Environmentally Induced Genes of Rhizobia

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Summary:

An *in vivo* selection strategy is being developed to investigate rhizosphere colonization and the early steps in symbiosis between *R.leguminosarum* and pea. *In vivo* expression technology (IVET) utilizes a mutant defective in survival in the environment that can be rescued for growth when complemented with a gene expressed downstream of a promoter probe library. We have shown that *purN* causes auxotrophy in the environment. *purN* deletion mutants of *Rhizobium leguminosarum* 3841 and *Sinorhizobium meliloti* 1021 are constructed. Vectors to contain the promoter probe library have been made containing the rescue gene and a marker gene to analyze gene expression.

Introduction:

Bacteria that form the family Rhizobiaceae can interact with leguminous plants, which allows a metabolic symbiosis to occur. The bacteria reduce atmospheric nitrogen to ammonia in specialised root organs called nodules. The nitrogen is then exported to the plant in exchange for root exudates. Much is known about the molecular basis for nitrogen fixation and the processes allowing nodulation and fixation to occur. However, little is known about the molecular basis for rhizobial survival in the rhizosphere. A strategy is sought to develop a system to isolate and characterise rhizosphere- and symbiotic-adaptation specific genes. *In vivo* expression technology (IVET) can be used to isolate environmentally induced genes[1]. In this work, an IVET strategy utilising a purine mutant is developed, as purine mutants of rhizobia are deficient in nodulation[2], nitrogen fixation and rhizosphere survival. This project's main objective is to develop and utilise an IVET strategy to gain a greater understanding of the molecular basis of adaptation of Rhizobia to their environment. Combining this strategy with 3 fully sequenced Rhizobia (*S. meliloti, M. loti, R. leguminosarum* (in progress)) will provide a powerful tool for understanding rhizobial-environment interactions.

Construction of a Purine Auxotroph:

To make a purine mutant, a deletion in the final gene in the purine biosynthetic pathway was made using crossover PCR[3] and homologous recombination, replacing the gene with an antibiotic resistance encoding interposon. See figure 1 for rationale of crossover PCR.

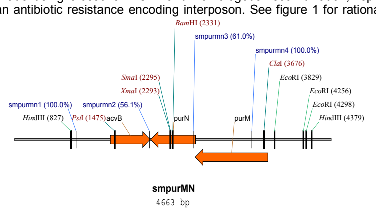


Fig 1: Map of *S. meliloti purMN* region. Arrows indicate genes and direction of transcription. In blue are primers designed for crossover PCR. Green lines indicate restriction sites present.

The 2 PCR products made using primer pairs either side of *purN* were mixed and amplified using the outermost primers to create a 2Kb product of the region without *purN*. This was then cloned into pCR2.1 TOPO (Invitrogen), and the fragment cloned into pJQ254 as a blunt *eco*RV *smaI* clone. A spectinomycin/streptomycin cassette was inserted via *Bam*HI sites in the middle of the crossover product. This was then cloned into pJQ200SK via NotI restriction sites and conjugated into *S. meliloti* 1021. Double recombinants were selected on TY Sucrose, spectinomycin, streptomycin plates. See figure 2 for cloning steps.

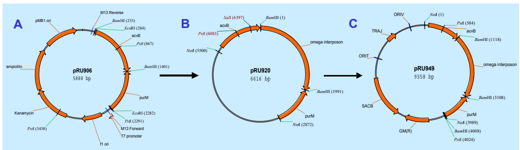


Fig 2: Construction of deletion vector. A: crossover product in pCR2.1 TOPO. B: pJQ254 base vector with crossover product and interposon to replace *purN*. C: Final vector, crossover and interposon transferred into plasmid pJQ200SK.

Construction of an IVET Vector:

A vector has been constructed to deliver the promoter probe library into *R. leguminosarum* 3841. An IVET vector may be integrating or non-integrating, and there are advantages and disadvantages to both. We constructed the main part of the vector in a broad host range, medium copy number, mobilisable plasmid, pOT2 (pBR322 derivative) to test the functionality of the construct; e.g, did the GFP express downstream of *purN* under the control of an inducible promoter. The contruct (polylinker, *purN*, GFP) was then tranferred to a mobilisable plasmid pRU1061, a GUS derivative of pK18mob, which cannot replicate in *Rhizobium*. Maps of these constructs are shown below (Figure 3).

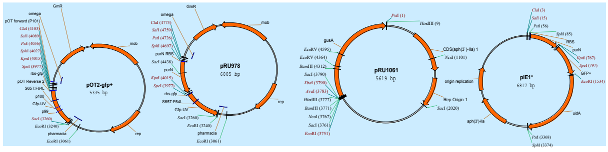


Fig 3: Construction of IVET vector. pOT2-gfp+ is a pBR based vector containing a modified GFP so that it is more bright than standard GFP-uv. In the polylinker, *purN* (rescue gene) has been cloned, containing its own ribosome binding site to create the plasmid pRU978. The polylinker, *purN*, GFP and translational stop codons were then taken and cloned into pRU1061 to create a plasmid that is the final IVET vector – pIE1.

Testing for correct expression of GFP under inducible conditions.

A dctA promoter from *R. leguminosarum* was cloned upstream of *purN* in the polylinker library cloning site. The dctA promoter is switched on in response to the presence of dicarboxylic acids. The plasmid was conjugated into *R. leguminosarum* 3841, and grown on minimal media with either succinate/ammonia or glucose/ammonia as carbon and nitrogen sources. GFP was expressed in bacteria grown on succinate, but not in bacteria grown on glucose. Figure 4 illustrates the difference in expression under inducible and non-inducible conditions. This experiment shows that GFP is not effected by being expressed downstream of a rescue gene, and its expression is inducible, not constitutive.

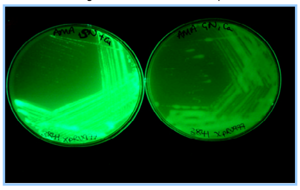
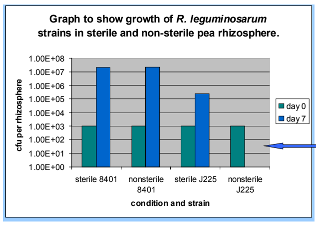


Fig 4: R. leguminosarum 3841, carrying a plasmid expressing *purN* and GFP downstream of an inducible promoter, dctAp (3841 pRU977). The left plate shows expression of GFP in inducible conditions (presence of dicarboxylates) and the right plate shows that in non-inducing conditions there is no background GFP expression.

Growth Characteristics of Purine Mutants of Rhizobia:

A purine transposon mutant of *Rhizobium leguminosarum* A34 was grown on pea in non-sterile and sterile conditions. It was found that after 7 days growth, the mutant was at undetectable levels in non-sterile conditions, but was comparable to wild type level growth in sterile conditions.

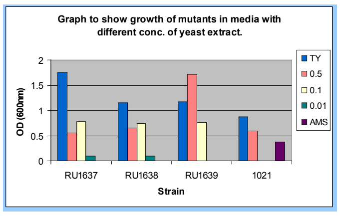


J225 below limits of detection (10^3cfu/ml)

From this data, and from published data, it was possible to conclude that a purine mutant would be a suitable auxotroph for an IVET strategy.

Growth of Purine Auxotroph in Rich and Purine Free Media:

The mutants isolated from the conjugation of pRU949 into S. *meliloti* 1021 were grown in rich liquid media (TY) or minimal liquid media containing various concentrations of a purine supplement (AMS +/- yeast extract) for 3 days. The optical densities were read and the graph below shows the data. It indicates that the mutants are impaired for growth without the presence of exogenous purine. This will help the selection of *in vivo* induced promoters rather than constitutively-on promoters.



Future Work:

[illegible] *in vivo* induced genes. Characterisation of the genes by mutagenesis and phenotypic analysis. Comparative genomics between sequenced rhizobial strains. Other work could include confocal microscopy to visualise the induction of genes in the rhizosphere, sectioning and staining of nodules to look for induction of genes.

References:

1. Rainey, P.B. (1999) Adaptation of *Psuedomonas fluorescens* to the plant rhizosphere. *Env. Microbiol.* **1:**243-257.
2. Djordjevic, S.P. et al. (1996) The addition of AICAR to nodulation-defective purine auxotrophs of NGR234 restores bacterial growth but leads to novel root outgrowths on Siratro. *MPMI* **9:** 114-124
3. Horton, RM. et al. (1989) Engineering hybrid gene without the use of restriction enzymes; gene splicing by overlap extensions. *Gene* **77:** 61-68.