Alternative Host Model To Evaluate Aeromonas Virulence

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Bacterial virulence can only be assessed by confronting bacteria with a host. Here, we present a new simple assay to evaluate Aeromonas virulence, making use of Dictyostelium amoebae as an alternative host model. This assay can be modulated to assess virulence of very different Aeromonas species.

Bacterial virulence designates the complex array of bacterial traits that allow pathogenic bacteria to cause a disease in an infected host. Virulence factors include, for example, secreted bacterial toxins or the ability to escape the host immune system. By definition, the virulence of a given bacterial strain can only be measured by confronting it with a host. To assess the virulence of bacterial pathogens, mice are often used, based on the premise that their immune defenses are similar to those of the human body. These experiments are, however, difficult to carry out, expensive, and ethically problematic, since they inflict significant suffering on infected animals. In addition, mice are not appropriate hosts for certain pathogens such as Aeromonas salmonicida that normally infect cold-blooded vertebrates living at low temperatures. These considerations have led to the development of nonmammalian host models to study the pathogenic potential of bacteria.

Alternative hosts such as the nematode Caenorhabitis elegans and the insect Drosophila melanogaster or even unicellular Acanthamoeba castellanii or Dictyostelium discoideum amoebae have proven useful to study bacteria virulence (9, 10). The relevance of these models is based on the observation that many pathogens have a low species specificity, due to the universality of virulence factors implicated in the infectious process. It is also likely that these alternative hosts are naturally confronted with the same pathogens in their natural environment and that many of the bacterial virulence factors were developed to fight these natural predators (1).

The use of unicellular amoebae allows a very simple assessment of bacterial virulence in many different pathogens. In a typical experiment, Dictyostelium cells form a phagocytosis plaque on a lawn of nonpathogenic bacteria (Fig. 1A) but not on a lawn of pathogenic bacteria. The virulence of bacteria can thus be extrapolated from their ability to sustain Dictyostelium growth, as shown previously for Klebsiella pneumoniae (2) or Pseudomonas aeruginosa (6, 14). These previous studies also reported an excellent correlation between virulence as evaluated in a Dictyostelium host model and in a mouse infection model.

Assessing virulence of Aeromonas bacteria is challenging since different Aeromonas species (e.g., A. salmonicida and A. hydrophila) infect different hosts (fish, leeches, mice, and humans), have different growth requirements (e.g., low or high temperature), and cause very different diseases (furunculosis and septicemia in fish and wound infections, meningitis, pneumonia, gastroenteritis, and septicemia in humans). In addition, some strains of A. salmonicida lose their virulence at temperatures above 21°C, due to the thermostability of a large pVirA virulence plasmid (15). Fish can be used as hosts to evaluate virulence of A. salmonicida at low temperature, but this requires specific installations and poses significant practical problems, such as disposal of contaminated water.

In order to assess the virulence of A. salmonicida against Dictyostelium, we tested the ability of 1,000 Dictyostelium cells to grow at 17°C on a lawn of A. salmonicida (JF2267) grown on an HL-5 agar medium (12). This pathogenic strain was isolated from an arctic char with typical furunculosis (3) and was able to establish a systemic and lethal infection in rainbow trout (4). This virulent strain (Table 1) did not allow growth of Dictyostelium amoebae (Fig. 1B). On the contrary, the JF2397 strain has lost its large pVirA virulence plasmid, is incapable of synthesizing type III secretion system (T3SS) components (15), and was permissive for Dictyostelium growth (Fig. 1B). Similarly, the mutant strain JF2747 was shown previously to be nonvirulent for trout (4), due to the deletion of the ascV gene encoding an inner membrane component of the T3SS. This deletion renders that bacterium incapable of secreting T3SS toxins and effector molecules. This strain was also permissive for Dictyostelium growth (Fig. 1B). The virulence against Dictyostelium was restored by complementation with a plasmid expressing AscV (strain JF3239), which restores secretion of T3SS proteins (7) (Fig. 1B). Together, these results indicate that the T3SS-dependent virulence of A. salmonicida can be evaluated in a Dictyostelium host model.

We next tried to use the same assay to test the virulence of the mesophilic Aeromonas hydrophila strain serotype O34. Under the conditions described above, the wild-type A. hydrophila strain AH-3 was not permissive for Dictyostelium growth. However, the ascV T3SS mutant, which was shown to be avirulent...
in rainbow trout and mice (18), was nonpermissive (virulent) for *Dictyostelium* (Fig. 2A, 100% NL-5 agar). We then reasoned that slowing down the growth of bacteria might change the threshold at which a bacterial strain is permissive for *Dictyostelium* growth. To test this hypothesis, we reduced gradually the richness of the growth medium by diluting it. We observed that, at lower nutrient concentrations (medium diluted 10 times or more), the AH-3 wild-type strain remained nonpermissive, while the avirulent ascV mutant was permissive for *Dictyostelium* growth (Fig. 2A and B). This appears to be an empirical manner of adjusting the threshold at which virulence of a bacterial strain is detected.

In order to test whether under these newly defined conditions other virulence factors would also be in play, we tested a few other well-characterized *A. hydrophila* mutants in which potential virulence mechanisms distinct from the T3SS were affected. There are several very conserved pathways regulating virulence in many bacteria, in particular the quorum-sensing and the PhoP/PhoQ regulatory systems. Quorum sensing is a mechanism controlling gene expression in response to an expanding bacterial population and is essential for virulence of many gram-negative pathogens. In *A. hydrophila*, quorum sensing was shown in particular to control the production of exo-proteases (17) and biofilm formation (11). The corresponding ahyI and ahyR mutants were permissive for growth of *Dictyostelium* (Fig. 2C). The two-component regulatory system involving PhoP (the transcriptional regulator) and PhoQ (the sensor kinase) transcriptionally controls some of the virulence determinants and is essential for virulence of *Yersinia pestis* (13) and *Salmonella enterica* serovar Typhimurium (8). Interestingly, an *A. hydrophila* phoP mutant also exhibited a loss of virulence against *Dictyostelium* (Fig. 2C). These results suggest that the *Dictyostelium* host model provides a meaningful assessment of bacterial virulence not restricted to T3SS-dependent cytotoxicity. More experiments will, however, be necessary to determine extensively which virulence traits can be assessed accurately in this *Dictyostelium* host model.

This study demonstrates that *Dictyostelium* can be used as a simple host model to assess the virulence of distinct *Aeromonas* species. It also describes an empirical method to adjust conditions in order to set the threshold of this assay for strains with

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

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<tr>
<th>Strain</th>
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<td>JF3239</td>
<td>JF2747 derived, ΔascV + pMMB66EH-ascV (Amp')</td>
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**FIG. 1. Virulence of *Aeromonas salmonicida* against *Dictyostelium*.** (A) The ability of *Dictyostelium* to grow on a bacterial lawn was assessed as described previously (2) by depositing 1,000 wild-type *Dictyostelium* DH1-10 cells (5) on a lawn of bacteria grown on HL-5 agar medium. A phagocytosis plaque was observed 7 days later when bacteria were permissive (nonvirulent). (B) The wild-type virulent *Aeromonas* medium. A phagocytosis plaque was observed 7 days later when bac-

**FIG. 2. Virulence of *Aeromonas hydrophila* against *Dictyostelium*.** (A) The ability of wild-type (WT) *A. hydrophila* (AH-3) and the isogenic T3SS-negative ΔascV mutant was tested on HL-5 agar, pure or diluted, as described in the legend to Fig. 1. Only low concentrations of nutrients (10% HL5 agar or lower) allowed the detection of T3SS-dependent virulence of *A. hydrophila*. (B) More extensive analysis of the phenotypes shown in panel A. (C) Loss of virulence of the ΔahyI and -R and ΔphoP mutants was also revealed in this assay (5% HL-5 agar).
very different growth requirements. This system could allow in the future a systematic analysis of *Aeromonas* virulence factors. Since *Dictyostelium* is amenable to genetic analysis, this system might also allow analysis of host resistance mechanisms.

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