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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*.

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 11–13.8% 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.

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Internet Resources <http://www.pseudomonas.com>

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

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 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 (Bradley, 1973; Minamishima et al., 1968) and is now used as a tool in *P. aeruginosa* vaccine studies, type III secretion, and general signal transduction and regulation (Brencic and Lory, 2009; Campodonico et al., 2010; Lee et al., 2005). 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 (www.pseudomonas.com) (Winsor et al., 2009), which is an excellent resource for researchers. Table 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 18mm glass tubes with 3 ml of media on a roller drum and generally note exponential doubling times for PAO1 of 1 to 1.5 hours in minimal media (such as MOPS Glucose, see *Reagents and Solutions*) and 25 to 35 minutes 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 medium 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.

BASIC PROTOCOL 1: GROWTH OF *P. AERUGINOSA* FROM A FROZEN STOCK

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 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 bacteria onto agar plate using sterile applicator sticks.
3. Incubate at 37°C for 16–24 hours.

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 Solutions and Reagents 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 grown on agar plates

LB broth (see *APPENDIX 4A*) or MOPS minimal medium (see Reagents and Solutions)

Antibiotics, if necessary (see Table 2)

Sterile wooden applicator stick or inoculating loop

Sterile glass tubes or flask

- Add the medium of choice to 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

- Pick a single colony or small amount of bacteria from the agar plate.
- Inoculate culture with wooden stick by suspending bacteria into the growth medium.
- Place the tube in the 37°C incubator on a shaker at 200 rpm or, preferably, a roller drum for 16–24 hours.

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 2)

50% Glycerol, sterile

1.2 ml sterile cryogenic vials

37°C incubator

–80°C freezer

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

4. Store bacterial glycerol stock in -80°C freezer.

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

REAGENTS AND SOLUTIONS

Minimal Medium: 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.

10X MOPS Stock—Mix components together and fill solution to 500 ml with water and filter-sterilize. This stock can be stored for 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.

Component	Stock	Volume for 500ml stock	Concentration at 1X
MOPS	1 M (pH 7.5)	200 ml	40 mM
Tricine	1M (pH 7.5)	20 ml	4 mM
FeSO ₄	18.4 mM	5 ml	0.01 mM
NH ₄ Cl	1.9 M	25 ml	9.52 mM
CaCl ₂	53 mM	50 μ l	0.5 μ M
MgCl ₂ (hexahydrate)	512 mM	5 ml	0.52 mM
NaCl	5 M	50 ml	50 mM
Micronutrients ^a	100X	5 ml	see table

^a see Recipe for 100x Micronutrient Stock below

100X Micronutrient stock for MOPS—To make the micronutrient mix, add the solid to 90 ml of deionized water, mix, and bring the total volume to 100 ml. Store at room temperature indefinitely.

Component	mg / 100ml	Stock Concentration
Ammonium molybdate tetrahydrate	0.3 mg	3 μ M
Boric acid	2.4 mg	400 μ M
Cobalt chloride	0.7 mg	30 μ M
Cupric sulfate	0.3 mg	10 μ M
Manganese chloride	1.6 mg	80 μ M
Zinc sulfate	0.3 mg	10 μ M

1X Modified MOPS Media—To make 1X MOPS medium, combine the components below. *Note: be sure to dilute the 10x MOPS with de-ionized water before adding the other components to avoid precipitation.* Filter sterilize 1X MOPS before use ^a. The storage time

for 1X MOPS depends on the carbon source added. To prolong shelf-life, make the 1X MOPS without a carbon source and add the source as needed. In this manner, 1X MOPS can be stored for up to 6 months at room temperature.

Component	Stock	Volume for 500ml of 1X	Final Concentration
10X MOPS Stock	see above	50 ml	see above
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 ₂ SO ₄	27.5 mM	5 ml	0.29 mM
K ₂ HPO 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 1x from sterile stock solutions with good aseptic technique. However, this final filtration step 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 (CaCl₂) and increase growth rate and yield on substrates requiring metabolism by iron-containing oxidases (FeCl₂). They are not required for growth on glucose and they are not part of the media from the original citation.

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 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 both 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 large 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 (Lee et al., 2006; Stover et al., 2000). 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 16–24 hours 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 is grown on a rich medium.

Time Considerations

P. aeruginosa takes 16–24 hours 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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Table 1Common *P. aeruginosa* strains

Strain	Source	Origin	Genome	Reference
PAO1	clinical; non-respiratory	Australia, 1954	complete	Stover et al. 2000
PA14	clinical; burn wound	US, 1990's	complete	Rahme et al. 1995
PA7	clinical; non-respiratory	Argentina	complete	Roy, et al. 2010
PAK ^a	unknown	Japan	incomplete	Minamishima et al. 1968
LESB58	Cystic Fibrosis isolate	UK, 1988	complete	Cheng et al. 1996
PA2192	Cystic Fibrosis isolate	US, 1980's	incomplete	Methee et al. 2008

^aPAK 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.

Table 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

^aWe 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.

^bThese 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.

^cWe 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.

^dTo add this amount of carbenicillin or ampicillin when making plates, we typically weigh the appropriate amount out, dissolve in water, and filter sterilize before addition.