A simple mung bean infection model for studying the virulence of *Pseudomonas aeruginosa*

Sneha Garge,¹ Sheyda Azimi²,* and Stephen P. Diggle²

**Abstract**

Here we highlight the development of a simple and high-throughput mung bean model to study virulence in the opportunistic pathogen *Pseudomonas aeruginosa*. The model is easy to set up, and infection and virulence can be monitored for up to 10 days. In a first test of the model, we found that mung bean seedlings infected with PAO1 showed poor development of roots and high mortality rates compared to uninfected controls. We also found that a quorum-sensing (QS) mutant was significantly less virulent when compared with the PAO1 wild-type. Our work introduces a new tool for studying virulence in *P. aeruginosa* that will allow for high-throughput virulence studies of mutants and testing of the in vivo efficacy of new therapies at a time when new antimicrobial drugs are desperately needed.

*Pseudomonas aeruginosa* is an opportunistic pathogen that can be isolated from diverse habitats, including water, soil, animals and plants [1–3]. In humans, it is a leading cause of infection, morbidity and mortality in cystic fibrosis (CF) lungs, and is a problematic pathogen in burn wounds, chronic diabetic wounds and immunocompromised individuals [1, 3, 4]. Because of the problems that *P. aeruginosa* causes in human hosts, researchers have developed a number of different *in vivo* infection models for assessing its virulence, pathogenesis and disease [5–7]. These include animal models such as wax moths [8, 9], fruit flies [10], nematodes, mice [11–16], pigs [17] and *ex vivo* pig lungs [18, 19]. Because *P. aeruginosa* uses a number of the same virulence factors to infect and cause disease in both animals and plants, plant infection models such as the *Arabidopsis* model have also been widely used [20, 21]. A number of factors are important to consider when designing and using a virulence model. These include cost-effectiveness, ethical considerations, ease of growth in a laboratory, measures of virulence and the ability to handle large sample sizes. In this study we describe the development of a mung bean infection model [22], and how this easy-to-establish model can be used to assess *P. aeruginosa* virulence.

We transferred the surface-sterilized seeds onto soft agar plates (0.8 % w/v agar) and added 2 ml of sterile water to the surface of the agar (Fig. 1a). We then incubated the plates for 24 h at 37 °C under humidified conditions to allow for the germination of seeds. We used eight germinated seeds per group for the infection studies. As a first test of our model, we wanted to determine whether quorum sensing (QS) is important for virulence in mung beans, because QS has previously been shown to be an important regulator of virulence in a number of different hosts, including mice, *Arabidopsis*, lettuce, nematodes and insects [11, 23–25]. We used a PAO1 *lasI– rhlI–* double mutant (PAO-JG1) [26] to determine the role of QS in pathogenesis, colonization and disease development in mung beans.

To infect the seeds, we inoculated the PAO1 wild-type strain and PAO-JG1 into 100 ml lysogeny broth (LB) from overnight start-up cultures, and incubated them at 30 °C/200 r.p.m. to reach a cell density of ~10⁹, 10⁶ and 10³ colony-forming units (c.f.u.s) ml⁻¹. We centrifuged stationary-phase cultures at 7000 r.p.m. for 10 min and we resuspended pellets into 4 ml of sterile phosphate-buffered saline (PBS) (pH 7). We added eight germinated mung bean seeds to the bacterial suspensions and then incubated them at 30 °C for 24 h under static conditions to achieve a good colonization of bacterial cells on the germinated seeds. We incubated equal numbers of seeds in 4 ml of PBS as uninfected controls. After the incubation, we took three seeds from each treatment group, resuspended them in 1 ml of sterile PBS and vortexed them for 30 s. We used these

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**Abbreviations:** AHL, N-acyl homoserine lactone; CF, cystic fibrosis; LB, lysogeny broth; QS, quorum sensing.
suspensions to perform viable counts (c.f.u. ml\(^{-1}\)) to determine the number of adhered bacteria that coated each seed. In our first trial runs, we used all three initial c.f.u. ml\(^{-1}\) for the bacterial cultures, but we saw no significant differences in the growth rate of plants infected with PAO1 wild-type below \(\sim 10^9\) c.f.u. ml\(^{-1}\) (Fig. 1b). The cell counts are shown in Table 1.

For plant growth, we prepared Murashige–Skoog agar media [4.4 g l\(^{-1}\) Murashige–Skoog (Sigma Aldrich) and 0.8% w/v agar (Oxoid)]. We poured 30 ml aliquots of media into large glass tubes (30 × 200 mm, Duran groups) and sterilized them by autoclaving for 20 min. We aseptically transferred both infected and PBS control mung bean seedlings (five seeds per group) into the tubes. We allowed the seedlings to grow in a natural daylight cycle for 10 days in gnotobiotic conditions. To determine the viable bacterial counts (c.f.u. mg\(^{-1}\) of root weight) of both strains colonizing the roots of the mung bean seeds during the 10 days of

**Fig. 1.** Mung bean infection method and comparison of growth defects in infected plants. (a) The procedure to infect mung bean seeds with *P. aeruginosa*. (b) A comparison of PAO1- and PAO-JG1-treated mung bean plants with different initial bacterial densities 3, 5 and 10 days post-treatment. The images are representative of three independent trials.
incubation, we cut the root of each plant with sterile scissors, and resuspended these in 1 ml of sterile PBS and vortexed them for 30 s. We used these suspensions to perform a viable count of bacteria to determine the number of bacteria colonizing the root of each plant. We normalized the c.f.u.s on each root by the respective root weight (mg) of each plant root. We showed that both the PAO1 and the PAO-JG1 strain were viable on the roots throughout the infection period (Table 1). We determined the health of the plants by measuring parameters such as the mortality rate of seedlings, shoot length, root length and the number of root branches. We defined the mortality rate as the percentage of seedlings that died in terms of the total number of seedlings treated with bacterial culture suspension in each trial. We found that the mortality rate of the seedlings treated with PAO1 was higher than that for the PAO-JG1-treated seedlings (Fig. 2). We observed that all of the seedlings infected with various c.f.u. ml\(^{-1}\) of PAO-JG1 developed into healthy plants (Figs 2a and 1b). We found that seedlings that survived infection with approximately 10\(^6\) c.f.u. ml\(^{-1}\) of PAO1 demonstrated severely attenuated growth (Figs 2a and 1b). These plants displayed disease symptoms, such as water-soaked lesions on their roots and blackening of the roots and shoots.

Our infection model examined three parameters to determine and score plant health: root length, shoot length and the number of root branches. In seedlings that survived PAO1 treatment, we observed a significant decrease in the root and shoot length measurements compared to seedlings treated with PAO-JG1 and healthy PBS control plants (Fig. 2b–d). We found that the root development of PAO1-treated plants was also impaired, as the number of root branches was reduced compared to PAO-JG1-treated plants and healthy PBS control plants (Fig. 2d).

In six independent trials, each with five technical replicates, we found that PAO1 either caused the death of the seedlings or the seedlings survived with impaired growth. In contrast, we found that PAO-JG1 did not cause mortality of seedlings in any of the trials and, furthermore, the seedlings developed into significantly healthy plants after 10 days (Figs 1 and 2e). These data support previous observations indicating that the las and rhl QS systems regulate the virulence of P. aeruginosa in plant tissues [27–29]. Although we found that QS is important for virulence and disease in mung beans, infections were not completely prevented, suggesting the role of other factors responsible for pathogenesis in mung bean plants that are QS-independent.

Our data demonstrate that we have developed a simple plant model to study the mechanisms of pathogenesis and virulence during P. aeruginosa infection. Our model has advantages compared to the popular Arabidopsis model, which takes 4–6 weeks to grow the plants and then 5 days post-inoculation to observe disease symptoms [20, 27]. The mung bean model takes a maximum of 12 days, which includes 1 day to obtain germinated mung bean seedlings, 1 day to infect the germinated mung bean seeds with bacterial cells and 7–10 days to grow the plants and allow for the development of disease symptoms. The methods involved in this model are easy to implement and also offer a number of convenient features. It is easy and cheap to acquire mung bean seeds, germinate them and maintain the plants in most laboratory facilities, and this can be done without any ethical considerations. Coating germinated seedlings with culture suspension to achieve infection is a simple microbiological technique, and coating allows researchers to evaluate infection in terms of the colonization and mortality of seedlings. This can be used as an important parameter for qualitative studies of the colonization of a host and the virulence and phenotypic traits involved in the pathogenesis of P. aeruginosa. You can also score the pathogenesis of P. aeruginosa in plants by measuring parameters such as root length, shoot length and the number of root branches. These are useful parameters where the levels of infection by different mutants or strains can reveal important information about virulence factors and virulence in general.

Large sample sizes are not a limitation for the model, so it can be used as a high-throughput virulence screen. Due to the clear effects of QS on virulence that we found, screening libraries of chemical compounds such as N-acyl homoserine lactone (AHL) mimics, analogues, inhibitors, antagonists, or quorum-quenching bacteria for controlling the infection could be tested easily. Functional studies of the genes and regulatory proteins involved in phenotypes such as motility, adhesion, colonization and pathogen survival, and their impact on virulence, can be conducted using the model. Finally, if seedlings are coated with sub-lethal doses of P. aeruginosa that do not cause the death of plants, this could allow for sociomicrobiology studies relating to social behaviours, to determine how spatial and temporal interactions within and between bacterial communities progress in a natural environment. In summary, we have developed a cheap, reproducible and simple-to-cultivate plant host model.

### Table 1. Viable counts of colonized bacteria in seedlings and roots for PAO1 or PAO-JG1

<table>
<thead>
<tr>
<th></th>
<th>Initial c.f.u. ml(^{-1})</th>
<th>c.f.u. ml(^{-1}) adhered to seedlings</th>
<th>c.f.u. ml(^{-1}) of roots 10 days post-inoculation</th>
<th>c.f.u. mg(^{-1}) of roots 10 days post-inoculation</th>
</tr>
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<tbody>
<tr>
<td>PAO1</td>
<td>1.16±0.65×10(^6)</td>
<td>1.92±1.64×10(^6)</td>
<td>5.4±5.0×10(^6)</td>
<td>1.62±0.6×10(^6)</td>
</tr>
<tr>
<td>PAO-JG1</td>
<td>1.36±0.68×10(^6)</td>
<td>1.80±1.65×10(^6)</td>
<td>5.1±6.4×10(^6)</td>
<td>3.5±1.4×10(^5)</td>
</tr>
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model that can be used to study *P. aeruginosa* virulence, and it also may also have relevance for other bacterial species.

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**Conflicts of interest**
The authors declare that there are no conflicts of interest.

**References**

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Fig. 2. Mung beans infected with QS mutant strain PAO-JG1 grew significantly better than seeds infected with PAO1. (a) Representatives of PAO1- and PAO-JG1-treated plants. (b) Root length. (c) Shoot length. (d) Number of root branches. The values represent the mean of six trials. The bars indicate the standard deviation of the mean. Each trial had five replicates. Statistical analysis was performed using one-way ANOVA and Bonferroni’s multiple comparison test (*, *P*<0.05; **, *P*<0.01; ***, *P*<0.0001). (e) Mortality rates in mung bean seedlings treated with PAO1 and PAO-JG1. There were five culture suspension-treated seedlings in each trial.

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