Why are the symbioses between some genotypes of *Sinorhizobium* and *Medicago* suboptimal for N_2 fixation?

by

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DECLARATION

I hereby declare that, unless otherwise stated, the work presented in this thesis is my own and has not been submitted for a degree at any other institution.

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Jason James Terpolilli

To all my family who

left their homes many years ago

with hopes, dreams and fears,

in search of a better life.

We are too much accustomed to attribute to a single cause that which is the product of several, and the majority of our controversies come from that.

Marcus Aurelius

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ABBREVIATIONS

A_{260}	Absorbance at λ = 260 nm
A_{280}	Absorbance at λ = 280 nm
AM3	Antibiotic medium 3
AHL	Acyl homoserine lactone
BLAST	Basic local alignment search tool
BP	Base pair
CR-YMA	Congo red-yeast mannitol agar
DEPC	Diethylpyrocarbonate
DI	Deionised
dpi	Days post inoculation
DsRed	*Discosoma* sp. red fluorescent protein
EDTA	Ethylenediamine tetraacetic acid
EPS	Exopolysaccharide
EPS I	Exopolysaccharide I, also known as succinoglycan
EPS II	Exopolysaccharide II, also known as galactoglucan
GFP	Green fluorescent protein
Gm	Gentamycin
HMW	High molecular weight
HPLC	High performance liquid chromatography
IS	Insertion sequence
LA	Lupin agar
LB	Luria-Burtani
LBMC	Luria-Burtani with Mg^{2+} and Ca^{2+}

LMW	Low molecular weight
LSD	Least significant difference
MES	Morpholinoethanesulfonic acid
MGT	Mean generation time
MW	Molecular weight
NA	Nutrient agar
OD_{420}	Optical density at $\lambda = 420$ nm
OD_{600}	Optical density at $\lambda = 600$ nm
ONPG	O-Nitrophenyl-β-galactoside
PCR	Polymerase chain reaction
Rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
Sp	Spectinomycin
SSC	Saline-sodium citrate
Sm	Streptomycin
Tn*5*	Transposon 5
Tc	Tetracycline
Tp	Trimethoprim
Tris	Tris(hydroxymethyl)aminomethane
TY	Tryptone yeast (equivalent to TYC)
YMA	Yeast mannitol agar

SUMMARY

The conversion of atmospheric dinitrogen (N_2) into plant available nitrogen (N), by legumes and their prokaryotic microsymbionts, is an integral component of sustainable farming. A key constraint to increasing the amount of N_2 fixed in agricultural systems is the prevalence of symbioses which fix little or no N. The biotic factors leading to this suboptimal N_2 fixation have not been extensively analysed. Using the widely studied and cultivated perennial legume *Medicago sativa* and the model indeterminate annual legume *Medicago truncatula* with the sequenced bacterial microsymbiont *Sinorhizobium meliloti* 1021 (Sm1021) as a basis, the work presented in this thesis examined the effectiveness of N_2 fixation in these associations and in other comparable systems and investigated factors which lead to the establishment of suboptimally effective symbioses.

The ability of Sm1021, *S. medicae* WSM419 and the uncharacterised *Sinorhizobium* sp. WSM1022 to fix N with *M. truncatula* A17, *M. sativa* cv. Sceptre and a range of other *Medicago* spp. was evaluated in N-limited conditions. As measured by plant shoot dry weights and N-content, Sm1021 was partially effective with *M. truncatula* A17 whereas WSM1022 and WSM419 were both effective with this host in comparison to nitrogen-fed (N-fed) control plants. In contrast, Sm1021 and WSM1022 were effective with *M. sativa* while WSM419 was only partially effective. Nodules induced by Sm1021 on *M. truncatula* A17 were more numerous, paler, smaller in size and more widely distributed over the entire root system than in the two effective symbioses with this host. On the contrary, nodule number, size and distribution did not differ between these three strains on *M. sativa*. WSM1022 was effective on *M. littoralis*, *M. tornata* and two other

cultivars of *M. truncatula* (Jemalong and Caliph) but Sm1021 was only partially effective on these hosts. These data indicate that the model indeterminate legume symbiosis between *M. truncatula* and Sm1021 is not optimally matched for N_2 fixation and that Sm1021 possesses broader symbiotic deficiencies. In addition, the interaction of WSM1022 with *M. polymorpha* (small white nodules but does not fix N), *M. murex* (does not nodulate), *M. arabica* (partially effective N_2 fixation) and *M. sphaeorcarpus* (partially effective N_2 fixation), and the sequence of the 16S rDNA, are all consistent with this isolate belonging to the species *S. meliloti*.

The colony morphology of TY, half-LA and YMA agar plate cultures of Sm1021, WSM419 and WSM1022 suggested differences in EPS profiles between these strains. Sm1021 is very dry, compared to the mucoid WSM419 and extremely mucoid WSM1022. Sm1021 is known to carry an insertion in *expR* rendering the gene non-functional and resulting in the dry colony phenotype. WSM419 has an intact copy of *expR*, while the *expR* status of WSM1022 is not known. Rm8530, a spontaneous mucoid derivative of Sm1021 with an intact *expR*, was significantly less effective with *M. truncatula* than Sm1021, but there was no difference in effectiveness between these strains on *M. sativa*. The effectiveness of Sm1021, when complemented with a plasmid-borne copy of *expR* from Rm8530, was significantly reduced on *M. truncatula* but not *M. sativa*, implicating a functional *expR* as being the cause of reduced N_2 fixation observed with Rm8530 on *M. truncatula*. ExpR could reduce the effectiveness of Rm8530 by acting as a negative regulator of genes essential for symbiosis with *M. truncatula*, or by altering the quantity or structure of succinoglycan and/or galactoglucan produced. These data support the emerging view of ExpR being a central regulator of numerous cellular processes.

The timing of nodulation between Sm1021 and WSM419 on *M. truncatula* and *M. sativa* was investigated. Compared to the other symbioses analysed, the appearance of nodule initials and nodules was delayed when *M. truncatula* was inoculated with Sm1021 by 3 and 4 days, respectively. To explore whether events during early symbiotic signalling exchange could account for these observed delays, leading to the establishment of a suboptimal N_2-fixing symbiosis, a novel system was developed to compare the response of the Sm1021 transcriptome to roots and root exudates of *M. truncatula* A17 and *M. sativa* cv. Sceptre. This system consisted of a sealed 1 L polycarbonate chamber containing a stainless steel tripod with a wire mesh platform on which surface-sterilised seeds could be placed and allowed to germinate through the mesh, into a hydroponic medium below. After germination, Sm1021 cells were inoculated into the hydroponic solution, exposed to the roots and root exudates for 16 h, harvested and their RNA extracted. Comparison of Sm1021 mRNA from systems exposed to *M. truncatula* or *M. sativa* revealed marked differences in gene expression between the two. Compared to the no plant control, when *M. sativa* was the host plant, 23 up-regulated and 40 down-regulated Sm1021 genes were detected, while 28 up-regulated and 45 down-regulated genes were detected with *M. truncatula* as the host. Of these, 12 were up-regulated and 28 were down-regulated independent of whether *M. truncatula* or *M. sativa* was the host. Genes expressed differently when exposed to either *M. truncatula* or *M. sativa* included *nex18, exoK, rpoE1* and a number of other genes coding for either hypothetical proteins or proteins with putative functions including electron transporters and ABC transporters. Characterisation of these differentially expressed genes along with a better understanding of the composition of *M. truncatula* root exudates would yield a clearer insight into the contribution of early signal exchange to N_2 fixation.

Comparison of the regulation of nodule number between Sm1021 and WSM419 on *M. truncatula* and *M. sativa* revealed nodule initials at 42 days post-inoculation (dpi) on *M. truncatula* inoculated with Sm1021. In contrast, no new nodule initials were present 21 dpi on any of the other interactions examined. Moreover, analysis of nodule sections revealed that the number of infected cells in *M. truncatula*-Sm1021 nodules was less than for comparable symbioses. These data suggest that nodule number is not tightly controlled in the *M. truncatula*-Sm1021 association, probably due to N_2 fixation being insufficient to trigger the down regulation of nodulation. Quantification of N_2 fixation activity in this and other more effective symbioses is required.

The poor effectiveness of the *M. truncatula*-Sm1021 symbiosis makes these organisms unsuitable as the model indeterminate interaction and the implications for legume research are discussed. The recently sequenced WSM419 strain, revealed here to fix N_2 more effectively with *M. truncatula* than Sm1021, may be a better model microsymbiont, although WSM419 is only partially effective for N_2 fixation with *M. sativa*. The sequencing of *S. meliloti* WSM1022, a highly effective strain with both *M. truncatula* and *M. sativa*, would provide a valuable resource in indentifying factors which preclude the establishment of effective symbioses.