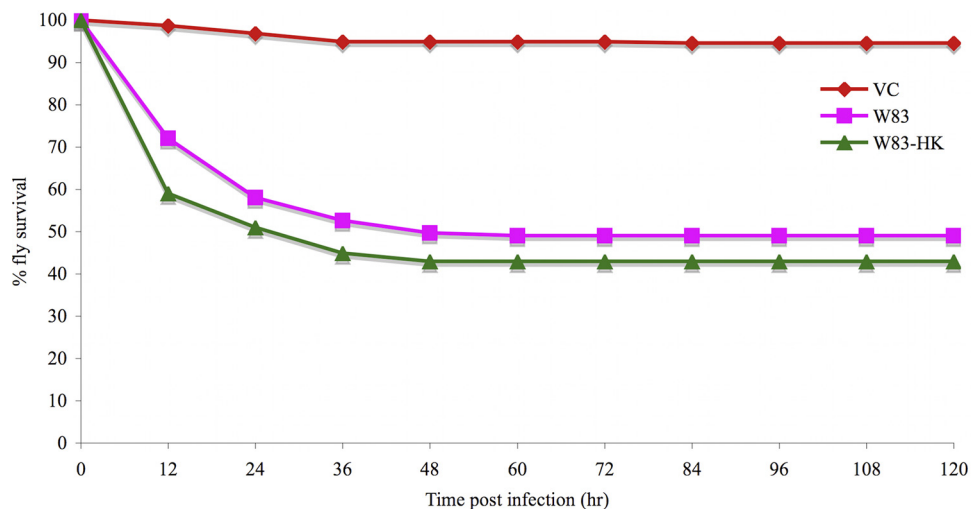
FIG. 6. Survival curves of adult female *Drosophila* flies infected with live or heat-killed (HK; by exposure to 60°C for 1 h) *P. gingivalis* strain W83.

Table 3). *Drosophila* flies infected with the *P. gingivalis mgl* mutant were as likely to die as *Drosophila* flies infected with the wt strain (Fig. 7F and Table 3). The experiments were repeated using a 1:2 dilution of each wt and mutant strain, and similar results were observed (data not shown).

DISCUSSION

The goal of this study was to examine the viability of *Drosophila melanogaster* as a model for examining *P. gingivalis*-host interactions. This model has been used to examine other bacteria-host interactions; however, *P. gingivalis* and other oral pathogens are obligate anaerobes and grow optimally at 37°C. The first step was to determine whether *P. gingivalis* was pathogenic in *Drosophila*. When the survival of animals infected with *P. gingivalis* (represented by strain W83) was compared to the survival of animals infected with a known pathogen (*P. aeruginosa* PA01) (19) and of a nonpathogen (*E. coli* DH5α) (15) for *Drosophila*, the *P. gingivalis*-infected animals were significantly more likely to die than *E. coli* DH5α-infected animals, although they were significantly less likely to die than *P. aeruginosa*-infected animals. A dose response was observed for *P. gingivalis* killing of *Drosophila* (Fig. 2), indicating that the bacteria could be more pathogenic at higher infective doses; however, at higher concentrations (ODs greater than 2.0) the bacteria clumped on the needle, making the delivery of higher doses unfeasible.

All *P. gingivalis* test strains were pathogenic to some degree in the *Drosophila* killing model (Table 2). Strain W83 was significantly more pathogenic than the five other *P. gingivalis* strains in this model, which correlates with the observations from studies using rodent models, in which W83 was also more pathogenic than the five other *P. gingivalis* strains (24, 28, 43, 56). W83 is also the strain that is most strongly associated with human periodontitis (30). These results suggest that there is a correlation between *P. gingivalis* pathogenesis in mammals and in *Drosophila*.

P. gingivalis is normally exposed to temperatures that range from 37°C to 38.8°C in the oral cavity (2) and grows optimally at 37°C *in vitro*, and unlike other bacteria studied using the *Drosophila* model, *P. gingivalis* is an obligate anaerobe. Thus, we determined whether *P. gingivalis* could grow in *Drosophila*. Colony counts were used to examine the growth of the *P. gingivalis* strains in the animals, and *P. gingivalis* DNA recovered from infected *Drosophila* was measured by real-time PCR. The results from the growth experiments indicated that although *P. gingivalis* does not multiply effectively in the *Drosophila* body cavity, the bacterium can persist for up to 60 h postinoculation in the animals. The aerobic environment of the *Drosophila* hemolymph and the temperature at which the infected animals are incubated are the likely factors that prevented the bacterium from growing optimally. The infected animals were incubated at 30°C, as their physiological processes deteriorate at temperatures higher than 30°C (42). When W83 growth at 30°C and 37°C was compared, the bacterium grew poorly at 30°C on solid medium (BBA) and in TSB (data not shown).

The six *P. gingivalis* factors tested for a role in *Drosophila* killing have been demonstrated to contribute to the bacterium's virulence in mammals (43, 59, 70, 72). In the current study, five of the components were shown to contribute to *P. gingivalis* virulence in the *Drosophila* model. The gingipains are proteases encoded by *rgpA* (arginine-specific protease), *rgpB* (arginine-specific protease), and *kgp* (lysine-specific protease) genes. They are considered to be major *P. gingivalis* virulence factors due to their ability to degrade a large variety of host proteins (reviewed in references 37, 58, and 60), and they are important to various degrees for pathogenesis in the mouse abscess model of infection (59). *P. gingivalis* major fimbriae (*fimA*) are involved in the invasion of gingival epithelial cells (71), and both major and minor (*mfa1*) fimbriae are important for *P. gingivalis* autoaggregation (50, 57) and biofilm formation with *Streptococcus gordonii* (44, 45). *mfa1* and *fimA* mutants are impaired in the ability to induce periodontal bone loss in a rat model of infection (70). The capsule, which is present on some strains of *P. gingivalis*, is important for virulence in the mouse abscess model of infection, as infection with encapsu-
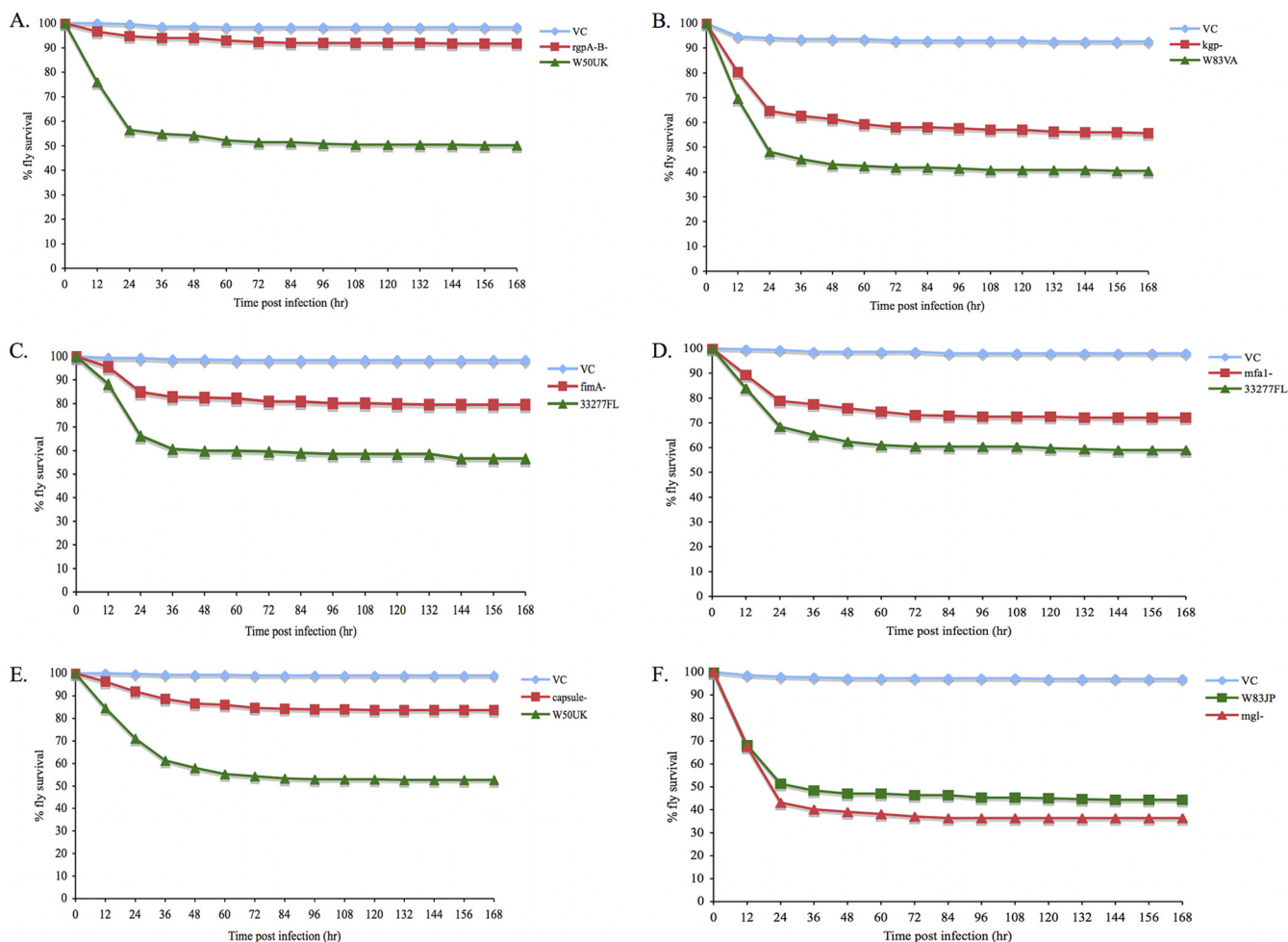
FIG. 7. Survival curves of wt *Drosophila* animals infected with wt or mutant strains of *P. gingivalis*. Green curves, wt-infected animals; red curves, mutant-infected animals; blue curves, mock-infected animals. (A) Survival of *Drosophila* flies infected with strain W50UK (wt) or an *rgpA rgpB* (arginine-specific proteases) mutant or mock infected (VC). (B) Survival of *Drosophila* flies infected with strain W83VA (wt), a *kgp* (lysine-specific protease) mutant, or mock infected (VC). (C) Survival of *Drosophila* flies infected with strain 33277FL (wt), a *fimA* (major fimbriae) mutant, or mock infected (VC). (D) Survival of *Drosophila* flies infected with strain 33277FL (wt), an *mfa1* (minor fimbriae) mutant, or mock infected (VC). (E) Survival of *Drosophila* flies infected with strain W50UK (wt), a capsule-deficient mutant, or mock infected (VC). (F) Survival of *Drosophila* flies infected with strain W83JP (wt), an *mgl* (L-methionine-α-deamino-γ-mercaptomethane-lyase) mutant, or mock infected (VC).

lated strains results in more soft tissue destruction and death than infection with unencapsulated strains (43). L-Methione-α-deamino-γ-mercaptomethane-lyase (*mgl*) catalyzes the conversion of L-methionine to the volatile sulfur compound methyl mercaptan (72). Methyl mercaptan is elevated in the mouths

(68) and crevicular air (12) of patients with active periodontitis, and exposure to methyl mercaptan has been shown to decrease DNA (38) and protein (38, 46) synthesis in gingival fibroblasts and to inhibit the migration of periodontal ligament cells (46). In the mouse abscess model, the *mgl* mutant was less

TABLE 3. Relative risk of mortality for pairwise comparisons of wt versus mutant *P. gingivalis* infections in *Drosophila*

P. gingivalis strains		RR (*P* value) for wt vs mutant[a]	Findings in other animal model studies (reference[s]) for:	
Mutant	wt		Periodontal bone loss	Abscess and death
rgpA rgpB mutant (arginine-specific proteases)	W50UK	**7.69 (<0.0001)**	—[b]	Correlation (15)
kgp mutant (lysine-specific protease)	W83VA	**1.63 (<0.0001)**	—	Correlation (15)
fimA mutant (fimbrillin, major fimbriae)	33277FL	**2.13 (<0.0001)**	Correlation (14, 46)	—
mfa1 mutant (minor fimbriae)	33277FL	**1.56 (0.0018)**	Correlation (46)	—
Capsule mutant	W50UK	**2.99 (<0.0001)**	—	—
mgl mutant	W83JP	0.87 (0.2)	—	No correlation (47)

[a] RR values with *P* values of <0.05 are shown in bold.
[b] —, the strains have not been compared in the indicated model.

able to cause soft tissue destruction and death than the wt strain (72). The results of this study demonstrate that several *P. gingivalis* factors (capsule, major and minor fimbriae, and gingipains) that are important for pathogenesis in mammals are also involved in the killing of *Drosophila*, suggesting that there is a strong correlation between *P. gingivalis* pathogenesis in mammals and in *Drosophila*. However, the noninvolvement of the *mgl* gene product in *P. gingivalis* killing of *Drosophila* suggests that the requirement for a particular virulence factor by *P. gingivalis* depends on the host involved. The observed differences in the *Drosophila*-killing abilities of the *P. gingivalis* strains suggest that additional bacterial factors are involved in pathogenesis in this model.

In contrast to other bacteria that have been studied in this model, *P. gingivalis* does not multiply effectively in *Drosophila*, yet the bacterium still kills the animals. Additionally, no gross differences in tissue integrity were observed between infected and mock-infected animals, indicating that the pathobiology of *P. gingivalis* in this model is not due to uncontrolled growth of the bacterium or overt damage to *Drosophila* tissues. Our observation that there was no difference in the survival of *Drosophila* infected with live- versus heat-killed strain W83 (Fig. 6) suggests that the pathology caused by the bacterium in this model is due to direct killing of the animals by a *P. gingivalis* component(s) or indirect killing by the host's own response. Schneider et al. observed that pathogens can kill *Drosophila* via mechanisms that are directly toxic or host mediated (63). The observation that several *P. gingivalis* factors play a role in *Drosophila* killing suggests that killing is multifactorial and not due to a single factor, e.g., a toxin. Host-mediated damage in *Drosophila* can occur either by the hyperactivation of the immune response, or the activation upon infection, of responses that are deleterious to the animals (63). It has been shown that overactivation of the Toll (26) and Imd (8) pathways (major regulators of *Drosophila* immune response genes), excessive production of nitric oxide (10) and reactive oxygen species (32), and the activation of the JNK pathway via Eiger (cytokine) signaling following *Salmonella enterica* serovar Typhimurium (9) or *Mycobacterium marinum* (63) infection results in *Drosophila* lethality. The molecular mechanisms by which these immune responses result in *Drosophila* lethality are currently unknown; however, Schneider et al. and others have suggested that they may be either energetically wasteful or directly toxic (26, 63). *P. gingivalis* major and minor fimbriae, the capsule, and gingipains have been demonstrated to be proinflammatory in mammals (16, 33, 34, 69), and it is known that the bacterium releases outer membrane vesicles (27, 53) that allow pathogen-associated molecular patterns (PAMPs) to be detected at sites distant from the bacterium. As *P. gingivalis* spreads systemically when inoculated into *Drosophila*, PAMPs present in cell surface-associated structures like fimbriae, gingipains, and capsular polysaccharide may induce a systemic hyperactivation of *Drosophila* immune responses, which is deleterious to the bacterium but also harms the animals. Although not systemic, an exaggerated host immune response is primarily responsible for the damage that occurs in periodontitis (61, 67) and in response to *P. gingivalis* infection in other animal models (4, 11, 35).

Despite the bacterium's inability to multiply effectively in *Drosophila*, *P. gingivalis* was still capable of killing the animals,

and several lines of evidence suggest that *P. gingivalis* killing of *Drosophila* is due to specific interactions between the bacterium and the host. First, *P. gingivalis* (W83)-infected *Drosophila* animals were more likely to die than *E. coli* DH5α-infected *Drosophila* animals (Fig. 1) that received an equivalent inoculum. Second, *P. gingivalis* (W83) killing of *Drosophila* was dose dependent (Fig. 2). Third, strains of *P. gingivalis* exhibited different killing abilities when inoculated into *Drosophila* (Fig. 4). Fourth, several *P. gingivalis* virulence gene mutants are attenuated in the *Drosophila* killing model (Fig. 7). Finally, several immune response-defective *Drosophila* mutants show increased susceptibility to killing by *P. gingivalis* (36).

In this study we have demonstrated that *Drosophila melanogaster* is a viable new animal model for examining *P. gingivalis*-host interactions. The introduction of this highly genetically manipulatable, high-throughput animal model with an innate immune system that is remarkably similar to mammals increases the repertoire of tools available to study *P. gingivalis*-host interactions, especially as the damage that occurs with periodontitis is primarily due to host effects. Although *Drosophila* is not a natural host for *P. gingivalis*, the results of this study suggest that there are many aspects of *P. gingivalis* pathogenesis that are evolutionarily preserved between mammals and *Drosophila*. Therefore, the *Drosophila* killing model could be used to identify *P. gingivalis* factors that are involved in pathogenesis, which is important, as the roles of a large number of *P. gingivalis* genes are unknown. This model could also be used to identify host factors that fight *P. gingivalis* infection or contribute to *P. gingivalis*-induced pathology and to examine the host-pathogen interactions of other oral pathogens. As it is clear that periodontitis is a polymicrobial infection, the *Drosophila* killing model could be exploited to examine the interactions among different oral species and their interactions with host defenses in a mixed bacterial inoculum.

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