Assessing selective plasmids for *Bradyrhizobium* sp. DOA9 and *Mesorhizobium loti*

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INTRODUCTION

- Plant growth is limited by the availability of usable forms of nitrogen, which they obtain from the soil where dinitrogen is reduced to ammonia by nitrogen-fixing bacteria.
- Synthetic nitrogen fertilizers produced through the Haber-Bosch process drove the green agricultural revolution throughout the 19th and 20th centuries, and have become essential for sustaining food supply for the growing global population.
- Excess production and use of nitrogen fertilizers can cause algal blooms, decreased coastal ocean oxygen levels, nitrous oxide greenhouse gasses, and soil acidification[1].
- Biological nitrogen fixation (BNF) in diazotrophic bacteria may provide a sustainable means of providing plants with nitrogen, but attempts to artificially engineer these features in plants have proven extremely difficult[2].
- Rhizobia are bacteria that intracellularly reside in specialized root nodules of legumes where they can perform BNF.
- The Karas team proposed that the engineering of Rhizobia to form intracellular symbioses with other eukaryotes could lead to synthetic nitrogen-fixing organelles, or SyNOrganelles[3].
- An important tool for engineering Rhizobia will be vectors that are compatible with Rhizobia, in addition to other model organisms for easier manipulation with existing tools.
- Brumwell et. al of the Karas team successfully developed multi-host shuttle (MHS) vectors that replicate in *Sinorhizobium meliloti* using origins from either of *S. meliloti*'s megaplasmids (Figure 1)[4].

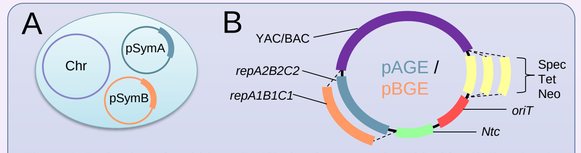


Figure 1. Derivation of multi-host (MHS) shuttle vectors for Rhizobia. A) The tripartite *S.meliloti* genome composed of a chromosome (Chr) and two megaplasmids (pSymA, pSymB). **B)** Components of MHS vectors derived from *S. meliloti repABC* origins, a Yeast and Bacteria Artificial Chromosome (YAC/BAC) backbone, an origin of transfer (*oriT*), a diatom selectable marker (*Ntc*), and Rhizobia selection markers (Spec, Tet, or Neo).

- The Karas team aims to approach the engineering of Rhizobia from different angles by experimenting with multiple strains. Like *S. meliloti*, *Mesorhizobium loti* was chosen for its existing research and potential as a model organism, and the less well-characterized *Bradyrhizobium* sp. DOA9 was chosen due to its unique ability to perform BNF under free-living conditions (outside of root nodules).
- Hence, the functionality of pAGE/pBGE in these additional strains was tested by determining appropriate antibiotic concentrations and delivering constructs by conjugation with the aid of pTA-Mob.

OBJECTIVE

Develop and assess tools for the engineering of additional Rhizobia species, *Bradyrhizobium* sp. DOA9 and *Mesorhizobium loti*, beginning with selective plasmids.

METHODS

- *Bradyrhizobium* sp. DOA9 was grown on Yeast Extract Mannitol (YEM) media[5], and *M. loti* on Tryptone Yeast Extract Mannitol (TY-M). 50 mL cultures were grown to an optical density (OD_{600}) of 1, centrifuged at 3000 rcf for 15 minutes, and resuspended in 1 mL of water.
- Wildtype sensitivity to antibiotics was tested by spot plating 5 µL aliquots from a 1 to 10^{-6} dilution series of 3 biological replicates for both Rhizobia species on various antibiotic concentrations. Plates were grown at 30℃ and examined after 1-5 days.
- Plasmid and antibiotic marker functionality was assessed by trans-conjugation from *E. coli* ECGE101 Δ*dapA* strains with the relevant constructs (Table 1) into Rhizobia.

Table 1. Constructs for testing delivery and antibiotic selection in *Bradyrhizobium* sp. DOA9 and *Mesorhizobium loti*. *Escherichia coli* ECGE101 Δ*dapA* strains were used to deliver constructs, and each contained pTA-Mob to encode conjugation machinery (except for with pTA-Mob2.0 which contains its own mobilization and can replicate in *S. meliloti*). Constructs contain *repABC* origins from *S meliloti.*

Construct	Antibiotic Resistance	Origin of replication
pAGE1.0	Spectinomycin (Spec)	pSymA
pAGE2.0	Tetramycin (Tet)	pSymA
pAGE3.0	Neomycin (Nm)	pSymA
pBGE1.0	Spec	pSymB
pBGE2.0	Tet	pSymB
pBGE3.0	Nm	pSymB
pTA-Mob2.0	Gentamycin (Gm)	unknown

- Conjugation experiments (Figure 2) were performed in groups based on antibiotic selection, with controls (per antibiotic) of a conjugation to DOA9 from *E. coli* with no construct (Mob negative), and a plate with water in place of *E. coli* (negative).

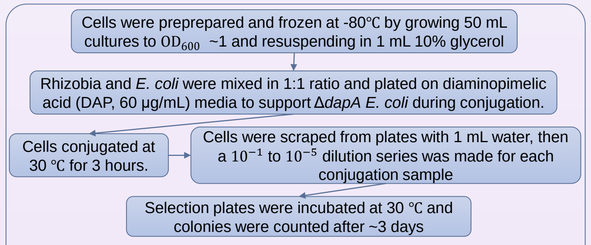


Figure 2. General protocol for Rhizobia conjugation experiments. Growth media were YEM for *B.* sp. DOA9 and TY-M for *M. loti.* Appropriate antibiotic concentrations were determined based on antibiotic assay results.

- Transconjugant colonies were passed on new plates three times, then screened by multiplex PCR (MPX) of DNA isolated from Rhizobia by alkaline lysis.
- MPX amplicons were analysed by agarose gel electrophoresis, with expected band sizes of 294 and 226 bp for the pAGE and pBGE origins, and 504, 456, and 390 bp for the Spec, Tet, and Neo markers, respectively.

RESULTS

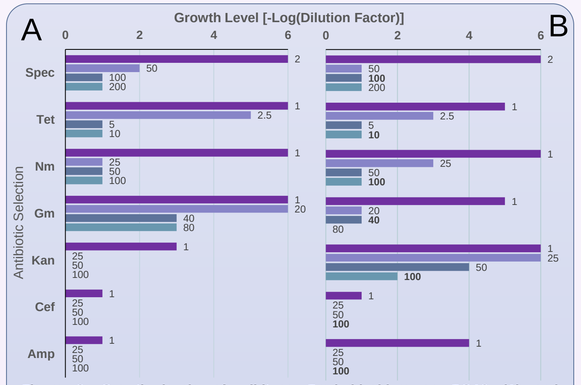
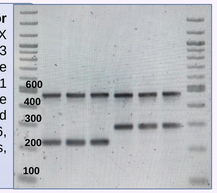


Figure 3. Growth levels of wildtype *Bradyrhizobium* sp DOA9 (A) and *Mesorhizobium loti* (B) under selection with various antibiotics. Spot plates (from a 1 to 10^{-6} dilution series of 3 biological replicates) were grown at 30℃ for ~4 days, and the highest dilution factor to which growth was observed is shown. Antibiotic concentrations (µg/mL) are indicated to the right of each bar.

Table 2. Colony counts for conjugation of Rhizobia multi-host shuttle (MHS) vectors from *Escherichia coli* to *Bradyrhizobium* sp. DOA9 (A) and Mesorhizobium loti (B), using various antibiotic markers. Controls consisted of conjugations with no MHS construct in *E. coli* ("Mob"), or no *E. coli* present ("-"). Confluent growth, colonies that are to high to count, and no growth are denoted by "conf", "thtc", and "✗".

	Antibiotic (µg/mL)	Sample	Colony Counts				
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A	Spec 200	pAGE1.0	8	✗	✗	✗	✗
		pBGE1.0	8	✗	✗	✗	✗
		Mob	✗	✗	✗	✗	✗
		-	✗	✗	✗	✗	✗
	Tet 10	pAGE2.0	conf	conf	thtc	thtc	76
		pBGE2.0	conf	conf	thtc	1040	128
		Mob	✗	✗	✗	✗	✗
		-	✗	✗	✗	✗	✗
	Nm 100	pAGE3.0	4	✗	✗	✗	✗
		pBGE3.0	7	✗	✗	✗	✗
		Mob	✗	✗	✗	✗	✗
		-	✗	✗	✗	✗	✗
	Gm 60	pTA-Mob2.0	167	11	✗	✗	✗
		Mob	50	20	8	✗	✗
		-	75	25	5	✗	✗
B	Spec 100	pAGE1.0	conf	conf	thtc	1369	214
		pBGE1.0	conf	conf	thtc	751	111
		Mob	✗	✗	✗	✗	✗
		-	✗	✗	✗	✗	✗
	Tet 5	pAGE2.0	conf	thtc	156	22	3
		pBGE2.0	conf	thtc	382	79	3
		Mob	✗	✗	✗	✗	✗
		-	✗	✗	✗	✗	✗

Figure 4. Agarose gel electrophoresis results for *Bradyrhizobium* sp. DOA9 Tet transconjugants. MPX was performed on DNA isolated by alkaline lysis from 3 colonies each (C1-3) on the 10^{-5} dilution plates of the pAGE2.0 and pBGE2.0 experimental samples. A Purple 1 kb plus DNA ladder is included on both sides, with the sizes indicated in bp. Expected band sizes are 294 and 226 bp for the pAGE and pBGE origins, and 504, 456, and 390 bp for the Spec, Tet, and Neo markers, respectively.



DISCUSSION

- Of the vector antibiotics tested in DOA9, Spec, Tet, and Nm all appear effective in both Rhizobia but slightly more-so in *M. loti*, except for Gm which was only partially effective in DOA9.
  - Kanamycin (Kan), Cefotaxime (Cef), and Ampicillin (Amp) were also tested in case of future use in constructs, and all 3 appeared very effective, except for Kan in *M. loti* which performed poorly.
- Hence, high antibiotic concentrations were used to sufficiently prevent background growth in DOA9 conjugations, amd lower concentrations were used with *M. loti* since they are more potent.
- For DOA9 conjugations, only Tet constructs resulted in substantial growth, suggesting that Spec200 and Nm100 were too strong. Future experiments will attempt conjugations with lower doses.
  - The pTA-Mon2.0 conjugation resulted in substantial growth on the negataive controls, in accordance with the poor effect of Gm on DOA9. Future experiments may use a different antibiotic marker.
  - Screening of Tet transconjugants results in bands matching the expected marker size, but the pAGE and pBGE origins appear reversed, possibly due to a switch while preparing cultures. Follow-up experiments are being performed to confirm this.
- Both Spec and Tet constructs appeared functional in *M. loti*. Nm and Gm constructs remain to be tested, and transconjugants remain to be screened.
- In summary, it has been shown that Rhizobia MHS vectors can be delivered by conjugation to *Bradyrhizobium* sp. DOA9 and *Mesorhizobium loti*, using Tet for DOA9, and Spec or Tet for M. loti as effective antibiotic markers.

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