Drosophila as a Model Host for Pseudomonas aeruginosa Infection

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Using the fruit fly Drosophila melanogaster as model host, we have identified mutants of the bacterium Pseudomonas aeruginosa with reduced virulence. Strikingly, all strains strongly impaired in fly killing also lacked twitching motility; most such strains had a mutation in pilGHIJKL chpABCDE, a gene cluster known to be required for twitching motility and potentially encoding a signal transduction system. The pil chp genes appear to control the expression of additional virulence factors, however, since the wild-type fly-killing phenotype of a subset of mutants isolated on the basis of their compact colony morphology indicated that twitching motility itself was not required for full virulence in the fly.

The extraordinary versatility of bacteria in the genus Pseudomonas is reflected in the diversity of studies using these organisms: studies of biochemical pathway evolution (27), biodegradation of toxic waste (31), and pathogenesis. There is a pressing need to incorporate the results of all these investigations into an improved understanding of the biology of Pseudomonas aeruginosa, a bacterium so versatile that it is not only a major cause of opportunistic human infection but also virulent toward plants (29, 30), insects (4, 17, 30), and the soil-dwelling nematode worm Caenorhabditis elegans (9, 23). Because of this broad spectrum of virulence, a variety of models that allow efficient screening of bacterial mutants have been developed (18). Recently, for instance, P. aeruginosa genes required for killing the nematode C. elegans have been identified (23, 41; L. A. Gallagher and C. Manoil, unpublished data), as has a worm gene required for sensitivity to killing (9).

Innate immunity in the fruit fly Drosophila melanogaster protects it from overwhelming infection in a life rich in interactions with microorganisms (3). The signal transduction cascade underlying this system is under intense investigation, and these studies have revealed striking similarities to the mammalian innate immune response (2, 16). In both insects and mammals, Toll family receptors signal through Rel family transactivators (2, 16), mediating responses that are specific to different classes of pathogens (22). These responses include secretion of antimicrobial peptides (3, 20). The fruit fly thus may be an especially good model organism to reveal the complex interactions between P. aeruginosa virulence factors and host defenses which underlie human disease such as the chronic respiratory infections of cystic fibrosis patients (15, 36).

P. aeruginosa PAO1 kills fruit flies. Previous studies showed that P. aeruginosa is a particularly virulent pathogen of fruit flies; pricking with a needle dipped into a culture of P. aeruginosa strain α1 is lethal: the bacteria grow exponentially within the fly until and even after the death of the fly (4). This observation suggested that the fruit fly could be used to screen for Pseudomonas mutants with reduced virulence. We therefore tested whether PAO1, the best-characterized strain of P. aeruginosa (38), was also proficient at fly killing.

PAO1 (from the laboratory of B. Iglewski) was grown with 1 mM succinate as the carbon source in M63 minimal medium (35) without the added iron and with 1 mM MgSO4. Cultures were grown for 12 h to stationary phase in a rotary shaker at 37°C. The fruit fly D. melanogaster Canton S was grown with standard cornflour-molasses medium at 25°C. Adult female flies 2 to 4 days old were pricked in the dorsal thorax with a 25-gauge needle dipped directly into the PAO1 culture; the females were then returned to standard fly culture vials with food. Flies generally died 16 to 28 h after infection with wild-type bacteria (Fig. 1) and became noticeably lethargic 1 h before death. Female flies were used since pricking with a sterile needle was consistently harmless while up to 15% of male flies died, presumably because of their smaller size.

Immediately after infection and at various subsequent time points, individual flies were ground with a Teflon pestle in an Eppendorf tube with 100 μl of 10 mM MgSO4 and serial dilutions of the homogenate were spread on Luria-Bertani (LB) agar to determine viable bacterial cell counts. These experiments showed that 400 to 2,000 PAO1 cells were introduced into and onto the fly by pricking and that the flies died when the bacterial titer reached 1 × 106 to 40 × 106 cells. These results are consistent with those of the previous study using a different strain of P. aeruginosa (4).

Screening for P. aeruginosa mutants impaired in fly killing. P. aeruginosa PAO1 was mutagenized using plasmid pUT carrying transposon ISphoA/hah essentially as described for Escherichia coli (24) but with the following modifications: the chloramphenicol resistance gene in the transposable element had been replaced with a tetracycline resistance gene (Gallagher and Manoil, unpublished), mating recipient PAO1 cells were grown in 5 ml of LB broth for 12 h at 42°C without shaking, the mating duration was 30 min, and PAO1 cells carrying a chromosomal insertion of the transposable element were selected on LB agar containing 10 μg of chloramphenicol per ml (to counterselect E. coli) and 60 μg of tetracycline per ml after incubation for 48 h at 30°C. Single colonies were patched to a selective master plate and also used to inoculate 200-μl cul-
Twitching-motility mutants impaired in fly killing. The 22 sequenced twitching-motility mutants also impaired in fly killing (Fig. 2) grew on plates with LB or M63-succinate media at a rate indistinguishable from PAO1 (based again on colony size and time of appearance). Of these 22 mutants, 15 had an insertion in pilGHIJKL chpABCDE (1, 10, 12, 38; C. Whitchurch, M. Young, A. Leech, A. Semmler, and J. Mattick, Pseudomonas 99: Biotechnology and Pathogenesis, abstr. S41, 1999), a cluster of genes most similar to the che genes for flagellum-mediated chemotaxis in E. coli and the frz and dif genes involved in type IV pilus-mediated social gliding motility in Myxococcus xanthus (25, 37, 40, 43, 48). The extent to which each of these 15 insertions affects the expression of genes downstream in the cluster is unknown (11). However, this study has identified multiple gene clusters required for full virulence in the fly (Fig. 2), and for the 15 pil chp mutants, the fly-killing phenotype is typified by that of the chpA37 mutant, with 50% fly killing delayed by approximately 6 h (Fig. 1). This strain was therefore used as a representative in subsequent experiments. An equivalent phenotype was observed for five mutants, each with a different insertion in fimV (Fig. 2), a newly discovered gene required for twitching motility (33). The amino acid sequence of FimV and the effect of FimV overexpression on cell shape suggest a role in peptidoglycan remodeling necessary for pilus biogenesis and function (33).

The remaining two mutants each had an insertion in a locus not described previously in the context of twitching motility: an open reading frame (ORF) that could encode a protein similar to various putative homologs of E. coli TonB (up to 36% amino acid identity over 277 residues) and an ORF that could encode a protein similar to various putative homologs of E. coli ExbD, an inner membrane protein that complexes with TonB for transport of receptor-bound substrates (up to 64% amino acid identity over 145 residues). The tonB-like ORF (designated orf406) is adjacent to the pil chp cluster and immediately upstream of algH (Fig. 2), encoding a global regulator affecting the expression of alginate as well as several quorum-sensing-controlled virulence factors (32); disruption of orf406 caused a delay of several hours in 50% fly killing (data not shown). The exbD-like ORF (designated orf2982) is immediately upstream of the PAO1 homolog of E. coli Ipak (Fig. 2), encoding lipid A 4'-kinase; disruption of this ORF caused a particularly strong phenotype, with 50% killing delayed by approximately 24 h (data not shown). For this mutant, as well as the chpA37 mutant and strains with a mutation in fimV or orf406, the number of viable bacterial cells used to infect flies (determined
with fly homogenates made immediately after infection) varied within the range observed for PAO1 (data not shown).

Twitching-motility mutants not impaired in fly killing. Genes with diverse roles in twitching motility (1) were disrupted in strains not impaired in fly killing (Fig. 2): pilR and fimS are regulatory genes representing one member of each of the two known two-component sensor-regulator pairs; pilQ, pilY1, and pilN are putative structural genes, as are pilW, pilX, and pilE, which encode pilin subunit (PilA)-like proteins; pilT and pilU encode putative nucleotide binding proteins which could mediate pilus retraction (5, 26, 47) as a basis for motility; and the role of pilZ in twitching motility is unknown. pilD encodes the leader peptidase, which processes components not only of the type IV pilus but also of the type II secretion apparatus, whose substrates include the exoenzyme virulence factors phospholipase C, elastase, and exotoxin A (39); it was therefore unexpected that the pilD mutant would be unimpaired in fly killing (Fig. 1). Also surprising was the observation that the pilU mutant had a unique colony morphology: cells grew slightly slower than did wild-type cells on LB plates and formed colonies with a dry wrinkled surface, distinct from the wet smooth surface of colonies of cells of each of the other mutants in this study. Although pilT and pilU encode homologous proteins, previous studies noted a difference in the virulence (7) and phage sensitivity (45) of pilT and pilU mutants.

A chpA mutant kills flies more slowly than PAO1 even though it grows at the same rate within the fly. The pil chp genes have recently been proposed (Whitchurch et al., Pseudomonas ‘99) to function as a signal transduction pathway adding a layer of regulation to PAO1 virulence factor expression. We therefore analyzed the chpA37 mutant in more detail in the fly-killing assay. Individual batches of five flies infected with either strain followed an identical course: the number of viable bacterial cells in fly homogenates decreased over the first 4 h after infection and then increased exponentially, doubling approximately every 1.3 h (Fig. 3). The fact that individual data points, essentially representing separate experiments, consistently indicated the same bacterial growth rate within the fly, testifies to the reproducibility of the assay. Furthermore, although the number of viable bacterial cells in single-fly homogenates varied between 400 and 2,000, this variability was not sufficient to obscure a delay in fly killing; indeed, for the data shown in Fig. 3, the initial difference between the number of viable cells of the chpA37 mutant and of PAO1 was completely eliminated by 16 h after infection. Based on the colony morphology of cells of the chpA37 mutant recovered from the fly, twitching motility was not regained over the course of the infection.
decreased from 3.7 h for wild-type flies (standard error of the mean, 0.62) to 0.8 h for Bc,imd flies (standard error of the mean, 0.73). Although the observed reductions are somewhat subtle, they are nevertheless consistent with ChpA being important for overcoming fly innate immunity.

Parallels between the biology of P. aeruginosa and that of M. xanthus. This study identified P. aeruginosa mutants with reduced virulence toward the fruit fly. All strains strongly impaired in fly killing also lacked twitching motility; most such strains had a mutation in pilGHIJKL chpABCDE, a gene cluster which could encode a signal transduction pathway. An additional set of twitching-motility mutants generated in this study, however, was not impaired in fly killing (these strains were identified by their compact colony morphology, indicating a lack of surface spreading). Therefore, twitching motility itself is not required for full virulence in the fly, in turn suggesting that the pil chp genes control the expression of as yet unknown virulence factors which are the true determinants of fly killing (Fig. 5).

The first description of the pil chp genes in PAO1 (10) emphasized their similarity to genes involved in gliding motility in M. xanthus, a nonflagellated soil bacterium that glides on surfaces as it hunts the bacteria that are its food. Subsequent studies have greatly expanded the parallels between these two organisms: twitching motility in P. aeruginosa has been shown to be genetically and morphologically equivalent to social gliding motility in M. xanthus (34); genes coordinately regulated with each motility include those for biosynthesis of exopolysaccharides, which could facilitate surface spreading (37), alginate for P. aeruginosa (46); cell-to-cell signaling is crucial for each motility in groups of bacterial cells (14, 37), quorum sensing for P. aeruginosa (14); and under stressful environmental conditions, each motility is required to form a structured community of resistant cells (28, 42), a biofilm for P. aeruginosa (8, 13, 28, 44). Incorporating models proposed for M. xanthus (40, 43, 48),
the chemotaxis-like pil chp genes in PAO1 could encode a signal transduction system that controls adaptations for surface growth, adaptations that include twitching motility as well as expression of factors required for full virulence in the fruit fly (Fig. 5).

The pil chp signal transduction system is likely to be important for virulence not only in the fruit fly but also in mammals. This conclusion is supported by a study of P. aeruginosa genes mediating epithelial cell injury (19); part of one such gene was identified as being similar to E. coli cheA, and, using DNA sequences not available at the time of that study, the only exact match to this sequence in the PAO1 genome is chpD. The fruit fly thus may be a particularly good model host to both reveal and characterize new components of P. aeruginosa pathogenicity: this study has shown that bacterial mutants can be efficiently screened in wild-type flies and that individual strains then can be tested in mutant flies with specific defects in an

FIG. 5. Model for the role of the pil chp gene cluster in both twitching motility and virulence in the fruit fly. The P. aeruginosa pilCHIKL chpABCDE gene cluster could encode a signal transduction system and is required for twitching motility mediated by type IV pili (1, 12) and possibly other adaptations for surface growth. Such adaptations appear to include the expression of as yet undetermined virulence factors, since pil chp mutants are impaired in fly killing even though twitching motility is not required for full virulence in the fly.

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