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UNIT 3D.1 Laboratory Maintenance of *Agrobacterium*

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Abstract

Agrobacterium species are plant-associated relatives of the rhizobia. Several species cause plant diseases such as crown gall and hairy root, although there are also avirulent species. *A. tumefaciens* is the most intensively studied species and causes crown gall, a neoplastic disease that occurs on a variety of plants. Virulence is specified by large plasmids, and in the case of *A. tumefaciens* this is called the Ti (tumor-inducing) plasmid. During pathogenesis virulent *agrobacteria* copy a segment of the Ti plasmid and transfer it to the plant, where it subsequently integrates into the plant genome, and expresses genes that result in the disease symptoms. *A. tumefaciens* has been used extensively as a plant genetic engineering tool and is also a model microorganism that has been well studied for host-microbe associations, horizontal gene transfer, cell-cell communication, and biofilm formation. This unit describes standard protocols for laboratory cultivation of *A. tumefaciens*.

Keywords

Growth media; Genetic analyses; Taxonomy; Opines; Plant association; Virulence; Plasmids; Attachment

Agrobacterium tumefaciens is a member of the α -Proteobacteria within the family *Rhizobiaceae*. It is a soil microbe and a facultative phytopathogen capable of causing crown gall disease on plants. *A. tumefaciens* is closely related to symbiotic species of nitrogen-fixing rhizobia. Its virulence depends on the presence of a specific plasmid known as the Ti (tumor-inducing) plasmid. For a comprehensive review of *Agrobacterium* biology, see Tzfira and Citovsky, 2008.

Due to the amount of genetic exchange and variation between species of *Agrobacterium*, the nomenclature of the group continues to be a point of controversy. Large plasmids such as the Ti plasmid, although not essential for survival in the laboratory, contribute enormously to the ecology and relevant phenotypes of the bacteria. In addition, these plasmids can be transferred horizontally between species, further complicating the issue. Historically, members of the genus *Agrobacterium* were classified at the species level according to their plasmid-determined phytopathogenic properties (i.e. *tumefaciens* for crown gall, *rhizogenes* for hairy root, *vitis* for grape infections, and *radiobacter* for non-pathogenic derivatives), and further subdivided into biovars I, II, and III (Table 1), based on other phenotypic and physiological characteristics. Genetic and genomic analyses have helped to more accurately define the phylogenetic relationship between different species so that in a more polyphasic method of nomenclature, *A. tumefaciens*, *A. vitis*, and *A. rubi* fall into one group, whereas *A. rhizogenes* and most *Rhizobium* species are associated with a second distinct lineage (Table 1). There is still some debate as to whether all *Agrobacterium* species should be

classified as the same genus *Rhizobium*, but for now the most widely accepted methodology for classification distinguishes between the two genera.

Within the *A. tumefaciens* species, strains and plasmids have also historically been classified by compounds called opines, which are produced by infected neoplastic tissue in response to the disease (Table 1). The incorporated T-DNA encodes genes that cause the plant to synthesize the opines, which can serve as sources of carbon, nitrogen and phosphorus for the infecting *A. tumefaciens* and certain other soil microbes. Opines are metabolized by *A. tumefaciens* via other genes encoded on the Ti plasmid that are not transferred to the plant. Historically plasmids have been named by their dominant opine, such as the broadly studied nopaline-type Ti plasmids and the octopine-type Ti plasmids (Table 1). Several other opine-based classification groups have also been commonly used. Upon isolation from infected plants, the isolates of *A. tumefaciens* were grouped by this scheme, and thus this classification is often also used to refer to the genomic background in which the Ti plasmid of a specific opine type was first isolated. For example, a commonly used laboratory strain is *A. tumefaciens* A348, which has a nopaline-type genetic background from which the resident nopaline-specifying Ti plasmid had been cured, and into which an octopine-type Ti plasmid was introduced. This informal opine-based classification remains in common usage today, despite the fact that most Ti plasmids specify the synthesis of more than one type of opine, and also that this catabolic capacity is encoded on the inherently unstable and horizontally transferrable Ti plasmid. One of the most commonly studied strains is *A. tumefaciens* C58 (isolated from a cherry tree in 1958 in upstate New York), which contains a nopaline-type Ti plasmid. Other common laboratory strains, 15955, R10, and A348 contain an octopine-type Ti plasmid. Different Ti plasmids possess the same incompatibility region and thus multiple types generally do not occur together in the same strain (Soberon, 2004).

The detection of a subset of opines by *A. tumefaciens* cells proximal to the infected tissue leads to the activation of the genes responsible for conjugal transfer of the Ti plasmid (*tra* and *trb*) and copy number (*rep*). This control is indirect, as opines induce the response to an externalized diffusible signal, *N*- β -oxo-octanoyl-L-homoserine lactone (3-oxo-C8 HSL), that functions in quorum sensing control of plasmid functions. This signal molecule interacts with specific transcription factors to increase copy number and conjugal transfer.

For nopaline-type Ti plasmids, such as in C58, the transcriptional regulator of the *tra* operons, *traR*, is repressed in the absence of the conjugal opines, agrocinopine A and B. When sufficient agrocinopines are present transcription of *traR* is derepressed, and if the local population density is high, a proportional increase in 3-oxo-C8-HSL leads to activation of TraR. TraR is stabilized by forming a complex with 3-oxo-C8-HSL molecules, and subsequently activates the transcription of Ti plasmid interbacterial conjugal transfer (*trb*) genes and increases its copy number. Because conjugation is completely dependent on the presence of opines, it is a process that only occurs in the tumor environment. For a second species of megaplasmid also harbored by many agrobacteria called the At plasmid, conjugation is not dependent on opines and contains regulatory elements more similar to *Sinorhizobium meliloti*'s mega plasmids, pSymA and pSymB (Blanca-Ordóñez et al. 2010)

All media described in this unit support the growth of *A. tumefaciens*. It is an obligately aerobic organism and thus, growing with proper aeration is critical. AT is a standard defined medium for growth, and thus is less likely to result in the development of auxotrophic mutants. The other media described in this unit are complex formulations or serve more specific purposes (e.g. biofilm-promoting and *vir*-inducing media), which are specified in the section.

Growth occurs optimally at 28°C. At temperatures above 30°C, *A. tumefaciens* begins to experience heat shock and is likely to result in errors in cell division (and is in fact an alternate strategy to Unit 3D.2: Protocol6 for plasmid curing).

If growth is slow in media containing sugar alcohols (*e.g.* mannitol), it may be due to iron limitation.

A. Growth Conditions

A. tumefaciens grows optimally at 28°C. The doubling time can range from 2.5–4h depending on the media, culture format and level of aeration.

AT Minimal Media (Tempe et al. 1977)

AT is a common laboratory medium used to support growth of *A. tumefaciens*. This minimal medium provides a non-stressful environment with all essential nutrients and limits the development of auxotrophic mutants. AT medium is the standard growth medium and will allow for a doubling time ranging from 2.5h 4h (dependent on specific carbon/nitrogen sources and antibiotics) and will approach stationary phase at an OD₆₀₀ of 0.9.

<u>20X AT Buffer</u>	<u>Grams added/L</u>	<u>Final conc. (1X)</u>
KH ₂ PO ₄	214	0.079 M
pH to 7.0 w/ NaOH	35	0.044 M
<u>20X AT Salts</u>		
(NH ₄) ₂ SO ₄	40	0.015 M
MgSO ₄ ·7H ₂ O	3.2	0.6 mM
CaCl ₂ ·2H ₂ O	0.2	0.06 mM
MnSO ₄ ·H ₂ O	0.024	0.0071 mM
<u>50X Iron Stock</u>		
FeSO ₄ ·7H ₂ O	34.75	0.125 M
<u>1X AT Medium</u>	<u>Volume/L</u>	<u>Final conc. (1X)</u>
20X Buffer	50 ml/L	1X
20X Salts	50 ml/L	1X
50% glucose	10 ml/L	0.5% (28 mM)
*50X Iron	20 ml/L	1X
+/-Bacto-Agar	15 g	1.5%

Prepare sterile, autoclaved stocks of 20X Salts, 20X buffer, iron solution and the carbon source (most often glucose). Add the appropriate amounts to either sterile deionized water (AT broth) or molten (55°C) sterile 1.5% water-agar (AT agar), mix gently but thoroughly, and use or pour plates.

The carbon and nitrogen sources can be varied. *A. tumefaciens* will utilize a variety of sugars, α-keto acids, amino acids and sugar alcohols as carbon sources, as well as a wide range of diverse nitrogen sources. AT minimal medium with glucose and (NH₄)₂SO₄ is generally referred to as ATGN. AT medium with octopine as both carbon and nitrogen source is called ATO, as carbon source alone ATON and as nitrogen source alone ATGO.

Iron should be added prior to pouring for solid medium, and in most cases to liquid medium as well. Only use an iron stock that does not have a great amount of precipitate.

Induction Broth (IB medium) (Winans et al., 1988)

This growth medium is thought to mimic aspects of the *vir*-inducing conditions of a plant wound. In this medium, Ti plasmid bearing cells grow markedly slower with a lower yield (due to the high cost of *vir* gene expression and phosphorous limitation) and will reach stationary phase at an OD₆₀₀ of approximately 0.2–0.4.

In shaken liquid cultures, clumps of cells can be easily visualized in the medium. It has been observed that even in very light cultures that appear clear, there are high numbers of cells likely attributable to this clumping and perhaps smaller cell size.

<u>1X IB Medium</u>	<u>volume/L</u>	<u>Final conc (1x)</u>
1M MES 2-(<i>N</i> -morpholino) ethane sulfonic acid (pH 5.6)	20 ml	20 mM
20X AT Salts	50 ml	1X
0.2 M acetosyringone	1 ml	200 µM
50% glucose	10 ml	0.5% (28 mM)
0.1 M phosphate buffer (pH 7.0)	500 µl	50 µM
mqH ₂ O	up to 1L	

Higg Medium (Poindexter, 1978)

This medium stimulates rapid growth and robust biofilm formation. HIGG will lead to shorter doubling times and can support higher cell densities.

<u>1X Higg Medium</u>	<u>Volume/L</u>	<u>Final conc. (1x)</u>
1M imidazole (pH 7.0)	5 ml	5 mM
Hunter Base Concentrate	20 ml	1X
1M CaCl ₂	1 ml	100 mM
50% glucose	3 ml	0.15% (83 mM)
10% Sodium Glutamate	15 ml	1.5% (89 mM)
1M NH ₄ Cl	8.9 ml	8.9 mM
0.5M Phosphate Buffer (pH 7.0)	20 ml	10 mM
mqH ₂ O	up to 1L	

* Filter sterilize and store in foil

Metals 44

Amount required	Component
250 mg	Disodium EDTA
1.095 g	ZnSO ₄ •7H ₂ O
500 mg	FeSO ₄ •7H ₂ O
154.0 mg	MnSO ₄ •H ₂ O
39.2 mg	CuSO ₄ •5H ₂ O

Amount required	Component
24.8 mg	$\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$
17.7 mg	$\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$
to 100 ml	mqH ₂ O

-mix components

-Add a few drops of H₂SO₄ to retard precipitation

-Filter sterilize

Hutner Base Concentrate (100 ml)	→ Prepare stock of ammonium molybdate (92.5mg/ml) in sterile mqH ₂ O and filter sterilize. Add 10 µl per 100 ml Hutner Base
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1.0 g	Nitrilotriacetic acid
1.445 g	MgSO ₄
0.3335 g	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
0.925 mg	$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$
5 ml	Metals 44
to 100 mL	H ₂ O

Dissolve nitrilotriacetic acid in water and neutralize with KOH (~10ml of 10N KOH)

Add remaining components, and adjust pH to 6.6–6.8

Adjust volume to 100 mL

Filter sterilize

Store in foil to prevent precipitation

500 mM Phosphate Buffer pH7.0	
61 ml	0.5M Na ₂ HPO ₄
39 ml	0.5M KH ₂ PO ₄

Mix and autoclave; pH should be ~7.0

Yeast Extract Mannitol (YEM)

This is a rich medium that supports *A. tumefaciens* growth very well. Alternatives include LB, although it has been demonstrated to lead to some issues with irregular cell division. *A. tumefaciens* grows particularly well using mannitol as a carbon source, but it should be noted that iron can become limiting during sugar alcohol catabolism. YEM will lead to shorter doubling times and can support higher cell densities.

1X YEM Medium	grams/L	Final conc (1x)
Mannitol	5	27 mM
Yeast Extract	0.5	--
MgSO ₄ × 7 H ₂ O	0.2	0.8 mM
NaCl	0.1	0.017 mM
K ₂ HPO ₄	0.5	0.027 mM
Sodium gluconate	5	22.9 mM
mqH ₂ O	up to 1L	

Prepare 16.6% CaCl₂ solution and autoclave.

Add 1 ml of the 16.6% CaCl₂ solution to 1L YEM medium.

COMMON ANTIBIOTICS – WORKING CONCENTRATIONS FOR AGROBACTERIUM

Concentrations of antibiotics that prevent growth of wild-type bacteria have been optimized for work in *A. tumefaciens* C58 and may vary with other strains of *Agrobacterium*.

Antibiotic	Working concentration (µg/ml)
<u>Kanamycin</u>	150
<u>Gentamycin</u>	300
<u>Streptomycin</u>	2500
<u>Spectinomycin</u>	150
<u>Ampicillin</u>	100
<u>Tetracycline</u> *	3–4
<u>Rifampicin</u>	25

Working concentrations provided are for liquid media conditions. They should be doubled when working with solid media.

* Tetracycline has been demonstrated for C58 to be problematic due to spontaneous resistance (see UNIT 3D.2, PROTOCOL 5 'Creation of Markerless Deletions by Allelic Replacement').

B. Storage Conditions

Short-term storage

For short-term storage of *Agrobacterium*, cells can be kept on agar media and stored at 4°C for several weeks. It is important to keep in mind however, that cells are still in a metabolically active state at these temperatures, so storage under these conditions should be limited. Cells should not be maintained by long-term passaging on plates because cells are likely to incur mutations.

Long-term storage

The ideal way to store *Agrobacterium* strains, ensuring that genetic changes due to laboratory selection are limited, is to keep them frozen at –80°C in 30% glycerol or DMSO (cryopreservants that prevent membrane damage caused by freezing and thawing). Once frozen permanents are generated, it is best to limit the amount of freezing and thawing as

much as possible. For starting cultures from frozen permanents, it is recommended to keep samples cold and use a sterile stick, loop or needle to remove a small clump of cells (1–5 μ l) and inoculate directly into liquid or solid media.

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Table 1Common strains of *Agrobacterium* by opine utilization and biovar type.

Species	Strain	Opines	Biovar
<i>A. tumefaciens</i>	C58	nopaline, agrocinopines A + B	1
	A6, 15955, B6, R10	octopine, mannityl opines	1
	A348 ^a	octopine, mannityl opines	1
	Bo542	agropine-succinamopine, agrocinopines C + D	1
	Chry5	chrysopine	1
<i>A. radiobacter</i>	K27	agrocinopines A + B	2
	K84	nopaline, agrocinopines A + B	2
<i>A. rhizogenes</i>	A4	agropine	2
<i>A. vitis</i>	S4	vitopine	

^aNopaline genetic background with pTiA6^{NC} Ti plasmid