AZOSPIRILLUM HANDBOOK

A GUIDE TO USING *AZOSPIRILLUM* AS A CHASSIS

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Azospirillum as a chassis

Azospirillum is one of the most broadly studied Gram negative, free living (facultative endophytic) plant growth promoting bacterium. *Azospirillum brasilense* Sp7 is an alpha-proteobacterium with a circular chromosome of size 3Mbp. It has six replicons (which includes chromids and plasmids) and around 5700 genes that encode for proteins. There is a well-documented foundation for experiments that involve genetic manipulations in *Azospirillum*. For transforming *Azospirillum* with genes of interest, standardized conjugation experiments are usually performed. These are biparental mating experiments that involve specific *E.coli* donor strains (like S17.1) and *Azospirillum* as the recipient. Protocols for these setups are established and are mentioned below.

One of its benefits include nitrogen fixation which is achieved by post-translational control of the enzyme nitrogenase. Through the activity of this nitrogenase complex, *Azospirillum* can convert atmospheric nitrogen to ammonia under microaerobic, low nitrogen conditions. Ammonium, glutamine, nitrite and nitrate are shown to repress the nitrogen fixing ability of *Azospirillum*. Azospirilla are surface colonizing bacteria and their attachment to the plant roots occur in two steps- the adsorption and the anchoring phase.

Azospirillum brasilense has an oxidative metabolism that is optimum under microaerophilic conditions. Their motility helps them seek low oxygen conditions by aerotactic mechanisms and monitoring the changes in the metabolic status by energy taxis.

Moreover, *Azospirillum* is capable of synthesizing phytohormones essential for moisture and nutrient uptake. These include Indole Acetic Acid (IAA) and abscisic acid (ABA) that are involved in drought resistance. *Azospirillum* can also contribute to high soil quality by solubilizing inorganic phosphorous and mitigating abiotic stress due to excessive compost and heavy metals. They limit the availability of required nutrients like iron to phytopathogens. They also have the potential to induce the synthesis of antioxidant enzymes in plants, thereby, reducing the deleterious effects of reactive oxygen species (ROS) produced under stress conditions.

Induced systemic resistance (ISR) is a plant defense mechanism that confers resistance to biotic stresses. It has been shown that inoculating rice plants with *Azospirillum brasilense* enhances the ISR via signaling pathways.

Shipping Azospirillum, handling and revival

We received *Azospirillum* cultures as Agar stabs which were shipped at room temperature. Upon receival, we streaked it on LBA plates containing the appropriate antibiotics. The antibiotic was ampicillin in our case because wild type *Azospirillum brasilense* has ampicillin resistant beta lactamase gene in its genome. Upon growth, glycerol stocks were made with 20% glycerol as back-ups.

Protocol to make Agar stabs:

1. In a 15 mL falcon tube, add 5 mL LBA or minimal malate media and 5 uL of the respective antibiotics.
2. Add 1 mL of this media to an eppendorf and allow it to cool down in a slanting position
3. Dry this for 30 mins under laminar flow
4. Take a 200 uL pipette tip and blunt the pointed end using a flame. Pick up a fresh wet colony using this tip and stab it once in the Agar.
5. Seal this eppendorf with a parafilm and incubate at the appropriate temperature overnight.
6. It is ready for shipment the next day!

The doubling time of *Azospirillum* is around 3 hours and hence, it takes a lot more time to grow on plates and liquid cultures compared to *E.coli*. Unlike *E.coli* plates which can be stored in 4 C cold rooms for long periods of time, *Azospirillum* can only be cultured at 30 C.This means that cultures of *Azospirillum* have to be continuously restreaked to maintain them. In the case of *Azospirillum brasilense*, we restreaked the colonies every 5-7 days.

Azospirillum lipoferum, on the other hand, had to be restreaked every alternate day for maintenance. It also requires low salt LBA for optimal growth which contains half the amount of sodium chloride as compared to normal LBA.

Media preparation

Azospirillum can be grown in *Luria Bertini* broth media and Minimal malate media.Malate media is used for culturing *Azospirillum* because it is one of the few organisms that can utilize malate as a preferred carbon source. Minimal Malate media is of two types: Nitrogen free broth (NFB-) and Nitrogen rich broth (NFB+).

Minimal malate media is made up of three components:

1. 20X salt solution
2. 20X malate solution
3. 20X phosphate solution

For preparing 300 mL of Nitrogen rich minimal malate media, the following are the compositions of the solutions:

- Salt solution composition

Chemicals	Amount
NH4Cl	5g
MgSO4.7H2O	2.34g
KCl	0.75g

CaCl2.2H2O	0.08g
FeSO4.7H2O	0.0185g

- Phosphate solution composition

Chemicals	Amount
K2HPO4	15 g
Nah2PO4	5g

- Malate solution composition

Chemicals	Amount
Malate	25g
NaOH	12.5g
5 M NaOH	

The malate solution should have a pH of around 6.8. We add 12.5 g of NaOH and check the pH using a pH meter. Then we add 5M NaOH as required till the pH reaches 6.8.

- Completing minimal media
  1. To prepare 300ml of complete minimal media, we add 15 mL of each of the components of minimal malate media in 255 mL of 1% agar solution.
  2. The 1% agar solution is prepared by adding 5.1 g of agar-agar in 255ml of ddH2O

- For preparing nitrogen free minimal malate media, we do not add ammonium chloride in the salt solution. The rest of the components are added as mentioned above.

For *Azospirillum lipoferum*, a special media called the low salt LB media is prepared. To prepare 300 mL of low salt LB media, the following composition is used.

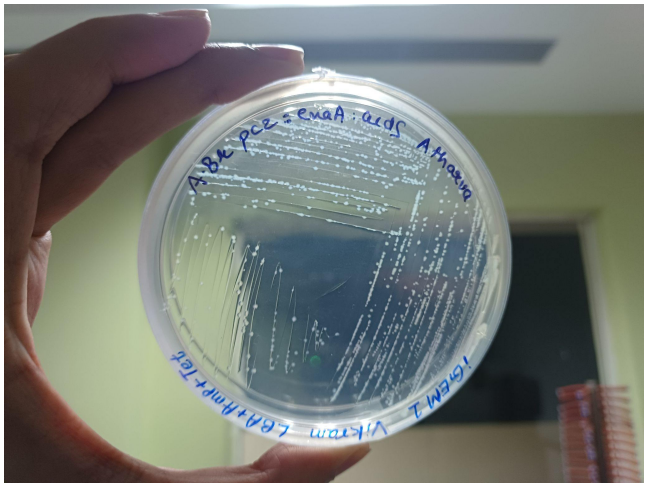
Chemicals	Amount
Tryptone type C	3 g
Yeast extract	1.5 g
Sodium Chloride	1.5 g
Agar (if solid media is required)	5.1 g

ddH2O	300 mL

Plating

Since *Azospirillum* plates are always kept in the 30 C incubator, they are parafilmed and kept so that the media faces upwards to prevent condensation. If tetracycline is used to make the plates, the plates are covered with aluminum foil as tetracycline is light-sensitive.

A five day old plate of wild type *Azospirillum brasilense*. The left side of the plate has been inoculated from a single colony from an old plate while the right side has been inoculated from the glycerol stock.



A one day old plate of trans conjugated *Azospirillum brasilense* containing the exaA:acdS construct in the pCZ plasmid.

Pellicle experiment

Pellicle experiments are used to monitor and study *Azospirillum* motility. It is used to study the aerotaxis of *Azospirillum* under different flagella mutations. The setups are done on a semi solid media in test tubes, where the hypoxic conditions gradually increase at increasing depths. We used these setups to generate a hypoxic environment to check our promoter activity. Hence, these setups can also be used for hypoxia-based experiments.

Step 1: Preparing primary culture

- ➔ Take 5 mL of complete minimal malate solution (NFB+) in test tubes
- ➔ Add appropriate antibiotics in appropriate concentrations.
- ➔ Scrape out single colonies from the respective plate using a pipette
- ➔ Discard the pipette tip inside the test tubes
- ➔ Keep the test tubes in an incubator at 30 C
- ➔ Wait until the OD becomes 1.

Step 2: Preparing secondary culture

- ➔ After the OD becomes 1, inoculate secondary culture
- ➔ Put 5 mL of minimal malate media in a test tube and add the appropriate antibiotics.
- ➔ Add 200 uL of primary culture to this test tube.
- ➔ Keep it in an incubator for around 8 hours.

Step 3: NFB- broth preparation

Add the following solutions in a 10 mL falcon:

1. 500 uL of phosphate solution
2. 500 uL of malate solution
3. 500 uL of N2 free salt solution
4. Add autoclaved ddH2O until the volume is 10 mL

Step 4: Inoculum preparation

- ➔ The secondary culture would have an OD of 0.8-0.9
- ➔ Collect 1 OD cells from the secondary culture
- ➔ Volume that is supposed to be collected = Desired OD/Actual OD
- ➔ Centrifuge the cells at 4000 rpm for 3 mins
- ➔ Discard the supernatant
- ➔ Take the pellet and resuspend it in 1 mL NFB- broth by tapping.

Step 5: 0.2% Agar (semi-solid media) preparation

- ➔ Dissolve 0.2 g Agar in 85 mL ddH2O. Autoclave this solution.
- ➔ Add 5 mL of each phosphate, N2 free salt and malate solutions

- ➔ To 30 mL solution of this, add 30 uL of ampicillin (the appropriate antibiotic)

Note: *Azospirillum brasilense* has ampicillin resistant beta lactamase genes in its genome.

Step 6: Inoculum setup

- ➔ In 5 ml of semisolid media that has still not solidified add the required antibiotics (appropriate with respect to the plasmid inserted in the chassis) and relevant reagents in appropriate amounts.
- ➔ Pipette 50 uL of cell mixture and very slowly and stably release it in the layer just below the top,
- ➔ Once inoculated, make sure that the test tube undergoes minimum shaking or movement and keep it at 30 C (for growth) or room temperature (if only movement has to be observed) in an extremely stable place.
- ➔ Check pellicle after 24-48 hours.

Genomic extraction protocol for Azospirillum

- ➔ Harvest cells from bacterial culture (2 mL) by centrifuging at 14,000 rpm for 2 mins. Collect the pellet and discard the supernatant.
- ➔ Resuspend cells in 567 uL of TE buffer.
- ➔ Add 30 uL of 10% SDS and 3 uL of proteinase K to the solution and invert mix it.
- ➔ Incubate at 37 C for 40-60 mins till the solution is colorless.
- ➔ Add 100 uL of 5M NaCl, mix by vortexing and add 80 uL of pre-warm CTAB solution.
- ➔ Mix it by vortexing and incubate at 65 C for 10 mins in a water bath.
- ➔ Add equal volume of PCI (Phenyl Chloroform Isoamyl alcohol). Mix it by vortexing for about 2 mins.
- ➔ Centrifuge at 14,000 rpm for 10 mins. Separate the upper aqueous layer in another eppendorf (take around 600 uL) and add 0.6x volume (usually 360 uL) of chilled isopropanol.
- ➔ Centrifuge at 14,000 rpm for 10 mins at room temperature.
- ➔ Discard supernatant and wash pellets with 1 mL of 70% ethanol.
- ➔ Add 0.7ml of 100% ethanol to 0.3 mL of water in a 1.5 mL eppendorf tube.
- ➔ Centrifuge at 14,000 rpm for 5 mins at room temperature.

- ➔ Discard supernatant and air dry the pellet in 37C for 5 mins.
- ➔ Add 50 uL of NFW, vortex properly and add 1 uL of RNAse.
- ➔ Incubate at 37 C for 1 hour. Store it in 4 C if required.

Conjugation experiment

Plating the donor and the recipient together:

- ➔ Day 1: Primary inoculation of *E.coli* S17.1 and *Azospirillum brasilense* Sp7 in LB
- ➔ Day 2: Secondary culture of S17.1 (50-100 ul in 5 mL LB) and Sp7 (250 uL in 5 mL LB)
- ➔ Monitor OD till it reaches 0.6- 0.8
- ➔ Collect 1:3 ratio of *E.coli* S17.1: *Azospirillum brasilense* and mix.
  For eg: Collect 300 uL of *E.coli* S17.1 and 900 uL of *Azospirillum brasilense* Sp7 and mix.
- ➔ Centrifuge at 4000 rpm for 3 mins. Discard the supernatant.
- ➔ Resuspend in 1 mL of 0.85% saline solution
- ➔ Add 100 uL of above mixture at the center of a blank plate dropwise
- ➔ Keep the plate without parafilm in a humid chamber at 30 C
- ➔ Check 2-3 days later for growth and then spread

Spreading and dilution:

- ➔ Take 1 mL of 0.85% saline solution in 1.5 mL eppendorf
- ➔ Using a 1 mL tip, take the central portion of *Azospirillum brasilense* Sp7 growth.
- ➔ It is observed that there is growth of *Azospirillum* in the center and surrounding its periphery, with a distinct margin, is *E.coli* growth
- ➔ Swirl and add to the 1 mL saline solution in the eppendorf
- ➔ Keep on scraping and swirling and mixing till nearly all *Azospirillum* has been removed from tip. Make sure not to touch *E.coli.*

- ➔ Pipette mix the saline solution and *Azospirillum* together using 1 mL tip.
- ➔ As the mixture will have some solid suspended, allow it to sediment by keeping it vertical for some time
- ➔ Meanwhile, take 4 eppendorf tubes for dilution and label them (-1,-2,-3 and -4)
- ➔ Make 5 plates having Ampicillin (for *Azospirillum*) and the corresponding antibiotic of the plasmid to be mobilized
- ➔ Label the 5 plates as Sp7:plasmid from 0 to -4
- ➔ Add 900 uL saline solution to the -1 tube and then add 100 uL of supernatant (having *Azospirillum*) formed from the previous tube.
- ➔ Continue doing the same for the subsequent tubes (serial dilution).
- ➔ For spreading, take 100 uL from each medium and drop it at various locations on separate plates and spread thoroughly.

PCR

The following modified master mix is used for PCR involving the *Azospirillum* genome.
Master Mix composition:

1. Taq polymerase - 1 ul
2. Taq buffer (10X)- 10 ul
3. Primers (forward and reverse)- 5 ul each
4. dNTP - 5 ul
5. DMSO - 5 ul
6. NFW- add so that the total volume is 100 ul
7. Template- 0.5 ul for genomic DNA and 2 ul of plasmid DNA
    - After adding all these components in a 1.5 ml eppendorf tube, pipette mix properly and distribute the master-mix in PCR tubes kept in ice
    - Centrifuge the PCR tubes for micro-seconds and keep the tubes in PCR machine
    - DMSO is added in the PCR master-mix because *Azospirillum* genome is GC rich.

PCR cycle
Initial denaturation: 95 C for 5 mins
Denaturation: 95 C for 1 min
Annealing: Temperature optimized by gradient PCR, Time: 1 min
Extension: 72 C for 1 min (for 1 kb gene), 2 min (for 2kb gene) and 3 min (for 3kb gene)
Storage: 4 C for infinity

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