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Daphnia magna, a Host for Evaluation of Bacterial Virulence

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We show that *Daphnia magna* can be used to assess acute virulence of pathogens relevant to human health, such as *Pseudomonas aeruginosa* or *Photorhabdus asymbiotica*. Analysis of bacterial mutants suggests that *P. aeruginosa* uses similar mechanisms to infect *Daphnia* and other hosts.

To evaluate the virulence of bacterial pathogens, a few nonhuman hosts can be used, such as rodents, fish, insects, or amoebae (9). Although mice are sometimes assumed to approximate better the conditions encountered in infected patients, nonmammalian models are often preferred for both practical and ethical reasons (10).

The small planktonic crustaceans of the genus *Daphnia* have been a model system in ecology for centuries (11). *Daphnia magna* can be cloned naturally, but crossing of clones is possible by environmental sex induction. Culturing *Daphnia* is easy and cost-efficient. The transparent body allows monitoring of internal body conditions. More recently, *Daphnia* has been developed as a model host to study interaction with bacterial or fungal parasites (reviewed in reference 6). These studies were however focused on parasites specific for *Daphnia* and incapable of infecting human patients. Surprisingly, virtually no attention has been devoted to the interaction of *Daphnia* with environmental pathogens also capable of mounting infections in human patients. To our knowledge, the only study of this facet of *D. magna* biology is a study of *Bacillus cereus* revealing that this bacterium is toxic to *D. magna* and that expression of *B. cereus* hemolysin II in *Bacillus subtilis* renders it pathogenic for *D. magna* (12).

Virulent *P. aeruginosa* strains cause rapid death of *D. magna*. In order to use *D. magna* to measure bacterial virulence, we exposed *D. magna* strain Xinb3 (7) to bacterial pathogens, and we assessed the strain's survival over a period of up to 36 h. For this, bacteria were cultured for 24 h at 37°C on standard medium (SM) plates (8), collected with a sterile loop, rinsed once with water, resuspended in ADaM buffer (Aachener Daphnien medium: $CaCl_2 \cdot 2H_2O$, 270 mg/liter; $NaHCO_3$, 55 mg/liter; SeO_2, 14 mg/ml; and sea salt [hw-Meersalz; Wiegandt GmbH], 333 mg/liter), and transferred at the indicated concentration in a 1.5-ml Eppendorf tube containing three *D. magna* daphnids (Fig. 1A). Tubes were observed at regular time intervals, to determine *D. magna* viability. Immobile *D. magna* daphnids falling to the bottom of the tube were recorded as dead, after checking that they did not move when the tube was inverted.

Pseudomonas aeruginosa is an environmental Gram-negative bacterium responsible for a wide range of infections, particularly in immunocompromised individuals and cystic fibrosis patients (5). *D. magna* incubated with the PT894 pathogenic strain of *P. aeruginosa* (1) at low concentrations (below an optical density at 600 nm [OD_{600}] of 0.4) all survived after 9 h, while the daphnids all died when exposed to high bacterial concentrations (above an OD_{600} of 3) (Fig. 1B). On the contrary, in the presence of a DP28 nonvirulent *pchH* mutant (1), *D. magna* survived at all bacterial concentrations tested (Fig. 1B). When exposed to a lethal dose of virulent PT894 (OD_{600}, 3) *D. magna* died over a period of 6 h (Fig. 2A). Loss of pyochelin synthesis in a DP32 *pchD* mutant attenuated slightly bacterial virulence (Fig. 2A), as observed previously in a *Dictyostelium* host (1). Similarly, in the presence of wild-type (WT) virulent strain PAO1 (OD_{600}, 3), *D. magna* died within 7 h (Fig. 2B), while death induced by a PT531 *lasR-rhlR* double mutant of PAO1 (3) was slower and less complete (Fig. 2B).

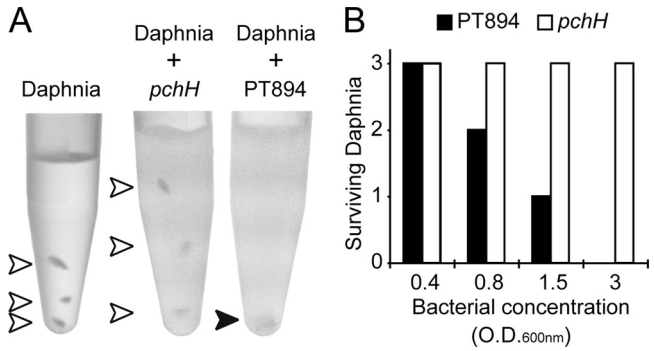

FIG 1 Virulence of *Pseudomonas aeruginosa* against *Daphnia magna*. (A) The ability of *P. aeruginosa* to kill *D. magna* was assessed by incubating three daphnids in 1 ml of ADaM with or without bacteria. Live daphnids swimming actively (white arrowheads) can easily be discriminated from dead immobile daphnids at the bottom of the tube (black arrowhead). Photos of Daphnia incubated for 9 h alone (left), in the presence of PT894 bacteria (right), or in the presence of avirulent *pchH* mutant bacteria (center) are shown. (B) Three daphnids were incubated for 9 h in the presence of various concentrations of PT894 or an avirulent *pchH* mutant. Dose-dependent death was observed at concentrations of PT894 above an OD_{600} of 0.8, while exposure to the avirulent *pchH* mutant did not affect *Daphnia* viability. Here and in the other figures, solid squares are used for virulent bacteria and empty squares for less virulent mutants.

The virulence of *P. aeruginosa* is caused in part by the secretion in the medium of various toxic compounds, such as rhamnolipids or elastase (13). In order to characterize the toxicity of bacterial exoproducts secreted in the culture medium, bacteria were cultured overnight in liquid SM, and then the culture supernatant

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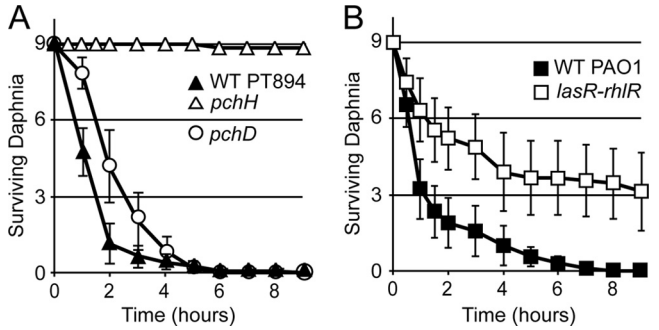

FIG 2 *P. aeruginosa* utilizes conserved virulence traits to kill *D. magna*. (A) Nine daphnids (in 3 tubes) were incubated in the presence of PT894 *P. aeruginosa* or in the presence of the isogenic *pchH* or *pchD* mutants (OD_{600}, 3). Survival was recorded at regular intervals for 9 h. The average and standard error of the mean (SEM) of at least 6 independent experiments are presented. (B) Virulence of the *P. aeruginosa* PAO1 strain and a *lasR rhlR* mutant was tested as described for panel A. The average and SEM of 9 independent experiments are presented. Mutants of *P. aeruginosa* previously described as avirulent in other systems killed *D. magna* less efficiently than the isogenic virulent WT strain.

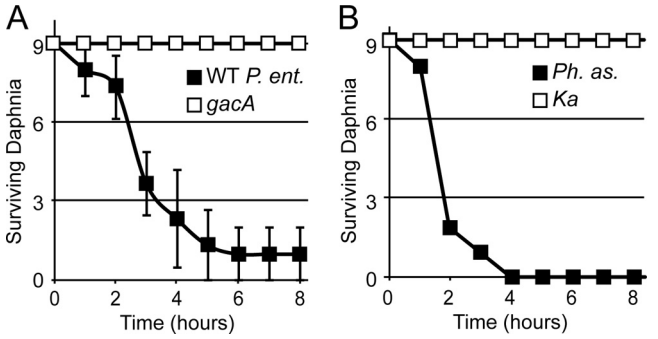

FIG 4 Various environmental bacteria can kill *D. magna*. We tested the abilities of several bacteria to kill *D. magna* as described in the legend to Fig. 2. (A) The *P. entomophila* WT strain (OD_{600}, 3) efficiently killed *D. magna*, while an isogenic *gacA* mutant did not. The average and SEM of 3 independent experiments are presented. (B) *Photorhabdus asymbiotica* (*Ph. as.*) (OD_{600}, 3) also efficiently killed *D. magna*. A laboratory strain of *K. pneumoniae* (*Ka*) failed to affect the viability of *D. magna*.

was recovered by centrifugation and added to *D. magna* at a final dilution of 1 in 4. Consistent with the notion that secreted exoproducts contribute to *P. aeruginosa* virulence, *D. magna* exposed to the supernatants of the two virulent bacteria died rapidly, while exposure to supernatants of avirulent mutants resulted in much less mortality (Fig. 3A and B).

Virulence of other opportunistic pathogens. In order to extend our observations to other pathogens, we tested the virulence of several other environmental pathogenic bacterial. A virulent strain of the entomopathogenic bacterium *Pseudomonas entomophila* (grown at 25°C) rapidly killed *Daphnia*, while a nonvirulent *gacA* mutant (11) did not (Fig. 4A).

Photorhabdus asymbiotica is an environmental Gram-negative bacterium capable of infecting a wide variety of hosts, from insects to humans (4). When exposed to *P. asymbiotica* (isolate Kingscliff), *D. magna* died rapidly (Fig. 4B), indicating that some of the virulence mechanisms of *P. asymbiotica* are effective against *D. magna*. On the contrary, two *Klebsiella pneumoniae* strains (a laboratory strain and a KP52145 strain) (2) failed to cause rapid death of *D. magna* (Fig. 4B) (data not shown), suggesting that the virulence of *K. pneumoniae* cannot be recapitulated in this system.

Overall, this study shows that *Daphnia magna* can be used as a model host with which to study the virulence of several bacterial environmental pathogens also capable of mounting opportunistic infections in humans. *D. magna* is simple to grow and to handle, and its use does not raise ethical or regulatory issues. Infection of *Daphnia* is particularly easy to perform since it only requires *Daphnia* to be coincubated with bacteria and provides a measure of bacterial virulence within a few hours. In combination, these features would allow researchers to carry out hundreds of tests within a week, allowing mass screening of mutants and isolates. Analysis of previously characterized bacterial mutants also reveals that *D. magna* is sensitive to virulence traits similar to those described in other host models.

Besides its use as a simple system to measure bacterial virulence, *Daphnia* represents a powerful model to analyze coevolution of hosts and pathogens in a natural context (6). The study of interactions between *D. magna* and pathogens is progressing rapidly, and we know more about *Daphnia*-pathogen coevolution than we know about any other model system (6). More detailed studies will be necessary to determine how interactions between environmental pathogens and *D. magna* may influence the virulence of bacteria and the host resistance mechanisms. A better understanding of the interaction between *Daphnia* and environmental pathogens may allow us to explore the evolutionary trajectories leading to bacterial adaptation and disease progression.

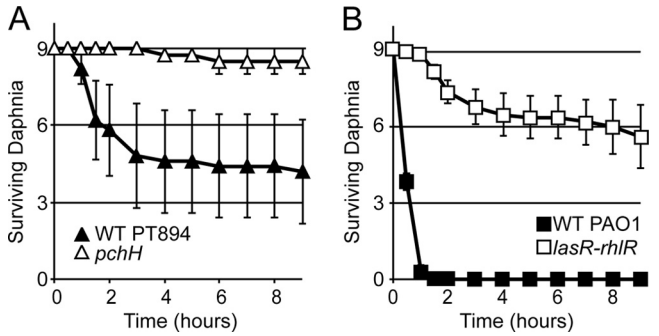

FIG 3 Secreted *P. aeruginosa* toxins can kill *D. magna*. *P. aeruginosa* bacteria were grown overnight in liquid SM. The supernatant of these cultures was added to *D. magna* at a final dilution of 1:4, and its effect on survival was assessed. The average and SEM of at least 5 independent experiments are presented. SM alone had no effect on the *Daphnia* survival (data not shown).

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