# Growth and Laboratory Maintenance of *Pseudomonas aeruginosa*

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## ABSTRACT

*Pseudomonas aeruginosa* is a common, free-living, Gram-negative bacterium that can cause significant disease as an opportunistic pathogen. Rapid growth, facile genetics, and a large suite of virulence-related phenotypes make *P. aeruginosa* a common model organism to study Gram-negative opportunistic pathogens and basic microbiology. This unit describes the basic laboratory growth and maintenance of *P. aeruginosa. Curr. Protoc. Microbiol.* 25:6E.1.1-6E.1.8. © 2012 by John Wiley & Sons, Inc.

Keywords: *Pseudomonas* • metabolism • growth • minimal media

## INTRODUCTION

*P. aeruginosa* is a ubiquitous Gram-negative bacterium with extensive metabolic diversity, allowing it to thrive in a wide variety of environments and nutrient sources. It is partly this metabolic flexibility that enables *P. aeruginosa* to succeed as an opportunistic pathogen. *P. aeruginosa* is a common cause of both community-acquired and hospital-acquired infections with impacts ranging from cosmetic to life-threatening. Community-acquired infections caused by *P. aeruginosa* include ulcerative keratitis of the eye, and skin/soft tissue infections such as folliculitis and those seen in diabetic wounds. *P. aeruginosa* can cause more serious disease in immunocompromised individuals and is a common cause of nosocomial infections including infection of burn wounds, urinary tract infections, bacteremia, and pneumonia (Sadikot et al., 2005). *P. aeruginosa* has been found to be responsible for 11% to 13.8% of all hospital-acquired infections (Driscoll et al., 2007). *P. aeruginosa* is also responsible for much of the morbidity and mortality in patients with the recessive genetic disorder cystic fibrosis (CF; Burns et al., 1998).

*P. aeruginosa* is a hardy bacterium that can be grown easily in a wide variety of conditions and temperatures. This unit describes the basic techniques to maintain and grow *P. aeruginosa* in the laboratory.

*CAUTION*: *P. aeruginosa* is a Biosafety Level 2 (BSL-2) pathogen. Follow your institutional guidelines for handling and safety while working with BSL-2 organisms. For general biosafety information, see *UNIT 1A.1*.

## STRATEGIC PLANNING

### Strain Selection

The clinical isolates turned laboratory strains, PAO1 and UCBPP-PA14 (PA14), are commonly used for the study of the basic biology and genetics of *P. aeruginosa*. The genome sequences for both strains are publicly available (*www.pseudomonas.com*) and an ordered transposon mutant library is also available for both strains, making them



*Current Protocols in Microbiology* 6E.1.1-6E.1.8, May 2012
Published online May 2012 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/9780471729259.mc06e01s25

**Table 6E.1.1** Common *P. aeruginosa* Strains

| Strain | Source | Origin | Genome | Reference |
|---|---|---|---|---|
| PAO1 | Clinical; nonrespiratory | Australia, 1954 | Complete | Stover et al. (2000) |
| PA14 | Clinical; burn wound | United States, 1990s | Complete | Rahme et al. (1995) |
| PA7 | Clinical; nonrespiratory | Argentina | Complete | Roy et al. (2010) |
| PAK[a] | unknown | Japan | Incomplete | Minamishima et al. (1968) |
| LESB58 | Cystic Fibrosis isolate | U.K., 1988 | Complete | Cheng et al. (1996) |
| PA2192 | Cystic Fibrosis isolate | United States, 1980s | Incomplete | Mathee et al. (2008) |

[a]PAK has been used for a large number of *P. aeruginosa* studies and was first exploited to explore phage biology. While its original source is unknown and the complete genome is not currently available, the prevalence of PAK in the literature merited its inclusion here.

very amenable to study (Jacobs et al., 2003; Liberati et al., 2006). PAO1 has undergone numerous serial passages and adaptation to the laboratory, and it has been demonstrated that sub-lines of the original isolate maintained in different laboratories across the world have changed significantly, including mutations in quorum sensing, drug efflux, and the type 3 secretion system (Klockgether et al., 2010). PA14 is a more recent isolate, and is more virulent than PAO1 in most disease models. PA14 has been used extensively for the study of biofilm formation (O'Toole and Kolter, 1998). Other differences for consideration are the observations that PAO1 is more electrocompetent and PA14 is much more efficient than PAO1 during conjugation. Other strains have various characteristics that may be of interest, for example the taxonomic outlier PA7 is an isolate that shows remarkable antibiotic resistance, and harbors 18 unique genomic islands not present in any other sequenced *P. aeruginosa* strains (Roy et al., 2010). Strain PAK has been used nearly as long as PAO1 and was originally used to study phage biology (Minamishima et al., 1968; Bradley, 1973) and is now used as a tool in *P. aeruginosa* vaccine studies, type III secretion, and general signal transduction and regulation (Lee et al., 2005; Brencic and Lory, 2009; Campodonico et al., 2010). Clinical isolates from specific diseases, such as CF, may be of interest in studying particular aspects of virulence and evolution of *P. aeruginosa* during infection. While the complete sequenced genome is not yet available for many *P. aeruginosa* isolates, work is ongoing and status of genome assembly can be found at the genome project page of the *Pseudomonas* Genome Database (*http://www.pseudomonas.com*) (Winsor et al., 2009), which is an excellent resource for researchers. Table 6E.1.1 lists several common strains used to study *P. aeruginosa.*

## Growth Conditions

*P. aeruginosa* grows well at 37°C, and can also survive at a wide range of temperatures from 4°C to 42°C. When selecting temperatures for growth, consider that temperature can affect virulence, and that below 30°C some virulence pathways are not active. Bacteria on plates can be stored at 4°C for future use with a recommended limit of one week. Cultures grow best with aeration, regardless of media, but *P. aeruginosa* is capable of growing anaerobically on some carbon sources when supplied with nitrate as the terminal electron acceptor. We typically grow *P. aeruginosa* in 18-mm glass tubes with 3 ml of medium on a roller drum and generally note exponential doubling times for PAO1 of 1 to 1.5 hr in minimal medium (such as MOPS, see Reagents and Solutions) and 25 to 35 min in a rich broth such as LB (see *APPENDIX 4A*).

## Media

*P. aeruginosa* grows well on LB broth, but can also utilize a wide range of compounds as sole carbon and/or nitrogen sources. To study growth on these sole nutrient sources,

various defined minimal media are used to grow *P. aeruginosa* such as MOPS [3-(*N*-morpholino) propane-sulfonic acid] medium, M9, or M63. Defined media formulations can be modified to study the role of various carbon, nitrogen, and sulfur sources on *P. aeruginosa* growth and virulence. We have found the most robust growth for a variety of carbon sources on MOPS medium. Other media that are used for *P. aeruginosa* include Pseudomonas isolation agar (PIA, Difco) that is used to select against *E. coli* after conjugations, and King A, which stimulates pyocyanin production.

## GROWTH OF *P. AERUGINOSA* FROM A FROZEN STOCK

***BASIC PROTOCOL 1***

*P. aeruginosa* can be preserved in frozen stocks of either 20% glycerol or 10% skim milk (Cody et al., 2008) that can be stored at −80°C. Growing bacteria on fresh plates from frozen stocks is important when starting experiments to ensure consistent results, particularly for clinical isolates from CF samples, as they can have very high mutation rates. When starting a strain from a frozen stock it is recommended that the bacteria be streaked out onto an LB agar plate, and a subsequent liquid culture started the next day from the fresh plate. Isolates can grow poorly when placed directly into liquid, particularly with antibiotics present. Recovery of strains directly onto MOPS agar plates is not recommended.

### *Materials*

*P. aeruginosa* frozen stocks (see Basic Protocol 3)
LB agar plates (see *APPENDIX 4A*), with antibiotics, if necessary (see Table 6E.1.2)

Sterile wooden applicator stick
37°C incubator

1. Scrape a small amount of bacteria from frozen stock.

   *Do not thaw frozen stock, scraping a small portion of frozen bacteria is sufficient, repeated freezing and thawing of stocks will result in reduced viability.*

2. Streak the bacteria onto agar plates using sterile applicator sticks.

3. Incubate for 16 to 24 hr at 37°C.

**Table 6E.1.2** Antibiotic Usage for *P. aeruginosa*[a]

| Antibiotic | LB liquid[b] | LB plate | MOPS liquid | MOPS plate | Stock |
|---|---|---|---|---|---|
| Gentamicin | 40 μg/ml | 50 μg/ml | 20 μg/ml | 25 μg/ml | 50 mg/ml |
| Kanamycin | 300 μg/ml | 300 μg/ml | 150 μg/ml | 150 μg/ml | 100 mg/ml |
| Carbenicillin[c] | 900 μg/ml | 900 μg/ml | 700 μg/ml | 700 μg/ml | 150 mg/ml[d] |
| Tetracycline | 50 μg/ml | 100 μg/ml | 50 μg/ml | 50 μg/ml | 50 mg/ml |

[a]We report here the antibiotic concentrations we use in our laboratory. However, there are wide ranges of antibiotic concentrations reported in the literature. Some of the variance depends on the age and source of the antibiotic. We recommend titrating your antibiotic during initial trials to get robust growth of resistant (i.e., plasmid carrying) bacteria and no spontaneous resistance from wild-type.

[b]These concentrations are for selection of cells carrying resistance plasmids. Antibiotic concentrations for plasmid maintenance can be lowered within experiments by half for all of these antibiotics. For transposon mutants, lower concentrations are often used after selection. For example, we use 10 μg/ml gentamicin for PA14 transposon mutants.

[c]We use carbenicillin in place of ampicillin to reduce satellite colonies during cloning with *E. coli*. Either antibiotic will work for *P. aeruginosa* and the *bla* resistance marker codes for detoxification of both compounds.

[d]To add this amount of carbenicillin or ampicilin when making plates, we typically weigh the appropriate amount out, dissolve in water, and filter sterilize before addition.

*BASIC PROTOCOL 2*

## GROWTH OF *P. AERUGINOSA* IN LIQUID MEDIUM

Liquid cultures of *P. aeruginosa* are commonly used for many applications. For general experiments, including electroporation (Choi et al., 2006), cultures are grown in LB. When studying effects of particular nutrient sources on *P. aeruginosa*, the bacteria can be grown overnight in a minimal medium such as MOPS that is supplemented with the selected nutrient sources (see Reagents and Solutions section). Growth in a minimal medium is also recommended for isolation of high-quality genomic DNA for plasmid and BAC library construction.

### *Materials*

*P. aeruginosa* freshly grown on agar plates (see Basic Protocol 1)
LB broth (see *APPENDIX 4A*) *or* MOPS minimal medium (see recipe)
Antibiotics, if necessary (see Table 6E.1.2)

Sterile glass tubes (e.g., 18-mm) *or* flasks (e.g., 125-ml)
Sterile wooden applicator stick or inoculating loop
37°C incubator

1. Add the medium of choice to a sterile tube or flask and appropriate amount of antibiotic when applicable.

   *Because P. aeruginosa grows better with increased aeration, we use 3 ml volumes in 18-mm tubes on a roller drum and 10 to 25 ml in 125-ml flasks.*

2. Pick a single colony or small amount of bacteria from the agar plate from Basic Protocol 1.
3. Inoculate the culture with a wooden applicator stick by suspending bacteria into the growth medium.
4. Place the tube in the 37°C incubator on a shaker at 200 rpm or, preferably, a roller drum for 16 to 24 hr.

*BASIC PROTOCOL 3*

## PREPARATION OF *P. AERUGINOSA* FROZEN STOCKS

*P. aeruginosa* strains should be stored long-term at −80°C. It is important to begin experiments using bacteria freshly streaked from the laboratory stock to ensure consistency.

### *Materials*

*P. aeruginosa* grown on LB agar plates (see Basic Protocol 1)
LB broth (see *APPENDIX 4A*), with antibiotics if necessary (see Table 6E.1.2)
50% glycerol, sterile

37°C incubator
1.2-ml sterile cryogenic vials
Vortex mixer
−80°C freezer

1. Inoculate *P. aeruginosa* into LB broth with appropriate antibiotics and grow culture of *P. aeruginosa* for 16 to 24 hr at 37°C.
2. Add 0.6 ml of the overnight culture into a 1.2-ml cryogenic vial with 0.4 ml of 50% (v/v) glycerol.
3. Mix the solution well by vortexing on a medium setting or by repeated inversions.

4. Store the bacterial glycerol stock up to 10 years in −80°C freezer.

*Viability is dependent on the frequency and number of freeze-thaw cycles. If the sample will be repeatedly removed from the freezer, generate four to six independent stocks.*

*It has been reported that viability of frozen stocks that have been thawed may be improved when they are prepared in 10% skim milk (Difco) instead of glycerol (Cody et al., 2008).*

## REAGENTS AND SOLUTIONS

*Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see* ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

### *MOPS (Neidhardt et al., 1974)*

We use MOPS medium to study the effects of particular nutrient sources on *P. aeruginosa* growth and virulence. In the authors' experience, *P. aeruginosa* grows better on a variety of carbon sources in MOPS than it does in M63 or M9. To make MOPS medium the following solutions will need to be prepared.

### *Micronutrient stock for MOPS, 100×*

To make the micronutrient mix, add the solid components (see Table 6E.1.3) to 90 ml of deionized water, mix, and bring the total volume to 100 ml. Store indefinitely at room temperature.

### *Modified MOPS medium, 1×*

To make 1× MOPS medium, combine the components shown in Table 6E.1.4. Dilute the 10× MOPS stock (see recipe) with deionized water before adding the other components to avoid precipitation. Filter-sterilize the 1× MOPS medium before use. The storage time for 1× MOPS depends on the carbon source added. To prolong shelf-life, make the 1× MOPS without a carbon source and add the source as needed. In this manner, 1× MOPS can be stored for up to 6 months at room temperature.

### *10× MOPS stock*

Mix the components from Table 6E.1.5 together and fill solution to 500 ml with water and filter-sterilize. Store up to a year at room temperature out of direct light.

*This stock may turn slightly yellow over time, but this does not appear to alter growth.*

**Table 6E.1.3** Solid Components for the Micronutrient Stock for MOPS

| Component | mg/100 ml | Stock concentration (μM) |
|---|---|---|
| Ammonium molybdate tetrahydrate | 0.3 | 3 |
| Boric acid | 2.4 | 400 |
| Cobalt chloride | 0.7 | 30 |
| Cupric sulfate | 0.3 | 10 |
| Manganese sulfate | 1.6 | 80 |
| Zinc sulfate | 0.3 | 10 |

**Table 6E.1.4** Components for 1× MOPS Medium[a]

| Component | Stock | Volume for 500 ml | Final concentration |
|---|---|---|---|
| 10× MOPS stock | See recipe | 50 ml | See recipe |
| Deionized water | N/A | 400 ml | N/A |
| Carbon source[b] | 1 M | 10 ml | 20 mM |
| $CaCl_2$[c] | 53 mM | 300 µl | 32 µM |
| $K_2SO_4$ | 27.5 mM | 5 ml | 0.29 mM |
| $K_2HPO_4$ | 172.8 mM | 5 ml | 1.32 mM |
| $FeCl_2$[c] | 8 mM | 500 µl | 8 µM |

[a]It is possible to make the 1× from sterile stock solutions with good aseptic technique. However, the final filtration step (see recipe) eliminates potential contamination issues from long-term stocks and is good practice particularly when studying mutants that may grow slower than wild-type for a given carbon source.

[b]We use most carbon sources at 20 mM. Depending on the quality and solubility of the carbon source tested, vary the concentration accordingly. A standard test for a carbon source of unknown quality and/or toxicity is to measure growth on 1, 5, 20, and 50 mM.

[c]These two additions have been made to stimulate biofilm formation on biotic surfaces ($CaCl_2$) and increase growth rate and yield on substrates requiring metabolism by iron-containing oxidases ($FeCl_2$). They are not required for growth on glucose and they are not part of the media from the original citation.

**Table 6E.1.5** MOPS Stock Components

| Component | Stock | Volume for 500 ml | Concentration at 1× |
|---|---|---|---|
| MOPS | 1 M (pH 7.5) | 200 ml | 40 mM |
| Tricine | 1 M (pH 7.5) | 20 ml | 4 mM |
| $FeSO_4$ | 18.4 mM | 5 ml | 0.01 mM |
| $NH_4Cl$ | 1.9 M | 25 ml | 9.52 mM |
| $CaCl_2$ | 53 mM | 50 µl | 0.5 µM |
| $MgCl_2$ (hexahydrate) | 512 mM | 5 ml | 0.52 mM |
| NaCl | 5 M | 50 ml | 50 mM |
| Micronutrients stock (see recipe) | 100× | 5 ml | See recipe |

## COMMENTARY

### Background Information

*P. aeruginosa* is a ubiquitous environmental bacterium known for its metabolic plasticity and ability to rapidly adapt to different environments. These features are probably a critical component enabling *P. aeruginosa* to be a successful opportunistic pathogen. *P. aeruginosa* remains among the top five bacterial species most commonly found in nosocomial infections; furthermore, incidence of hospital-acquired *P. aeruginosa* infections is on the rise. The reported cases of hospital acquired pneumonia caused by *P. aeruginosa* has increased from 9.6% to 18.1% from 1975 to 2003 (Gaynes and Edwards, 2005). *P. aeruginosa* is of particular interest as an opportunist in the lung environment. While *P. aeruginosa* is the causative agent in only about a quarter of ventilator-associated pneumonia (VAP) cases, it is responsible for about half of the associated morbidity and mortality (Chastre and Fagon, 2002). *P. aeruginosa* infections are also extremely common in patients with CF. Chronic infection occurs early in CF patients; one study that used broth culture of respiratory samples combined with serologic assessment found more than 95% of patients to be positive for *P. aeruginosa* infection by the age of 3 years (Burns et al., 2001). *P. aeruginosa* is also the most common pathogen causing chronic

infection in the CF lung and contributes to the morbidity and mortality in CF (Emerson et al., 2002; Rajan and Saiman, 2002). Due to the low permeability of its outer membrane, numerous antibiotic efflux pumps, and ability to acquire resistance mechanisms by genetic exchange, treatment of *P. aeruginosa* infections is becoming more challenging (Fischbach and Walsh, 2009).

The genome of *P. aeruginosa* varies in size between 5.5 and 7 Mbp and is among the largest of the sequenced bacterial genomes (Klockgether et al., 2010); this large size results from genetic complexity rather than gene duplication (Stover et al., 2000; Lee et al., 2006). Over 500 regulatory genes and a disproportionately large number of genes involved in nutrient import, antibiotic efflux, protein secretion, and chemo-sensing were identified in the genome of the common laboratory strain PAO1 (Stover et al., 2000). The large genome size and complexity reflect the physiologic adaptability of *P. aeruginosa* that allows it to thrive in a large variety of environments. The elucidation of mechanisms employed by *P. aeruginosa* to thrive in niches such as the lung could provide new insights to treatment strategies for *P. aeruginosa* infections.

## Critical Parameters and Troubleshooting

*P. aeruginosa* is a hardy bacterium that grows well under the conditions described in this unit. *P. aeruginosa* is highly viable when streaked from frozen glycerol or milk stocks. The most likely reason for failing to see growth after streaking from the frozen stocks is use of an incorrect antibiotic plate. It is also possible that not enough bacteria were taken from the stock or that the stock is old. Older glycerol stocks should be remade periodically, especially if they are removed from the freezer often. *P. aeruginosa* can be restreaked from the initial plate stored at 4°C. However, we recommend only restreaking from this original agar plate to start fresh plates as needed within that week. After the initial plate is one week old, we recommend restreaking a fresh plate from the frozen stock rather than conducting serial passage of the strain on plates. Multiple serial passages should be avoided due to probability of mutations arising that could result in inconsistent data.

## Anticipated Results

Basic Protocol 1 describes the growth of *P. aeruginosa* from a frozen stock. After incubating the streaked plate for 16 to 24 hr colonies will be large, opaque, and convex with a slightly rough edge and light tan in color. *P. aeruginosa* can produce pyocyanin, which can give the agar a greenish-blue color. The amount of pigment produced varies between strains and a sweet grape-like odor, caused by 2-aminoacetophenone, can sometimes be smelled when the bacteria are grown on a rich medium.

## Time Considerations

*P. aeruginosa* takes 16 to 24 hr to grow from streaking onto plates and in rich medium. Growth on minimal medium can take longer depending on nutrient sources provided and concentration of the carbon source. The protocols described in this unit should take only several minutes to complete, although making the MOPS stocks requires more time.

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## Internet Resources

http://www.pseudomonas.com

*The Web site for the Pseudomonas aeruginosa genomes, as well as other sequence species within the Pseudomonas genus.*