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Culture and Maintenance of *Agrobacterium* Strains

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Summary

As aerobic chemoorganotrophs, most *Agrobacterium* strains will grow on a wide range of complex and defined media. Methods commonly used for the culture and storage of other chemoorganotrophs will usually work for agrobacteria as well. Problems with culture or strain maintenance will occur more frequently because of careless technique than because of strain difficulties. Here we describe a few of the complex and defined media that have been successfully used in the growth of agrobacteria including some that are semiselective for agrobacteria. Finally, we present methods suitable for short- and long-term storage of *Agrobacterium* strains.

Key Words: Complex media; defined media; minimal media; selective media; enrichment media; stab cultures; dessication; vermiculite.

1. Introduction

Most strains of *Agrobacterium tumefaciens* and its avirulent cousin *A. radiobacter* are able to grow on minimal media with salts and a simple carbon source. *A. rhizogenes*, *A. rubi*, and some other natural isolates are auxotrophs that require the addition of growth factors such as biotin, nicotinic acid, pantothenate, and (or) glutamate to minimal medium. Carbon sources that are not readily used, even by the more nutritionally proficient members of the genus, include cellulose and starch. Nitrate or ammonium salts are sufficient nitrogen sources for *A. tumefaciens* and *A. radiobacter*, but not for *A. rubi* or *A. rhizogenes* ***(1–3)***.

There are many methods for storage of bacteria, and most will be suitable for maintenance of *Agrobacterium*. Important considerations in choosing a storage method include the length of time cells can be expected to remain viable, the genetic stability of the stored population, the number and value of the cultures to be maintained, and the frequency with which access to the cultures will be required ***(4)***. Here, we describe three methods suitable for

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short-term (3 mo to a year) or long-term (indefinite) storage of *Agrobacterium*. The methods described include maintenance on stab cultures, desiccation on vermiculite, and low-temperature freezing.

Stab cultures are a simple and inexpensive method for maintenance of *Agrobacterium* strains with transfer to fresh medium at regular intervals. If one has few cultures to maintain, it may be the best choice, but note that there is some risk of genetic change through mutant selection and plasmid loss. In addition, the potential for contamination rises with successive transfers.

An easy and inexpensive method of strain storage consists of desiccation in a protective environment. Pesenti-Barili et al. ***(5)*** found an 80% survival rate for *A. radiobacter* 13 mo after inoculation of sterile vermiculite and storage at 4°C. In that study, the author's intent was to identify a method for preservation of a biologically active strain appropriate for commercial purposes. We present a scaled-down version of their system as a suitable method for the short-term storage of *Agrobacterium* strains.

For laboratories with a large number of strains, the best long-term (indefinite) method of preservation is the freezing of small cultures at –70 or –80°C. Freezing a strain eliminates the possibility of genetic change, and the method requires no handling for maintenance purposes after the initial storage event. On the other hand, the cost of a low-temperature freezer is substantial, and arrangements will be needed to protect against failure of the freezer owing to electrical interruptions or mechanical breakdown.

2. Materials

2.1. Complex Media (see *Notes 1–4*)

1. Yeast-mannitol medium: 10 g/L mannitol, 1 g/L yeast extract, 0.5 g/L K_2HPO_4, 0.2 g/L $CaCl_2$, 0.2 g/L NaCl, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 10 mg/L $FeCl_3$.
   a. Dissolve all ingredients in 900 mL of water.
   b. Adjust pH to 7.0.
   c. Bring the volume to 1 L.
   d. For *A. rubi*, the addition of biotin, nicotinic acid, and calcium pantothenate, each at 200 µg/L, will improve growth. For *A. rhizogenes* and *A. vitis*, add biotin.
2. Nutrient-yeast medium: 8 g/L nutrient broth powder, 2 g/L yeast extract.
   a. Dissolve ingredients in water.
   b. Bring volume to 1 L and autoclave.
   c. When making nutrient broth or agar, simply leave out the yeast extract.
3. YDPC medium: 4 g/L peptone, 4 g/L yeast extract, 5 g/L $(NH4)_2SO_4$, 10 g/L $CaCO_3$, glucose (20% solution).
   a. Dissolve first four ingredients in 900 mL water.
   b. Bring the volume to 1 L.

c. Check that pH is close to 7.0.
d. Autoclave.
e. Autoclave the glucose separately and add 100 ml/L to the cooled medium.

4. MG/L medium: 5 g/L tryptone, 2.5 g/L yeast extract, 5 g/L NaCl, 5 g/L mannitol, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.25 g/L K_2HPO_4, 1.2 g/L L-glutamate, thiamine (10% solution, filter-sterilized).
   a. Dissolve all ingredients except thiamine in 900 mL water.
   b. Check that pH is close to 7.0.
   c. Bring volume to 1 L.
   d. Autoclave.
   e. When media cools to 60°C or below, add 120 µL/L of the thiamine solution.
5. LB: 10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract.
   a. Dissolve ingredients in water.
   b. Bring volume to 1 L.
   c. Check that pH is close to 7.
   d. Autoclave.
6. YEB medium: 5 g/L tryptone, 1 g/L yeast extract, 5 g/L nutrient broth, 5 g/L sucrose, 0.49 g/L $MgSO_4 \cdot 7H_2O$.
   a. Dissolve ingredients in water.
   b. Adjust the pH to 7.2 and bring volume to 1 L.
   c. Autoclave.

2.2. Defined Media: AB (AB*I) Medium (***see** ***Notes 1, 2, 5, and 6)***

1. (*see* **Note 6**): prepare and autoclave separately a 20X AB* salts solution (20 g/L NH_4Cl, 6 g/L $MgSO_4 \cdot 7H_2O$, 3 g/L KCl, 0.2 g/L $CaCl_2$, and 15 mg/L $FeSO_4 \cdot 7H_2O$), a 500 m*M* phosphate solution (60 g/L K_2HPO_4 and 20 g/L NaH_2PO_4, pH 7.5), an AB* buffer [3.9 g/L 2-(*N*-morpholino)ethane sulfonic acid (MES; pH to 5.5 with KOH)], and a 20% carbon source (glycerol, arabinose, glucose, or sucrose) solution.
2. Filter-sterilize a 10% solution of thiamine and (optional) a 10% solution of casamino acids.
3. Cool autoclaved solutions to room temperature.
4. For 1 L of medium, add 50 mL AB* salts and 2.4 mL of the phosphate solution to the MES buffer.
5. Add 100 µL thiamine.
6. Add the carbon source to 0.2%.
7. If using casamino acids, add them to 0.05%.
8. If you are preparing *vir* gene induction medium, add a phenolic inducer, e.g., 3,5-dimethoxy-4-hydroxy-acetophenone (acetosyringone) dissolved in dimethyl sulfoxide (DMSO). The amount of phenolic inducer will depend on your strain and the desired level of *vir* gene induction.

2.3. Enrichment and Selective Media

1. Medium 1A for *A. tumefaciens* and related strains (*3*; *see* **Notes 1**, **2**, and **7**): 3.04 g/L L-arabitol, 1.04 g/L K_2HPO_4, 0.54 g/L KH_2PO_4, 0.16 g/L NH_4NO_3, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.29 g/L sodium taurocholate, 2 ml/L 1% crystal violet, and 15 g/L agar.
   a. Dissolve the first seven ingredients in the water.
   b. Bring the volume to 1 L.
   c. Add to the agar and autoclave.
   d. Cool to 50°C and add 10 mL each of the following: cycloheximide (2% solution) and $Na_2SeO_3 \cdot 5H_2O$ (1% solution).
   e. Optional: K_2TeO_3 (100 mg/mL stock in ultrapure water) can be added to 80 µg/mL to improve the selectivity of the medium for *Agrobacterium* strains *(11)*.
2. Medium 1E for *A. rhizogenes* (*3*, *see* **Notes 1**, **2**, and **7**): 3.05 g/L erythritol, 1.04 g/L K_2HPO_4, 0.54 g/L KH_2PO_4, 0.16 g/L NH_4NO_3, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.29 g/L sodium taurochlorate, 1 ml/L 1% solution of yeast extract, 5 ml/L 0.1% solution malachite green, and 15 g/L agar.
   a. Dissolve the first eight ingredients in water.
   b. Bring volume to 1 L.
   c. Add to the agar and autoclave.
   d. Cool to 50°C.
   e. Add 10 mL each of the following: cycloheximide (2% solution) and $Na_2SeO_3 \cdot 5H_2O$ (1% solution).
   f. Optional: K_2TeO_3 (100 mg/mL stock in ultrapure water) can be added to 160 µg/mL to improve the selectivity of the medium for *Agrobacterium* strains *(11)*.

2.4. Antibiotics for Agrobacterium Selection and Culture (see Table 1 and Note 8)

2.5. Strain Storage

1. Sterile vials with screw-cap tops, e.g., Corning, cat. no. 430489 (*see* **Note 9**).
2. Fresh plate or liquid culture of strain to be stored (*see* **Note 10**).
3. Protective solution: 5% sucrose in sterile skim milk (autoclaved).
4. Fine or extrafine vermiculite, expanded type (*see* **Note 11**).
5. Precision balance.
6. Oven.
7. Low-temperature freezer (*see* **Note 12**).
8. 50% Glycerol in MG/L, YEB, or other liquid medium (autoclaved).

3. Methods

3.1. Media Preparation

In general, the pH of media for the growth of *Agrobacterium* should be between pH 6.8 and 7.2. A noted exception is AB*I (*vir* gene induction)

Table 1
Antibiotics Used in the Culture and Selection of Resistant *Agrobacterium* Strains[a]

Antibiotic	Stock concentration (mg/mL)	Solid medium (μg/mL)	Liquid medium (μg/mL)
Carbenicillin	100 in water	100	30–50
Chloramphenicol[b]	3 in ethanol	3	3
Erythromycin	100 in ethanol	150	100
Gentamicin	100 in water	100	100
Kanamycin	50 in water	50	10–20
Rifampicin	10 in methanol	10	10
Spectinomycin	100 in water	100	25–50
Tetracycline[b]	3 in 50% ethanol	3	1.5

[a]*See* **Note 8**. Note that antibiotic concentrations may need to be adjusted depending on the strain and the copy number of the gene determining resistance.
[b]Some *Agrobacterium* strains have natural resistance to chloramphenicol and tetracycline.

medium, in which the acidic pH helps maximize *vir* gene induction ***(10)***. Liquid medium can be converted to plate media by adding it to 15 g/L agar before autoclaving. Polystyrene Petri dishes (100 × 15 mm) are suitable for most plate media and are available from several biological supply companies, including Fisher Scientific.

The ideal growth temperature for *Agrobacterium* is 25–30°C. Repeated subculture or growth at high temperature (e.g., 37°C) risks loss of the megaplasmid that determines virulence ***(1,14)***. Depending on the strain and the composition of plate medium, it will take 2–4 days for colonies to appear at 25°C. The growth of even prototrophic strains of *Agrobacterium* will proceed faster when amino acids and vitamins are supplied either as a component of complex media or with supplementation of defined media. Another determinant of growth rate is the "age" of the inoculum. This is most apparent with liquid cultures: the inoculation of media with cells from a smaller overnight culture still in the exponential or early stationary phase of growth will shorten the lag phase that precedes exponential growth in the larger culture. For large liquid cultures, good aeration may be achieved by shaking (at 220 rpm) growing cells in Erlenmeyer flasks with a volume four or five times that of the culture, e.g., 200 mL in a 1-L flask. For smaller cultures (e.g., 2–3 mL), autoclavable glass tubes (16 × 125 mm) are available from Fisher Scientific.

3.2. Strain Storage

3.2.1. Storage of Agrobacterium Strains through Stab Cultures

1. Prepare 100 mL of sterile nutrient agar (*see* **Note 13**). Cool the medium to between 50 and 60°C for ease of handling.
2. Aseptically transfer 1 to 2 mL of the medium to each sterile tube. Loosely cap the tubes. When the agar sets, tighten caps and cover the tubes with foil or plastic wrap to prevent spores and fungi from settling on the tubes. Stab medium can be stored for several months at 4°C.
3. To inoculate a stab culture, use a flame-sterilized loop to pick up a single colony of *Agrobacterium* cells and stab it into the center of the medium. Repeat for one or more backup cultures. Immediately cap the tube tightly, and seal it with parafilm.
4. Incubate the inoculated stab culture tube at 25°C for 2 d.
5. Stab cultures can be stored at room temperature or (best) at 4°C for 4–6 mo.
6. Transfer the strain to new stabs every few months after streaking for single colonies and confirming genetic characteristics with antibiotic resistance, polymerase chain reaction, and/or virulence tests (*see* **Note 14**).
7. To access the stored culture, use a sterile inoculating loop to remove a small amount of the culture to a nutrient agar plate. Streak to obtain independent colonies.

3.2.2. Storage of Agrobacterium Strains through Desiccation

1. Begin preparation for strain storage by washing the vermiculite with distilled water. Drain it well, and place it in a warm (or very warm) oven 80 to 150°C (*see* **Note 15**). Periodically, check the weight of the vermiculite. When its weight stabilizes, the vermiculite is dry.
2. Place 0.2 g of vermiculite into several 2-mL screw-cap tubes. Autoclave the tubes with vermiculate for 30 min (*see* **Note 16**).
3. Dry vermiculite in tubes in the oven 80–150°C until dry, as indicated by stable weight.
4. Prepare overnight culture(s) of strains to be stored in nutrient broth with any antibiotics necessary for plasmid maintenance.
5. Use the overnight culture(s) to inoculate separate flasks containing 150 mL YDPC with the appropriate antibiotics. Grow to early or middle stationary phase (*see* **Notes 17** and **18**).
6. Pellet the cells by centrifugation. Remove the supernatant. Resuspend the cells from 150 mL of YDPC in 0.8 to 1.0 mL protective solution. Use a 200-µL aliquot of the cells to inoculate each tube of vermiculite. Vortex or shake briefly to distribute the culture through the vermiculite (*see* **Note 19**). Incubation is not required.
7. The vermiculite culture can be stored in room temperature for 4–6 mo, but longevity is increased by storage at 4°C (*see* **Notes 14** and **20**).

8. To access the culture, aseptically place a bit of the vermiculite into a microcentrifuge tube containing 100 µL sterile 0.8% NaCl. Vortex well to wash the bacteria into the saline. Remove about 25 µL of the saline to an MG/L (or other) agar plate with appropriate antibiotics and streak for single colonies.

3.2.3. Storage of Agrobacterium *Strains by Low-Temperature Freezing*

1. Inoculate 2 or 3 mL of media (MG/L or YEB are a good choices) that contains the appropriate antibiotics for plasmid maintenance with your strain (*see* **Note 21**).
2. Grow the culture to early or middle stationary phase (usually overnight). Chill the culture and the sterile medium containing 50% glycerol on ice.
3. Add an equal volume of the medium with glycerol to make the culture 25% glycerol. Mix culture and medium completely.
4. Transfer the cells to a labeled 2-mL tube, and place it in the freezer (*see* **Notes 12** and **14**). The frozen culture can be stored indefinitely.
5. To recover the strain, simply scrape a bit of the frozen culture onto plate medium containing the appropriate antibiotics. This can be done with a sterile, cooled, inoculating loop or with a sterile pipet tip. It is important to handle the culture quickly to prevent thawing and refreezing. If handled properly, the same culture can used repeatedly for retrieving the bacteria strain.

4. Notes

1. Media ingredients are available from a number of biological supply companies including Fisher Scientific, BD-BBL/Difco, and Sigma-Aldrich.
2. Autoclave-generated steam at a temperature of 121°C and pressure near 15 lb/in^2 is the most common method of sterilizing bacterial growth media. Recommended autoclave times range from 15 min to 1 h. It should be noted that agar conducts heat poorly. The temperature in the center of 500 mL of a 1.2% agar solution was found to reach 121°C 40 min after the internal chamber of the autoclave reached that temperature ***(6)***. Thus, even a 30-min autoclave time would be insufficient to kill heat-resistant microbial spores. If the growth of fungi and spore-forming organisms on autoclaved medium is a problem, increase the surface area of the media. One may do this by changing the size and shape of the container or by dividing the media or solutions into multiple containers.
3. Media sterilization through autoclaving requires consideration of the effect of heat on certain nutrients. Sucrose and other glycosides with furanoside groups will hydrolyze when heated at acid pH ***(6)***. The heating of reducing sugars, e.g., glucose, and/or phosphate, and/or amino acids or peptides together, may create toxic compounds or make nutrients unavailable ***(7)***. The reaction of sugar with other media components becomes a problem when concentrated solutions are heated at alkaline pH. Thus, sugars and peptones should be dissolved (not lie together at the bottom of the autoclave container). Media consisting of a sugar-peptide solution should not be prepared in concentrated form, and pH should generally be less than 8.0 ***(7)***. Alternatively, one can autoclave (or filter-sterilize) sugar solutions separately for addition to other media components after autoclaving.

4. All *Agrobacterium* strains should be able to grow on complex media. A survey of the literature indicates that *Agrobacterium* is frequently grown in Luria-Bertani (LB), nutrient broth (8 g/l), with or without the addition of yeast extract or glucose, and many other complex media. Potato dextrose agar plates are a common medium for the growth of *Agrobacterium*. These media are commercially available, and their formulas are readily available in microbiology handbooks and by Internet searches.
5. Putting together a defined medium requires the separate sterilization of its various components. For example, magnesium, potassium, ammonium, sodium, and phosphate ions can become unavailable when heated together owing to the formation of various insoluble magnesium phosphate salts. Thus, the magnesium source should be prepared and autoclaved separately from the phosphate source ***(6)***. Also, note that an acidic solution of sucrose is subject to hydrolysis when heated ***(6)***. This is of particular importance when preparing media for analysis of *A. tumefaciens* virulence genes, as glucose, but not sucrose will act as a stimulant of *vir* gene induction ***(8)***.
6. AB* medium is a derivative of AB medium ***(9)***. With the addition of a phenolic inducer, AB* becomes AB*I (AB* induction medium) for use as an inducing medium for *A. tumefaciens* virulence genes. For expression of the virulence genes at high levels, choose arabinose or glucose as the carbon source ***(8)*** and limit the addition of AB* phosphates to 1.2 m*M* ***(10)***. Otherwise, phosphate can be added to 10 m*M*. Casamino acids will increase the growth rate of *Agrobacterium* in AB* medium, particularly when the carbon source is glycerol. The addition of casamino acids does not effect *vir* gene expression in *A. tumefaciens* as measured by a *virB-lacZ* fusion (our personal observation). If making AB*I agar plates, it is necessary to autoclave the agar separately. Heating agar in acidic medium prevents it from becoming sufficiently solid upon cooling. Prepare the MES buffer solution as a threefold concentrate, i.e., 3.9 g MES in 300 mL water, and adjust the pH to 5.5. Autoclave 15 g agar in enough water (approximately 600 mL) to make the total volume 1 L after the additions of salts, phosphate, carbon source, and so on. The acidity (pH 5.5) of AB*I medium increases expression of the *vir* genes ***(10)***. However, the medium can also be made with pH near 7.0 by buffering with 25 m*M* Bis-Tris rather than MES when a nonacidic defined medium is desired.
7. The selective nature of enrichment media depends primarily on the presence of substances that are toxic to fungi and some bacteria. *Agrobacterium* forms shiny, white, raised colonies that may become mucoid or turn orange-brown after extended incubation on media 1A and 1E ***(3)***. The addition of tellurite at 80 µg/mL for 1A medium and at 160 µg/mL for 1E medium improves selectivity for *Agrobacterium*, although *Rhodobacter* and other *Rhizobium* species are also tellurite resistant ***(11)***. *Agrobacterium* will form shiny, convex, black colonies on medium amended with tellurite. The identity of selected strains should be confirmed with 16s RNA analysis and probes specific to pTi or pRi ***(11)***.

8. In general, antibiotic stock solutions are filter-sterilized and stored at –20°C. Antibiotics should be added to medium at temperatures below 60°C. Plate media that contain antibiotics should be labeled with the date of preparation and stored at 4°C. For liquid media, add the antibiotic just before inoculating the culture. Note that the half-life of many antibiotics in plate media is relatively short. If in doubt as to the efficacy of the antibiotic, test the plate with a strain that you know to be sensitive to the antibiotic. Ampicillin cannot be used for selection of *Agrobacterium* carrying a plasmid with the β-lactamase (*bla*) gene in rich medium. Carbenicillin may be used instead. *A. tumefaciens* C58 contains *tetA* and *tetR* genes similar to the Tet(A) class of determinants of tetracycline resistance ***(12)***. A high concentration of tetracycline induces tetracycline resistance in *A. tumefaciens* C58 and hinders identification of colonies that are tetracycline resistant owing to plasmid transformation. At concentrations of tetracycline below 5 μg/mL, the number of spontaneously resistant colonies is reduced or eliminated. *A. tumefaciens* strains C58, A136, and BG53 are chloramphenicol resistant owing to the presence of the *catB* gene ***(13)***. *A. tumefaciens* LBA4404 and GV3101 are chloramphenicol-sensitive strains.
9. The choice of tubes and labels will depend on the method chosen for storage. It is convenient to use small (2-mL) tubes. Tubes may be plastic or glass with caps that fit snugly to limit air exchange and prevent contamination. It is convenient for the tubes to have a surface designed for ease of labeling with a pen. However, depending on the storage method, tape will provide an adequate label. For strains to be stored frozen, note that tape may fall off after a few years.
10. Cultures of strains to be stored should be inoculated from healthy single colonies or a fresh liquid culture grown in the same medium to be used in the preservation step.
11. Vermiculite can be purchased at nurseries or garden centers. Vermiculite sold in the United States has been tested and confirmed to be free of asbestos.
12. Temperatures for freezing bacteria should be below –30°C owing to uneven freezing of eutectic mixtures ***(4)***. Storage at temperatures between –65 and –80°C is common and gives good results for very long (indefinite) periods of storage. It is not necessary to fast-freeze with dry ice and ethanol or liquid nitrogen.
13. Storage of stab cultures (like other methods) depends, in part, on reducing the cell's metabolic rate. *Agrobacterium* will survive at room temperature for many months on stabs made with nutrient agar. Yeast-manitol agar is another good choice for stab culture media. Longest survival times occur when storage of inoculated stabs is at 2 to 4°C ***(3,4)***.
14. Clear labels and good records are important components of all storage methods. Labels should note the strain name and any plasmids it contains. Records and labels should be numerically coordinated so that someone can easily find the strain and information regarding its source, genetic characteristics, and antibiotic resistances.
15. Drying the vermiculite after washing and after autoclaving is necessary to control the proportion of the vermiculite carrier to moisture during storage of the bacteria.

16. Extra tubes can be prepared with vermiculite, autoclaved, and kept at room temperature for later use. Pesenti-Berili et al. *(6)* found that a second autoclaving of tubes and vermiculite 24 h after the first autoclaving eliminated the small amount of contamination found in an earlier trial of the method.
17. The amounts of nutrient broth and YPDC given here are for storage of one strain with backups.
18. Stationary phase cells develop resistance to stress that exponential cells may not have. However, if your cells grow too long after stationary phase, they will be in "death phase" and begin to die off. For cells grown in YDPC, try for an OD_{600} between 1.8 and 2.0. Depending on the inoculum and your strain, this may take up to 2 d. A growth curve (OD_{600} vs time) is useful to predict the length of time it takes for a specific strain to reach stationary phase in any particular media.
19. The vermiculite will still seem very dry after inoculation. Tight capping of the tube will allow moisture to be distributed throughout the vermiculite gradually, desiccating the bacteria.
20. The protective effects of vermiculite are believed to be owing to its ability to buffer pH shifts and absorb inhibitory metabolites. At room temperature, storage time will be on the order of 6 or more months, whereas it is reasonable to expect viable cells after more than 1 yr of storage at 4°C. It is prudent to perform a viability count occasionally to obtain an indication of when it is wise to reestablish the stored strains.
21. Prepare one or two backup cultures for storage. If feasible, store them in different freezers (make an arrangement with a colleague) to provide security against freezer failure.

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