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Bacteria-fungal Confrontation and Fungal Growth Prevention Assay

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[Abstract] There are some bacteria which can grow and multiply at the cost of living fungal biomass. They can potentially utilize fungi as a source of nutrients to forage over them. Such phenomenon is known as bacterial mycophagy, however, its mechanistic insights need to be explored to identify the molecules involved in mycophagy for potential utilization in controlling various fungal diseases. Recently we have demonstrated that a rice-associated bacteria *Burkholderia gladioli* strain NGJ1 exhibits mycophagous ability on several fungi, including *Rhizoctonia solani*, the necrotrophic fungal pathogen causing sheath blight disease in rice. We hereby describe our validated and efficient methods used to study *B. gladioli* strain NGJ1-*R. solani* interactions. These methodologies would be useful for designing assays to study the confrontation between bacteria and fungi which in turn enable discovery of novel antifungal molecules from such bacteria.

Keywords: *Burkholderia gladioli,* Bacterial mycophagy, Bacterial-fungal interaction, *Rhizoctonia solani,* Sheath blight disease

[Background] *Rhizoctonia solani* is an important plant pathogenic fungi with a wide range of hosts. It causes sheath blight disease in rice; the second most devastating fungal disease of rice after Blast disease (Fisher *et al*., 2012; Ghosh *et al*., 2014; Ghosh *et al*., 2017). In wake of developing control methods of sheath blight disease of rice, we isolated a bacterium with a broad spectrum antifungal activity from rice seedling. Based upon the rDNA and draft genome sequencing, the bacterium had been identified as *Burkholderia gladioli* strain NGJ1 (Jha *et al*., 2015). Beside antifungal property, we observed that the NGJ1 has the ability to forage over fungi and exhibit mycophagous ability on *R. solani* as well as various other tested fungi. Upon interaction with *R. solani* mycelia, NGJ1 growth was drastically enhanced. The NGJ1 imparted cell death response in *R. solani* and caused disintegration of fungal mycelia. We further established that the bacteria utilize type III secretion system to deliver a prophage tail-like protein (Bg_9562) to feed on *R. solani* (Swain *et al*., 2017).

Materials and Reagents

1. 1.5 ml and 2.0 ml microcentrifuge tubes (Tarson, catalog numbers: 500010 and 500020)
2. Pipette tips (10 µl) (Tarson, catalog number: 521000)
3. Pipette tips (200 µl) (Tarson, catalog number: 521010)

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4. Pipette tips (1,000 µl) (Tarson, catalog number: 521020)
5. 15 ml culture tubes (Riviera, catalog number: 71200155)
6. 30 ml culture tubes (Riviera, catalog number: 71200305)
7. Microscopic glass slides (GEM, catalog number: 051)
8. Toothpicks
9. Petri dishes (90 mm) (Tarson, catalog number: 460090)
10. *Rhizoctonia solani* AG1-IA strain (Lab collection)
11. *Burkholderia gladioli* strain NGJ1 (Lab collection)
12. Milli-Q water
13. Ethanol (Merck)
14. Potato dextrose agar (PDA) (HiMedia Laboratories, catalog number: GM096-500G)
15. Potato dextrose broth (PDB) (HiMedia Laboratories, catalog number: GM403-500G)
16. Sodium nitrate ($NaNO_3$) (Fisher Scientific)
17. Dipotassium phosphate (HiMedia Laboratories, catalog number: MB044-500G)
18. Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) (HiMedia Laboratories, catalog number: GRM684)
19. Potassium chloride (KCl) (HiMedia Laboratories, catalog number: MB043)
20. Ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) (HiMedia Laboratories, catalog number: GRM1377)
21. Dimethyl sulfoxide (MP Biomedicals, catalog number: 02196055)
22. Czapek dox broth (CDB) (HiMedia Laboratories, catalog number: M076-500G)
23. Czapek dox agar (CDA) (HiMedia Laboratories)
24. (Optional) KBA (HiMedia Laboratories, catalog number: M1544-500G)
25. Potato Dextrose Agar (PDA) (see Recipes)
26. Potato Dextrose Broth (PDB) (see Recipes)
27. Czapek dox agar (CDA) (see Recipes)
28. Czapek dox Broth (CDB) (see Recipes)

Equipment

1. Forceps
2. Stainless steel forceps (HiMedia Laboratories, catalog number: LA710)
3. Pipettes (Eppendorf-that can accommodate pipette tips of 10 µl, 200 µl, 1,000 µl)
4. Milli-Q water purification system (Merck, model: Milli-Q® Advantage A10,catalog number: Z00Q0V0WW)
5. Autoclave (INDFOS-110-PB)
6. Incubator and shaker (28 °C) (mrc, catalog number: LOM-65)
7. Microcentrifuge (TOMY DIGITAL BIOLOGY, model: MX-301)
8. Laminar Air Flow (Azbil Telstar, model: Telstar Bio II Advance, catalog number: EN 12469)

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9. Light microscope (Nikon, model: Eclipse E100)

Procedure

A. Bacterial-fungal confrontation assay on solid media

Note: All steps should be carried out in sterile conditions.

1. Pick a *Rhizoctonia solani* AG1-IA strain BRS1 sclerotium from approx. 2-week-old PDA plate using sterile toothpick/forceps, spot it at the center of fresh PDA plate (see Recipes) and incubate at 28 °C for a week. The *R. solani* sclerotium germinates to form mycelia which upon incubation produce plenty of black colored secondary sclerotia, the spore-like resting structures.
2. Pick a single colony of *B. gladioli* strainNGJ1 from PDA plate and inoculate it in 10 ml PDB broth (see Recipes) and incubate it for 48 h at 28 °C at 200 rpm. The bacterial number is quantified using serial dilution plating and colony counting.
3. Using sterile toothpick/forceps, place *R. solani* sclerotium at the center of PDA or CDA plate (see Recipes) and spot the *B. gladioli* strain NGJ1 culture (20 µl of 10^9 cells/ml) at four corners of the plate, equidistant from the center.
4. Further, incubate the plate at 28 °C and routinely monitor for bacterial spread over fungal mycelia (Figure 1; Please note the NGJ1 spreads over fungal mycelia, induces cell death response and feed on them).

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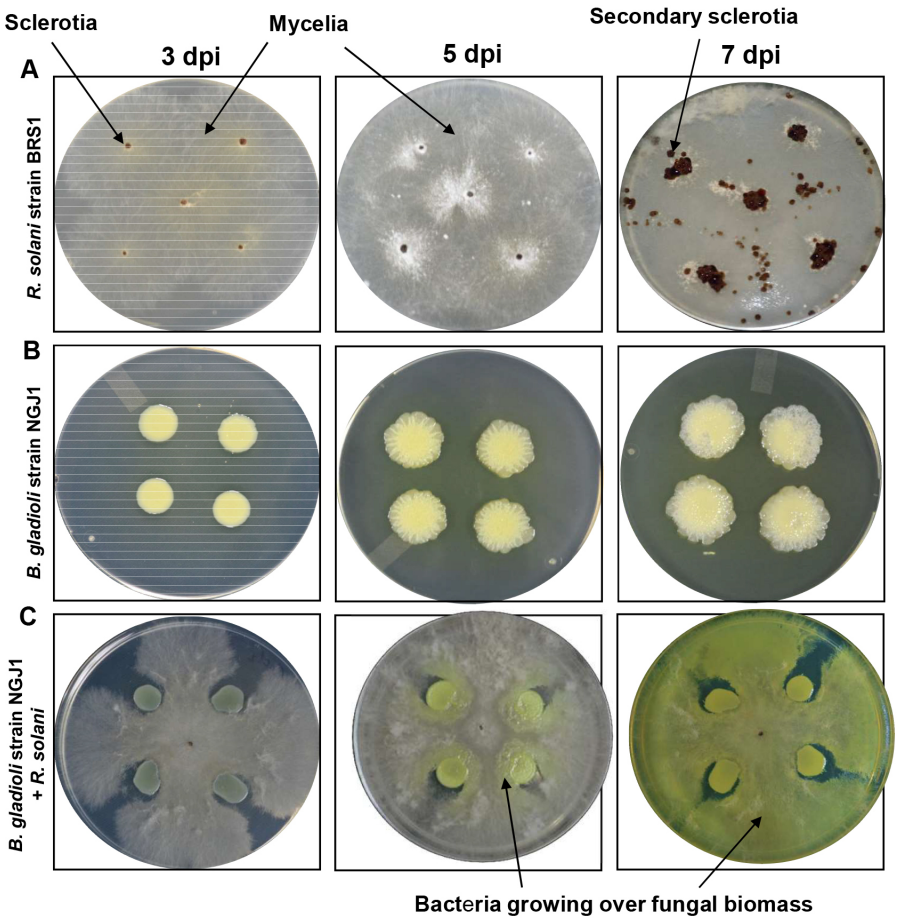

Figure 1. *B. gladioli* strain NGJ1 demonstrates mycophagy on *R. solani*. A. Growth pattern of *R. solani* sclerotia on PDA plates. During 3 and 5 dpi of growth, the fungal mycelia spread over petri-plate and by 7 dpi, plenty of secondary sclerotia are produced. B. Growth pattern of *B. gladioli* strain NGJ1 on PDA plates. Even during 7 dpi of growth, the bacteria failed to cover the entire plate (C) Confrontation of NGJ1 with *R. solani* on PDA plates. Initially by 3 dpi, the bacteria show limited antifungal activity but during 5 dpi, the bacteria started spreading over fungal biomass and by 7 dpi, the bacteria spread over entire fungal biomass.

B. Bacterial-fungal confrontation assay in liquid media

1. Collect fresh sclerotia from one-week-old plate of *R. solani* AG1-IA strain BRS1 using sterile toothpick/forceps. Inoculate 5-10 sclerotia in 10 ml PDB/CDB broth (see Recipes) and allow them to grow for 48 h at 28 °C with constant shaking (200 rpm) to obtain mycelial mass.
2. Pick a single colony of *B. gladioli* strain NGJ1 and inoculate it in 10 ml PDB/CDB broth at 28 °C for 48 h at 200 rpm.
3. 100 µl of 10^9 cells/ml culture of *B. gladioli* strain NGJ1 was inoculated into 10 ml PDB/CDB media containing 48 h pre-grown fungal mycelial mass.
4. Collect 100 µl of the bacterial solution at three different time points (24 h, 48 h and 72 h) of co-cultivation with pre-grown *R. solani* mycelia and upon serial dilution (10^5, 10^6, 10^7) plate 100 µl

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of bacterial solution on PDB/CDB plates. Use NGJ1 grown in absence of *R. solani* mycelia in PDB/CDB broth as a control.

5. Incubate plates at 28 °C for 24 h and count the bacterial colonies appeared on the plate.

C. Sclerotial growth prevention assay

1. Pick a single colony of *B. gladioli* strain NGJ1 and inoculate in 10 ml PDB broth and incubate at 28 °C for 48 h at 200 rpm.
2. Prepare 10 ml of 10^9, 10^7, 10^5, and 10^3 cells/ml dilutions of bacterial culture using sterile PDB.
3. Add 5-10 *R. solani* sclerotia in each of the diluted bacterial culture.
4. Incubate for 4 h at 28 °C with constant shaking at 200 rpm.
5. After 4 h, take out the treated sclerotia and individually place each sclerotium on fresh PDA plates.
6. For control, incubate the sclerotia for 4 h in PDB broth (without NGJ1).
7. Further, incubate plates at 28 °C and routinely monitor for mycelial growth.

D. Agar slide confrontation assay

1. Clean the microscopic glass slides with ethanol and autoclave them.
2. Prepare 1% agar solution (autoclaved) and gently pour 4 ml of it drop wise on the sterile glass slide to obtain thin agar layered slides. Let the agar solidify for 30 min in laminar air flow.
3. Place 2 freshly collected sclerotia on both ends of the thin agar layered slides, incubate the slides in a closed sterile Petri-plate having moist tissue paper (to maintain humid condition) and allow the sclerotia to germinate at 28 °C for 24 h.
4. Using a pipette, spot 20 µl of pre-grown culture (10^9 cells/ml) of *B. gladioli* strain NGJ1 at the center of the agar slides containing pre-grown *R. solani* mycelia.
5. Incubate at 28 °C under aseptic condition and using a light microscope, observe the slides (for bacterial spread and damage of mycelia) after 24 h, 48 h and 72 h of confrontation.

Data analysis

All the information about data processing, statistical tests, details of replicates and independent experiments was already included in original research paper (Swain *et al*., [2017]. A prophage tail-like protein is deployed by *Burkholderia* bacteria to feed on fungi. *Nature Communications.* 404(8). doi:10.1038/s41467-017-00529-0).

Notes

1. The *R. solani* sclerotial germination rate is not 100%, and this should be kept in mind while designing experiment.

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2. Apart from PDA and CDA; the bacterial-fungal confrontation can also be studied on King's Medium B base (KBA, HiMedia).

Recipes

1. Potato dextrose agar (PDA) for 400 ml
   40 g potato infusion form
   4 g dextrose
   6 g agar
   Add Milli-Q water up to 400 ml and autoclave the media
   Pour into sterile Petri plates, let the plates completely solidify and store them at 4 °C
2. Potato dextrose broth (PDB) for 400 ml
   40 g potato infusion form
   4 g dextrose
   Add Milli-Q water up to 400 ml, autoclave the media
3. Czapek doxagar (CDA) 400 ml
   12 g sucrose
   1.2 g sodium nitrate
   0.4 g dipotassium phosphate
   0.2 g magnesium sulfate
   0.2 g potassium chloride
   0.004 g ferrous sulfate
   6 g agar
   Add Milli-Q water up to 400 ml and autoclave the media
   Pour into sterile Petri plates, let the plates completely solidify and store them at 4 °C
4. Czapek dox broth (CDB) 400 ml
   12 g sucrose
   1.2 g sodium nitrate
   0.4 g dipotassium phosphate
   0.2 g magnesium sulfate
   0.2 g potassium chloride
   0.004 g ferrous sulfate
   Add Milli-Q water up to 400 ml and autoclave the media

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