Published in final edited form as:

Nat Protoc. 2009 ; 4(2): 117–124. doi:10.1038/nprot.2008.224.

Modeling *Pseudomonas aeruginosa* pathogenesis in plant hosts

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Abstract

A pathogenic model in which both the pathogen and its host are amenable to genetic manipulation can greatly facilitate the understanding of bacterial pathogenesis. Plants are genetically tractable and can be used as experimental models for human microbial pathogenesis. We present protocols for both lettuce and *Arabidopsis* leaf infection models using the opportunistic human bacterial pathogen, *Pseudomonas aeruginosa*. The lettuce model allows for high-throughput qualitative analysis of virulence and is suitable for screening large numbers of bacterial strains, whereas the *Arabidopsis* model provides a quantitative approach and permits the tracking of bacterial cell proliferation *in planta*. The lettuce model takes ~24 h including bacterial growth using store-bought lettuce, and the *Arabidopsis* model takes 4–6 weeks to grow the plants and a similar time as with lettuce to infect the plants. Both models are monitored for up to 5 d post-infection. These methodologies can and have been used to identify novel and critical *P. aeruginosa* pathogenicity agents, as virulence factors are often conserved across phylogeny.

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous opportunistic human bacterial pathogen. Research into the *P. aeruginosa* host–pathogen interaction has both scientific and practical importance. The bacterium infects a remarkably broad array of species, including plants, insects and vertebrates, and it is a major drug-resistant agent causing serious infections in immunocompromised individuals and in cystic fibrosis patients^{1,2}. *P. aeruginosa* uses a shared subset of virulence factors to infect and cause disease in both plants and mice, suggesting the existence of conserved universal mechanisms of pathogenesis across divergent evolutionary lineages³. Therefore, genetically tractable model hosts can be used in the development of therapeutics to treat infections.

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Several models have been developed that facilitate systematic genetic screens to identify both *P. aeruginosa* virulence factors and the host defense mechanisms⁴ operating during infection^{5–7}. These models include the *P. aeruginosa*–plant leaf infiltration model, and other divergent hosts ranging from amoebae to invertebrates and mammals^{1,8,9}. *Drosophila melanogaster* has also been used to study an antagonistic interaction between *P. aeruginosa* and its host^{6,10}. Both plants and insects have been informative in the study of *P. aeruginosa* pathogenesis, but *Drosophila* is more permissive in modeling mammalian pathogenesis due to conservation of an innate immunity pathway present between mammals and flies^{11,12}. Nevertheless, studies indicate that the plant response to pathogen attack may be evolutionarily conserved, strengthening the argument for studying animal pathogens in plants³; the utility of such studies will most likely increase as our knowledge of shared plant and mammal host–pathogen responses increases.

Similarities in plant and animal innate immune responses and pathogen strategies

Despite the evolutionary distance between plants, invertebrates and mammals, several studies have revealed conservation of innate defense mechanisms among these diverse hosts, which suggests a potential common ancestry of these systems³. Pathogens often use the same virulence factors, such as proteases and phospholipase, to attack plant or animal hosts. Microbial-associated molecular patterns, such as lipopolysaccharide and flagellin, can elicit an immune response from plants or animals, although many secreted effectors are host specific.^{3,13} In both plants and animals, immune responses can be initiated by host transmembrane receptors, which respond to microbial-associated molecular patterns (e.g., Toll-like receptor proteins in animals and Leucine-rich repeat proteins in plants)³. Animals and plants also respond to bacterial infection by activating a membrane-generated oxidative burst, using NADPH as the source of electrons^{14–17}, and programmed cell death appears to have an important function in infection¹⁸. Finally, plants, invertebrates and vertebrates all produce a class of antimicrobial peptides called defensins through a pathogen-inducible process^{19,20}. The commonalities among these different groups of organisms suggest that the study of plant host responses to animal pathogens should help to elucidate some of the different events that occur during pathogenesis, in both the pathogen and its host²¹.

Plants as infection models

Since the use of *Arabidopsis* to identify common themes of pathogenesis between plants and animals¹, plants have been used successfully to study pathogenesis in both Gram-negative and Gram-positive human pathogens, including *P. aeruginosa*, *Enterococcus faecalis* and *Staphylococcus aureus*^{1,22,23}. Several genes relevant to mammalian pathogenesis, each of which exhibits diverse function, have been discovered by using plants as model hosts, including the *P. aeruginosa* hydroxy-quinolone quorum sensing system and the multiple virulence factor regulator, MvfR^{24,25}. There are many benefits of using plants to model pathogenesis, such as the cost effectiveness of easily growing and maintaining the host and the ease of screening many bacterial strains in a short amount of time. *Arabidopsis* is an ideal choice as a plant host. It is genetically tractable, the genome is fully sequenced and many different mutant strains are available. Finally, using plant infection models eliminates the regulations and ethical considerations associated with mammalian experiments.

Arabidopsis has been used as a model to study plant biology for decades, and, as the *Arabidopsis* genome is fully sequenced, the system is genetically tractable. The first description of leaf infiltration of a plant pathogen into *Arabidopsis* leaves was reported in 1991 (see ref. 26) and a very thorough review of *Pseudomonas syringae*–*Arabidopsis* interactions and pathogenesis, including details on various leaf-infiltration methods can be found in *The Arabidopsis Book*²⁷. Details of *P. aeruginosa* strain PA14 invasion and infection of *Arabidopsis* have also been reported²⁸. In addition to *Arabidopsis* leaf infections, this plant host has been used for root colonization assays and the study of biofilm formation *in planta*²⁹. Several other plant models have also been described for modeling *P. aeruginosa* infections, including lettuce leaf infection, alfalfa seedling model and recently poplar and barley infection models^{30–32}. This protocol presents a high-throughput lettuce infection model and an *Arabidopsis* leaf infection model.

Experimental design

Here, we present protocols for both lettuce and *Arabidopsis* leaf infection models, on the basis of methods used routinely in our lab³⁰. The lettuce leaf infection model allows for high-throughput qualitative analysis of virulence and is suitable for screening large numbers of bacterial strains, such as mutant libraries (Fig. 1). The procedure involves infecting lettuce leaf midribs with bacterial cultures and visually evaluating the resulting disease symptoms. By using detached midribs, the assay can also be performed without the use of a growth chamber and is thus convenient for many researchers. In the *Arabidopsis* leaf infection model, bacteria are forced through the stomatal openings on the leaves; symptoms and bacterial cell counts are tracked for 5 d following inoculation. A qualitative assessment of bacterial virulence is achieved in this system by visually inspecting symptoms, and a quantitative evaluation is achieved by recording bacterial cell numbers *in planta*. This model is also suitable for gene expression studies on either the bacteria or the *Arabidopsis* host. Special considerations for the plant host and pathogen are addressed separately below.

Plant considerations.—Many different *Arabidopsis* ecotypes (strains isolated from various geographical areas) are available, and the ecotype chosen as the infection host can have a pronounced effect on the outcome of pathogenesis. Different *P. aeruginosa* isolates may respond more or less effectively to specific ecotypes¹. We use the common *P. aeruginosa* strain PA14¹ with *Arabidopsis* ecotype Llagostera (LI-O) or Colombia (Col). Both ecotypes show visible and severe symptoms and strain LI-O exhibits more extreme water-soaking symptoms. When working with other bacterial isolates, it is important to find the best conditions for a model host by experimenting with various plant ecotypes.

Begin the experimental procedure by choosing plants free from signs of stress, including reddish leaves, other discolorations and/or stunted growth. Plants should be grown under controlled light and temperature conditions. The oldest leaves on the plant (typically 3–4), located near the bottom of the basal rosette (Fig. 2) are more susceptible to infection, and are therefore selected for inoculation. Furthermore, leaves at a similar developmental stage are critical for reducing variability of infection symptoms and bacterial counts. Mark the inoculated leaves with a water-resistant marker to record which leaves have been infected. Some leaves can fall on the soil, which exacerbates disease symptoms, so we recommend

using plastic toothpicks for support. Wooden toothpicks draw moisture from the soil, causing the leaves to rot, and hence should not be used. When collecting leaf samples for bacterial counts, choose leaves with similar symptom severity (See Fig. 3 for examples of leaf symptoms).

Maintaining humid conditions is critical for establishing and sustaining effective infection. We recommend using an incubator capable of maintaining highly humid conditions, and setting the humidity level on the maximum setting (which translates to approximately 70–80% actual humidity in the incubator), and covering the flats with clear plastic domes. If there is too much condensation on the lid, puncture holes on the top to release moisture. Fewer bacteria counts or more attenuated symptoms with a wild-type strain could be due to inadequate humidity.

Bacterial culture considerations.—It is advisable that the *P. aeruginosa* strain you choose contains an antibiotic resistance marker on the chromosome. This ensures only growth of *P. aeruginosa* on plates, avoiding contamination for a more accurate assessment of colony-forming units (CFUs). For example, we use strain PA14 tagged with a rifampicin resistance gene. An alternative to chromosomally tagging bacterial strains is to plate samples on *Pseudomonas*-specific media (e.g., *Pseudomonas* Isolation Agar) to avoid contamination with other microorganisms. If screening a bacterial mutant library, it is necessary to remove potential auxo- trophs. Auxotrophs will be asymptomatic in the assays, and their true virulence phenotype cannot be assessed using these infection models. Be sure to include a control of the wild-type *P. aeruginosa* strain each time you do an infection assay.

The starting culture OD_{600 nm} is crucial to the outcome of the experiment. We recommend using a culture in late-log to early stationary phase (OD_{600 nm} = 2.7–3.0), as most of the virulence factors are maximally expressed at this stage. For instance, if you are studying quorum sensing, we recommend that cells are as close as possible to an OD_{600 nm} = 3.0, when quorum sensing (QS) genes are maximally activated. Another consideration is the number of cells used to infiltrate the plant (see Step 1A(x) in the PROCEDURE below). For example, inoculating with fewer cells can be useful for distinguishing moderate virulence differences between strains, or using a low inoculum concentration (5×10^3 to $1\text{--}5 \times 10^4$ CFU ml⁻¹) may reveal subtle differences between strains, whereas a higher inoculum (1×10^5 up to 2×10^6 CFU ml⁻¹) may help to distinguish highly attenuated strains from those that cause moderate symptoms (Fig. 4). In addition, the use of both high and low concentrations of inoculum is valuable in the study of different hosts or in this case *Arabidopsis* ecotype specificity. For instance, when a high-concentration inoculum of *P. aeruginosa* is used, some ecotypes exhibit no symptoms and others may display weak symptoms. This indicates that one specific *Arabidopsis* ecotype is resistant to infection. Moreover, the results and interpretations from these studies can be further extended to assess host responses during infection.

Statistical analysis of data

Experiments with mutants that exhibit attenuation in virulence should be repeated at least three times to ensure validity of results. At least four different samples per mutant per time

point should be obtained (see Step 1A(xvi) in PROCEDURE). To determine whether or not CFUs are significantly different between wild-type and mutant bacterial strains, a standard *t*-test can be used to compute *P*-values. Alternatively, statistical analysis of the bacterial growth curves *in planta* can be performed using the Kaplan–Meier³³, in the SPSS software package (version 15, SPSS Inc.). Differences between mutant and wild-type growth kinetics can be assessed using the log-rank (Mantel–Haenszel) test to compare the Kaplan–Meier growth curves of mutant and wild type.

Potential limitations

In *Arabidopsis*, different *P. aeruginosa* isolates elicit varying degrees of disease symptoms. To model the antagonistic interactions of *P. aeruginosa* with *Arabidopsis*, and gain insights into *P. aeruginosa* mammalian pathogenesis, the following criteria must be met: the strain must elicit severe symptoms, proliferate in plant leaves over time and be virulent in mice. For example, *P. aeruginosa* human isolate PA14 is highly virulent in mice and establishes a progressive and lethal infection in *Arabidopsis* leaves with some of the same virulence factors required for mammalian pathogenesis^{1,24,25}. PAO1, another *P. aeruginosa* clinical isolate, also causes disease in *Arabidopsis* and lettuce. Although PA14 uses many of the same virulence factors to infect *Arabidopsis*^{1,24}, *Drosophila*¹⁰, *Caenorhabditis elegans*,^{7,25} and *Galleria mellonella*³⁴ that it uses to mediate pathogenesis in mammalian hosts, some of the virulence genes may be important in plants but not mammals. For example, although *P. aeruginosa* type III secretion is important for mammalian pathogenesis, it is not involved in *P. aeruginosa* plant virulence³⁵. However, *P. aeruginosa* type III secretion is integral in *Drosophila* virulence³⁶, as well as for the pathogenesis of other bacterial plant pathogens.

MATERIALS

REAGENTS

- Luria Bertani (LB) broth (e.g., Fisher Scientific, cat. no. BP1427)
- LB agar plates (e.g., Fisher Scientific, cat. no. BP1425, prepared with antibiotics if used) or *Pseudomonas* Isolation Agar (Difco, cat. no. BD292710)
- Antibiotics (e.g., rifampicin 100 µg ml⁻¹ if used)
- 10 mM MgSO₄ (sterilize before use)
- 200 proof ethanol
- Potting soil (e.g., Metro Mix 200 or Fafard no. 2; Griffins, cat. no. 65-3125 or 65-1523)
- *Arabidopsis thaliana* Llagostera (L1-O) seeds (*Arabidopsis* Biological Resource Center, <http://www.biosci.ohio-state.edu/pcmb/Facilities/abrc/.abrchrom.htm>)
- 4-to 6-week-old *Arabidopsis thaliana* Llagostera (L1-O) plants (12 plants per bacterial strain tested)

- Commercially available Romaine lettuce seeds or Romaine lettuce purchased from a supermarket (if plants are not grown from seed, more replicates may be required)
- Phytoblend (Caisson Labs, cat. no. PTC001) Prepare a 0.1% (wt/vol). solution in water and autoclave (if available) or heat to fully dissolve the agar
- Selected strains of *P. aeruginosa*

EQUIPMENT

- 1-ml syringes without needles (1 per strain) (e.g., cat. no. BD-309602)
- 1.5-ml sterile microcentrifuge tubes (e.g., Fisher Scientific, cat. no. 02-681-272)
- Paper towels
- Pestle for grinding (e.g., Fisher Scientific, cat. no. K749521-1500)
- Cordless grinder (optional; e.g., Fisher Scientific, cat. no. K749540-0000)
- No. 2 cork borer (e.g., Fisher Scientific, cat. no. 07-865-10B)
- Forceps
- Whatman no. 1 filter paper
- Marker (preferably red or orange to mark leaves)
- Plastic toothpicks
- Plastic flats (trays) with clear domed lids (e.g., T.O. Plastics Inc., cat. no. 05023 and 05015, http://www.toplastics.com/horticulture/.horticulture_products.php)
- Plastic insert boxes for plant growth (to be placed in a flat) (e.g., T.O. Plastics Inc., cat. no. 01051, http://www.toplastics.com/horticulture/.horticulture_products.php)
- Microfuge (e.g., Fisher Scientific, cat. no. 13-100-510)
- Spectrophotometer capable of reading absorbance at 595 or 600 nm
- Test tubes with caps
- Aluminum foil
- Incubator at 37 °C with shaker or rotating drum for growing liquid bacterial cultures and for plate incubation
- Plant growth chamber with humidity control (e.g., Percival Scientific model E30B)

PROCEDURE

1| Two separate infection assays are depicted below. Option A describes the *Arabidopsis* leaf infection assay, which is both a qualitative and quantitative procedure. Option B describes

the lettuce leaf infection assay, which is qualitative and more suitable for high-throughput analyses.

A. *Arabidopsis* leaf infection assay

- i.** Dispense *Arabidopsis* seeds in a tube or small Petri dish containing 0.1% (wt/vol) Phytoblend agar. Cover with foil and incubate at 4 °C for 2–5 d. The soaking of the seeds will increase their germination rate and the agar will space out seeds for planting.
- ii.** Using a Pasteur pipette, plant seeds evenly spaced in each box with potting soil.
- iii.** Place flats containing boxes in a 20 °C growth chamber with 50–70% humidity and 12 h of light per day.
- iv.** Once seedlings have germinated (~ 1 week after sowing), thin plants to achieve 2–4 cm of space between plants (typically five plants per 5-inch box).
- v.** Incubate for 3–4 more weeks before inoculation. By the third week post-germination, check that the leaves are green and healthy looking and schedule experiment (Fig. 2 and Experimental design).
- vi.** The evening before infecting the plants, inoculate bacteria in LB broth, from a single colony, and incubate using a shaker at 200–250 r.p.m. or roller drum at 37 °C overnight (~ 16 h).
- vii.** The next morning, prepare a subculture from the overnight culture by diluting it 1:100 in fresh LB broth. Grow to the desired OD_{600 nm} (e.g., 3.0) at 37 °C.
- viii.** Centrifuge 2 ml of culture at room temperature for 2 min at 8,000*g*. Discard supernatant, and if a viscous material is visible on top of the pellet, carefully retain it, as it may include bacterial polysaccharides.
- ix.** Wash once with 1 ml of 10 mM MgSO₄ to remove traces of LB broth. Centrifuge as mentioned in Step 1A(viii).
- x.** Resuspend the cells in 1 ml of 10 mM MgSO₄ and determine the OD_{600 nm}. Prepare an inoculum of OD_{600 nm} 0.002 by serially diluting the cells in 10 ml of MgSO₄. Begin by preparing 10 ml at an OD_{600 nm} of 0.2, and dilute 1:10 twice to a final OD_{600 nm} of 0.002 (2 × 10⁶ CFU ml⁻¹) in 10 ml of buffer.

? TROUBLESHOOTING

- xi.** Prepare to inoculate a total of 12 plants per strain, infecting 4 leaves per plant. Before infection, mark the leaves selected for inoculation to distinguish infected leaves later in the experiment.

▲ **CRITICAL STEP** Keep plants under light to promote stomata opening, which will facilitate infiltration.

- xii.** Fill a 1-ml syringe with the bacterial suspension, taking care to avoid bubbles. Gently press the underside (abaxial surface) of the leaf with the syringe and depress the plunger while keeping the leaf between the tip of the syringe and your thumb (Fig. 5). If leaves do not readily absorb the liquid, repeat this process several times per leaf.

! CAUTION Wear eye protection when infiltrating leaves with the syringe.

▲ CRITICAL STEP Exclude leaves mechanically damaged during infiltration; mix tube containing bacterial suspension often to ensure that an equal concentration of bacteria is applied to the leaves.

? TROUBLESHOOTING

- xiii.** Following inoculation, dab off excess liquid from the leaves with a Kimwipe or paper towel. Leaves should not touch the soil and can be supported with plastic tooth-picks as needed.
- xiv.** Immediately after inoculation, place the plants in a tray containing ~500 ml of water, cover the tray with a clear plastic dome and incubate. If leaves appear dry before incubation, symptoms will be weak and bacterial numbers will be low.

▲ CRITICAL STEP Use a different tray for each bacterial strain tested to avoid cross-contamination.

- xv.** Incubate the plants at 29–30 °C at the maximum humidity setting (although set at maximum level, most incubators can realistically maintain only between 70 and 80% humidity) and a 12 h photoperiod for up to 5 d; record symptoms daily.
- xvi.** On day 0, collect two samples from each of four infected leaves to assess the number of inoculated bacteria and ensure that similar numbers of bacteria were infiltrated into the leaves. Samples can be taken every day or every other day from days 2 to 5. Symptoms begin to appear ~2 d post-inoculation. Collect plant samples by punching two holes from each of the four leaves per bacterial strain using the no. 3 cork borer (Fig. 6). To facilitate leaf punching, it is possible to remove the leaves from the plant. Support the cork borer by pressing against the abaxial leaf surface with your thumb (holding a piece of paper towel), while pushing the cork borer to abaxial leaf surface. The sampled leaves should not be used again in the experiment. Rinse the cork borer in ethanol and dry between samples.

▲ CRITICAL STEP When taking leaf samples for bacterial counts, choose leaves with visible symptoms (Fig. 3).

- xvii.** Place each pair of leaf disc samples into 1.5-ml microcentrifuge tubes (1 tube per pair of leaf discs) containing 1 ml of 10 mM MgSO₄ and vortex to remove any bacteria on the leaf surface.
- xviii.** Using forceps, remove the leaf discs from the microcentrifuge tube and dab off excess liquid on a paper towel. Transfer the samples into a new microcentrifuge tube containing 300 µl of 10 mM MgSO₄.
- xix.** Grind with a plastic pestle until nearly homogenous and add 700 µl of 10 mM MgSO₄.
- xx.** Perform serial dilutions (1:10) and plate 20 µl of each dilution onto a separate LB agar plate with appropriate antibiotic if used. A single plate can be divided into four quadrants to plate four dilutions per plate and conserve resources. Incubate overnight at 37 °C.
- xxi.** Count the colonies and calculate CFUs per ml.

? TROUBLESHOOTING

B. Lettuce leaf infection assay

- i.** Prepare plants for the lettuce leaf infection assay according to Box 1. Use option A if using potted plants or option B if using detached leaves, depending on experimenter preference and equipment availability.
- ii.** Prepare bacterial inoculum according to Box 2 or Steps A(vi-x) above. Bacteria can be taken directly from colonies on agar plates (option A, Box 2), or prepared in liquid culture in tubes (as described above in Steps 1A(vi-x)) or in microtiter plates (option B, Box 2) on the basis of experimenter preference. Care should be taken to avoid penetrating the middle space of the rib, as depicted in Fig. 7.
- iii.** Following inoculation, incubate infected lettuce plants or Petri dishes containing the inoculated midribs in a growth chamber at 29–30 °C with relative humidity of 90–100%, and a 12 h photoperiod for up to 5 d. If a growth chamber is not available, detached midribs can be incubated at room temperature (20–25 °C) on the bench top, but symptoms will take longer to develop and might not be as severe.
- iv.** Record symptoms daily for 4–5 d. Approximately 30 h post-infection, initial symptoms in the wild-type strain will appear as light-brown spots at the site of inoculation. Fully developed symptoms are expected to occur 4–5 d following infection and consist of strong brown and soft-rot areas surrounding the inoculation site. The soft-rot symptoms will eventually spread to the entire midrib, resulting in complete maceration and tissue collapse (Fig. 8).

TIMING

Arabidopsis assay:

Steps 1A(i-v), preparation of *Arabidopsis* plants: 4–6 weeks

Steps 1A(vi-x), preparation of bacterial inoculum: 20–24 h

Steps 1A(xi-xiv), infiltration of *Arabidopsis* leaves with bacterial suspension: 15–20 min per box of 5–6 plants

Steps 1A(xvi-xxi), assessment of bacterial numbers: 24 h including incubation and ~30 min to count colonies (e.g., 20 plates with four dilutions each)

Lettuce leaf infection assay:

Step 1B(i) and Box 1 (option A), preparation of lettuce leaves from store-bought lettuce: 30 min (e.g., 20 leaves)

Step 1B(i) and Box 1 (option B), preparation of potted lettuce plants: 12 weeks

Steps 1B(ii-iii) and Box 2, preparation of bacterial inoculum and infection: 20–24 h

Step 1B(iv), assessment of symptoms: 20 min daily (e.g., for 5 total days)

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

ANTICIPATED RESULTS

As early as 2 days post-infection, *Arabidopsis* leaves will start to elicit symptoms, including water soaking and yellow discoloration of infected leaves (see Fig. 3 for a range of symptoms expected throughout infection). Although symptoms are typically indicative of bacterial counts, it is important to note that symptoms might not always correlate with CFUs. For instance, healthier looking leaves could potentially have more bacteria than yellowish counterparts. The appearance of symptoms is also closely linked to the humidity levels maintained during the experiment. Increased humidity results in plants with more severe symptoms.

Colony-forming units for wild-type strains PA14 and PAO1 typically increase from days 0 through 2, with minor increases from days 2 to 5. It is not uncommon to see a drop in bacterial counts from days 4 to 5. The CFU data for each strain tested can be plotted on a graph versus time post-infection, and standard deviation should be included at each point on the graph. Alternatively, the individual CFU data points per strain can be plotted for a given time point with the calculated mean also represented on the graph. An important consideration is that even small differences in CFUs can be biologically significant if they are repeatable. For example, a repeated one-log difference in bacterial numbers from the wild type can be enough to consider the mutant strain potentially defective in virulence and would warrant further testing in other model systems. A *t*-test can be performed to calculate *P*-values to determine statistical significance, and/or statistical analysis of the bacterial growth curves *in planta* can be performed using the Kaplan–Meier as described in INTRODUCTION.

As indicated earlier, the lettuce assay permits qualitative assessment, and it is most likely that isogenic mutant strains that cause moderate symptoms may be overlooked using this screen. Nevertheless, this approach permits a ‘high-throughput’ approach and can therefore identify many novel virulence factors, including quorum-sensing mutants.

However, the availability of *P. aeruginosa* nonredundant mutant libraries for both PA14³⁷ and PAO1³⁸ strains now provides an alternative option to use the *Arabidopsis* leaf infection assay in a ‘high-throughput’ manner. An advantage of using the *Arabidopsis* model is that the host–pathogen interactions can be assessed not only from the bacterial side, but also from the side of the plant host, as it is genetically tractable and commonalities between plant and animal innate immunity mechanisms exist³. Many resources are available for the study of *Arabidopsis*, e.g., the website <http://www.Arabidopsis.org/index.jsp>, which provides information, such as genome annotation and the availability of various *Arabidopsis* seed stocks.

ACKNOWLEDGMENTS

This work was partially supported by the research grant R01AI063433. M.S. was supported by the Shriners Research Fellowship no. 8506. Thank you to Jenifer Bush for growing and maintaining our *Arabidopsis* plants.

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BOX 1 |**PREPARATION OF LETTUCE PLANTS**

- A.** Detached lettuce leaves
- i.** Purchase romaine lettuce from a supermarket and detach all healthy-looking leaves from the core (number and size varies based on size of the individual lettuce). Do not use the very young leaves as they are small and their midrib is very tender.
 - ii.** Wash the leaves with 0.1% (vol/vol) bleach and subsequently rinse twice with distilled water.
 - iii.** Trim excess leaf material from around the center and place the midribs of detached leaves in 15-mm-diameter Petri dishes containing a piece of Whatman no. 1 filter paper soaked in 10 mM MgSO_4 (see Fig. 1).
- B.** Potted lettuce plants
- i.** Treat lettuce seeds as in Steps 1A(i) and 1A(ii) of the main PROCEDURE.
 - ii.** Cultivate romaine lettuce plants in potting soil with a 12 h light cycle at 26 °C for 12 weeks.

BOX 2 |**PREPARATION OF THE BACTERIAL INOCULUM AND INFECTION**

- A.** Bacterial inoculum from agar plates (colonies)
- i.** Using a toothpick, pick individual colonies from agar plates and gently stab into the midribs of the lettuce leaves at least 3.0 cm apart. Always include at least one wild-type bacterial control on each midrib used in the experiment.

▲ **CRITICAL STEP** During inoculation, the toothpick should not penetrate the midrib middle space (Fig. 7). If that occurs, the midrib should be discarded.
 - ii.** Mark both the plate and the lid for orientation of the strains used for the infection.
 - iii.** Maintain the filter paper wet in 10 mM MgSO₄ (soaked in solution) throughout the duration of the experiment.
- B.** Bacterial inoculum from microtitre plates (liquid)
- i.** Grow mutant strains in LB broth overnight at 37 °C in a 96-well microtiter plate.
 - ii.** Dilute the wells 1:10 in LB broth and grow until early stationary phase, B6 h. If available, the OD_{600 nm} of the plate can be measured using a microtiter plate reader.
 - iii.** Centrifuge the plates to pellet the bacteria and wash with 10 mM MgSO₄. If observed, reserve the viscous material on top of the pellet that may contain bacterial polysaccharides.
 - iv.** Resuspend the pellets in 10 mM MgSO₄ and dilute to an OD_{600 nm} of 0.2 (2×10^7 CFU ml⁻¹). Serially dilute further to OD_{600 nm} of 0.02 and 0.002 (2×10^6 and 2×10^5 CFU ml⁻¹).
 - v.** Using a pipette, gently inoculate the midribs of each lettuce leaf with 10 µl of all three dilutions, spaced at least 3.0 cm apart. Pierce only the tip of the pipette tip into lettuce and slowly dispense the inoculum into the depression.

▲ **CRITICAL STEP** During inoculation, the pipette tip should not penetrate the midrib middle space (see Fig. 7 for illustration of the correct and incorrect inoculation techniques using a purple dye).

Figure 1 |.
Overview of the plant infection assays described in this article.

Figure 2 |.
Arabidopsis leaves grow as a rosette; choose only older leaves closest to the bottom, as indicated by arrows.

Figure 3 |.

Infected *Arabidopsis* leaves can exhibit a range of symptoms from mild chlorosis and water soaking to severe soft rotting of the entire leaf.

Figure 4 |.

The amount of cells in the inoculum can impact disease progression in *Arabidopsis* plants. The graphs show the growth of bacteria *in planta* over the course of the infection. Note that the mutant (moderate virulence defect) only appears less virulent than wild type when a lower inoculum was used (OD_{600 nm} of inoculum = 0.0004 = 4 × 10⁵ CFU ml⁻¹ compared with inoculum OD_{600 nm} of inoculum = 0.002 = 2 × 10⁶ CFU ml⁻¹). The graphs are also representative of typical CFUs obtained from leaf samples during the infection.

Figure 5 |.
Inoculate the underside of each leaf by gently pressing the syringe against your thumb. The movement of the bacterial suspension through the leaf is evident in the close-up picture.

Figure 6 |.
A cork borer (no. 3) is used to collect leaf disc samples for bacterial CFU counts.

Figure 7 |.

When inoculating lettuce midribs, avoid penetrating the middle space of the plant. Here we used a blue dye to visualize correct inoculation (top panel) compared with the incorrect penetration of the middle space (bottom panel). Arrows indicate the inoculation site at the top of the midrib and the middle space hole. Note that in the correct inoculation, the blue dye is confined to the inoculation site and remains above the middle space. In the incorrect inoculation, once the middle space has been penetrated, the blue dye can be seen in the surrounding area and has diffused throughout the local lettuce tissue.

Avirulent mutant

PA14 (wt)

Figure 8 |.

Infected lettuce leaves show severe rotting (black coloration) of the entire midrib when infected with the wild-type strain PA14. In contrast, an avirulent *P. aeruginosa* mutant shows only some signs of damage and/or infection near the inoculation site (arrows). A version of this image has been used, with permission, from ref. 25.

TABLE 1 |

Troubleshooting table.

Step	Problem	Possible reason	Solution
1A(x) and 1A(xxi)	Few symptoms, low CFUs	Lack of humidity throughout infection	Increase humidity in the growth chamber if possible. Make sure that the flats are tightly covered and move infected plants to the incubator as quickly as possible. In addition, make sure that the water level in each tray is checked daily and water is added as needed
	Excessive symptoms, rotting	Too much humidity	Pierce holes in the domed lids to allow for evaporation
	No differences observed between mutants and wild type	Sometimes high inoculum densities can mask subtle differences between strains	Try infiltrating leaves with less cells (e.g., 10 or up to 100 times less cells in the inoculum)
1A(xii)	Leaves are difficult to infiltrate	Stomata are closed	Provide more direct light to the plants before and during inoculation to maintain open stomata. Placing a clear domed lid on top of the plants a day before inoculation might also allow for easier infiltration