Requirement for Exopolysaccharide in the *Mesorhizobium-Lotus* Symbiosis

Simon John Kelly

MSc

A thesis submitted for the degree of
Doctor of Philosophy
The University of Otago, Dunedin
New Zealand

June 2012
Abstract

The establishment of a successful symbiotic interaction between rhizobia and legumes requires complex molecular communication between the two partners in order to determine compatibility and co-ordinate symbiotic developmental responses. Although a symbiotic association, the invasion of plant nodules by rhizobia bears parallels to plant pathogen interactions with a successful outcome requiring either avoiding recognition by the host plant’s innate immune system or suppressing its activation. Exopolysaccharide (EPS) production is ubiquitous amongst rhizobia and the aim of this work was to determine its function in the *Mesorhizobium-Lotus* symbiosis. Evidence is presented that wild-type EPS may suppress a defence response and, in its absence, truncated EPS molecules produced by certain EPS mutants may cause the bacterium to be recognised as a foe. Other EPS mutants may remain blind to the defence system.

Colony and symbiotic phenotypes of a range of *M. loti* R7A EPS mutants were examined on *Lotus corniculatus* and *L. japonicus* Gifu. Strains disrupted in the early stages of EPS biosynthesis were symbiotically proficient (e.g. *exoB* and, to a lesser extent, *exoA* mutants), whilst strains affected in later stages (e.g. *exoU* mutants) were severely impaired at the stage of infection thread initiation.

EPS extracts isolated from wild-type *M. loti* and mutant strains were chemically characterised and a proposed structure and biosynthetic pathway for R7A EPS was determined. R7A produces an O-acetylated acidic EPS that is an octasaccharide consisting of glucose, galactose, glucuronic acid and riburonic acid residues. R7A EPS mutants produced EPS fractions that contained varying glycosyl linkages, indicating variations in low-molecular-weight EPS production and/or alternative polysaccharides depending on the particular mutant strain.

Strains R7AexoB and R7AexoU that exhibit contrasting symbiotic phenotypes were thoroughly examined. The results suggested that R7AexoB was symbiotically proficient due to either complementary signalling by an alternative polysaccharide in the absence of EPS or the absence of surface polysaccharides that normally elicit a plant defence response. R7AexoU was severely impaired in nodulation, most likely
due to the production of a truncated EPS molecule that is actively perceived by the plant resulting in the activation of a defence response. Pre-inoculation results suggested that the defence response elicited by R7AexoU could be dampened by wild-type R7A but not by R7AexoB. Co-inoculation studies with a ΔnodA mutant suggested that EPS is required both for infection thread initiation and for release from the infection thread into the nodule primordia.

Several symbiotically-proficient exoU suppressor mutants were isolated following transposon mutagenesis. The insertions in these strains were in the exoA or exoL genes involved in the early stages of EPS biosynthesis, or in four genes not previously linked to EPS biosynthesis. Mutagenesis in an R7A background indicated that the four novel genes were not involved in wild-type EPS production; however, the colony phenotypes of the suppressor mutants suggested that their symbiotic proficiency was due to the prevention of truncated EPS production by R7AexoU.

Various mutation and complementation approaches were employed to investigate if EPS produced by *M. loti* NZP2037 is involved in the strain’s unique symbiotic abilities. Results suggested that it was not EPS but perhaps lipopolysaccharide or capsular polysaccharide produced by NZP2037 that was responsible for the observed differing symbiotic ability compared to other *M. loti* strains.

The results obtained in this study support a signalling role for *M. loti* EPS that acts to suppress host defence responses, allowing for infection thread formation and the development of nitrogen-fixing nodules.
Acknowledgements

Firstly I would like to thank my supervisor Professor Clive Ronson for his support and encouragement throughout my years in lab 607. I am forever grateful for his knowledge and the time spent discussing my project and helping me along the way.

I would also like to thank all of the staff and students of lab 607 and the 6th floor of the Department of Microbiology and Immunology for their support during my PhD. A special thanks to Dr John Sullivan for his endless assistance during my years at the lab bench.

I am extremely grateful to Professor Jens Stougaard for the opportunity to work in the laboratory of gene expression, Aarhus, Denmark and to be involved in the CARB programme. I have enjoyed attending the CARB retreats and would like to thank everyone involved in CARB, especially Drs Niels Sandal and Yasuyuki Kawaharada, for their assistance, discussion and ideas that have helped me throughout my PhD.

I was also fortunate to spend time in Professor Russell Carlson’s laboratory at the Complex Carbohydrate Research Center, Athens, GA, USA. I am appreciative of his and Dr Artur Muszynski expertise and guidance during my time at the Center and in subsequent email/ Skype communications.

Financial support provided by the University of Otago (PhD Scholarship) was invaluable and I am also thankful for the financial assistance provided by the Department of Microbiology and Immunology and CARB that allowed me to attend conferences and work in labs overseas, these were experiences I will never forget.

Finally I would like to thank my family and my extremely patient partner Rebecca for all their love and support that have helped me through my PhD.
# Table of Contents

Abstract ........................................................................................................................................... i

Acknowledgements ........................................................................................................................ iii

Table of Contents ............................................................................................................................... iv

List of Tables ........................................................................................................................................ ix

List of Figures ...................................................................................................................................... x

List of Abbreviations ........................................................................................................................ xiii

1 Introduction ................................................................................................................................. 1

1.1 Introduction ................................................................................................................................. 2

1.2 *Mesorhizobium loti* .................................................................................................................... 3

1.3 *Lotus* legumes ........................................................................................................................... 4

1.4 The symbiotic infection process ................................................................................................. 5

1.4.1 Nodule structure ..................................................................................................................... 7

1.5 Molecular communication .......................................................................................................... 9

1.5.1 Legume signals that induce rhizobial *nod* gene expression ............................................... 9

1.5.2 Nod factor structure and synthesis ....................................................................................... 11

1.5.3 Nod factor receptors ............................................................................................................. 13

1.6 Rhizobial polysaccharides ......................................................................................................... 14

1.6.1 Exopolysaccharide (EPS)......................................................................................................... 16

1.6.2 Lipopolysaccharide (LPS) ...................................................................................................... 27

1.6.3 K-antigens (KPS) / capsular polysaccharide ......................................................................... 30

1.6.4 Cyclic-β-glucans (CBGs) ........................................................................................................ 33

1.6.5 Cellulose ................................................................................................................................ 35

1.7 The involvement of plant pathogen surface polysaccharides in evasion of innate immunity ................................................................................................................................. 38

1.8 Summary of MSc research ......................................................................................................... 41

1.8.1 R7A EPS biosynthesis ............................................................................................................. 41

1.8.2 Symbiotic phenotypes of R7A EPS mutants .......................................................................... 43

1.9 The aim of this study .................................................................................................................. 45

2 Materials and methods ................................................................................................................ 46
2.1 Bacterial strains and plasmids ................................................................. 47
2.2 Media and growth conditions ................................................................. 47
2.3 Storage of bacterial strains ...................................................................... 47
2.4 Enzymes and chemicals .......................................................................... 47
2.5 Spectroscopy .......................................................................................... 54
2.6 DNA isolation and manipulation ............................................................. 54
  2.6.1 Plasmid DNA extraction .................................................................. 54
  2.6.2 Genomic DNA isolation .................................................................. 54
  2.6.3 Colony-crack DNA isolation ......................................................... 55
  2.6.4 Restriction enzyme digestion ....................................................... 55
  2.6.5 Agarose gel electrophoresis .......................................................... 55
  2.6.6 Southern hybridisation .................................................................. 56
  2.6.7 Polymerase chain reaction (PCR) .................................................. 57
  2.6.8 DNA sequencing ........................................................................... 61
  2.6.9 Computer analysis .......................................................................... 61
  2.6.10 Cloning of DNA fragments ........................................................... 61
  2.6.11 Preparation of electrocompetent cells .......................................... 62
  2.6.12 Electrotransformation ................................................................... 62
  2.6.13 Preparation of an M. loti NZP2037 cosmid library ....................... 63
  2.6.14 Transfer of plasmids by conjugation ............................................ 64
2.7 Mutagenesis ............................................................................................ 65
  2.7.1 Insertion duplication mutagenesis (IDM) ...................................... 65
  2.7.2 In-frame markerless deletion mutagenesis ................................... 65
  2.7.3 Transposon mutagenesis .............................................................. 69
2.8 Cellulase assay ....................................................................................... 69
2.9 Hydrophobicity assay ............................................................................ 69
2.10 Plant studies .......................................................................................... 69
  2.10.1 Seedling preparation ...................................................................... 70
  2.10.2 Split-root seedling preparation ...................................................... 70
  2.10.3 Plant inoculation ........................................................................... 70
  2.10.4 Plant growth conditions ............................................................... 71
  2.10.5 Plant nodulation observations ...................................................... 71
  2.10.6 Isolation of rhizobia from nodules .............................................. 71
2.10.7 Infection thread and nodule occupancy assays ........................................ 72
2.10.8 Visualisation of LjNin expression in LjNin-Gus transgenic L. japonicus Gifu .... 72

2.11 Chemical analysis of M. loti EPS extracts .................................................. 73
2.11.1 EPS extraction ............................................................................................ 73
2.11.2 Gel permeation chromatography .................................................................. 73
2.11.3 Phenol-Sulfuric acid colourimetric assay .................................................. 74
2.11.4 Trimethylsilyl derivatisation of EPS samples .............................................. 74
2.11.5 EPS glycosyl-linkage analysis ...................................................................... 75

3 Characterisation of R7A EPS mutants ............................................................... 77
3.1 Introduction ....................................................................................................... 78
3.2 Results .............................................................................................................. 78
3.2.1 Characterisation of previously isolated R7A EPS mutant strains ................. 78
3.2.2 Construction of an R7A exsH mutant ......................................................... 85
3.2.3 Attempts at isolation of further in-frame markerless deletion R7A EPS mutants 88
3.2.4 Attempted isolation of R7AΔexoK using the FRT/ Flp system ....................... 89
3.2.5 Complementation of R7AexoU, R7AΔexoA and R7AexoK mutants ............... 96
3.2.6 EPS and Nod factor gene expression during symbiosis ............................... 97
3.2.7 Expression of LjNin in response to R7A and R7AexoU and purified EPS extract 102
3.3 Discussion ....................................................................................................... 103

4 Chemical analysis of R7A EPS extracts ........................................................... 109
4.1 Introduction ....................................................................................................... 110
4.2 Results ............................................................................................................. 111
4.2.1 EPS extraction ............................................................................................ 111
4.2.2 Gel permeation chromatography .................................................................. 111
4.2.3 Glycosyl composition of EPS samples ...................................................... 113
4.2.4 Glycosyl-linkage analysis ........................................................................... 115
4.2.5 Analysis of EPS extracts from M. loti strains MAFF303099 and NZP2037 .... 118
4.3 Discussion ....................................................................................................... 120

5 Investigation R7AexoB and R7AexoU EPS mutants with contrasting symbiotic proficiencies ................................................................. 125
5.1 Introduction ....................................................................................................... 126
5.2 Results .......................................................................................................................... 126
5.2.1 Nodulation competition assays ............................................................................. 126
5.2.2 Isolation and characterisation of an R7A \textit{exoB exoU} double mutant ............ 127
5.2.3 Analysis of R7A\textit{exoB} LPS .............................................................................. 130
5.2.4 Symbiotic complementation of R7A\textit{exoU} by a Nod factor mutant strain .... 132
5.2.5 Complementation of R7A\textit{exoU} symbiotic impairment with R7A EPS .......... 137
5.2.6 Effects of R7A\textit{exoU} pre-inoculation on symbiotic proficiency ................. 139
5.2.7 Specificity of R7A\textit{exoU} symbiotic proficiency ........................................... 142
5.3 Discussion .................................................................................................................... 145
5.3.1 R7A EPS acts as a signal to modulate host defence responses. ....................... 151

6 Isolation of R7A\textit{exoU} nodulation phenotype suppressor mutants .................. 153
6.1 Introduction ............................................................................................................... 154
6.2 Results ....................................................................................................................... 154
6.2.1 Construction of an in-frame markerless deletion R7A \textit{exoU} mutant .............. 154
6.2.2 Construction and screening of an R7A\textit{Delta}exoU/ mTn5 mutant library ....... 156
6.2.3 Re-construction of the novel R7A\textit{Delta}exoU/ mTn5 mutations ..................... 167
6.2.4 Complementation of R7A\textit{Delta}exoU/ 2385 with a cosmid containing \textit{mll2385}...... 169
6.2.5 Complementation of R7A\textit{Delta}exoU/ 2385 with an R7A cosmid library ........ 170
6.2.6 Symbiotic proficiency of complemented R7A\textit{Delta}exoU/ 2385 isolates ........ 173
6.2.7 LPS production by R7A\textit{Delta}exoU/ mTn5 isolates ....................................... 173
6.2.8 Potential involvement of cellulose in EPS mutant phenotypes .................... 174
6.2.9 Bioinformatic analysis of cellulose production by \textit{M. loti} ................................. 176
6.2.10 Construction of an R7A \textit{celA} mutant ............................................................ 178
6.2.11 Construction of an R7A \textit{celA exoU} double mutant ................................... 180
6.2.12 Colony characteristics and symbiotic proficiency of R7A\textit{Delta}celA and R7A\textit{Delta}celAexoU .................................................................................................................... 181
6.3 Discussion ................................................................................................................... 182

7 Characterisation of \textit{M. loti} NZP2037 and its \textit{exo} mutants .................................. 188
7.1 Introduction ............................................................................................................... 189
7.2 Results ....................................................................................................................... 190
7.2.1 Construction of in-frame markerless deletion NZP2037 \textit{exoU} and \textit{exoB} mutants 190
7.2.2 Construction of an NZP2037 cosmid library .................................................... 194
7.2.3 Complementation of R7AexoB and R7AexoU non-mucoid colony morphology with the NZP2037 cosmid library ................................................................. 196
7.2.4 Complementation of R7A and R7A EPS mutant strains symbiotic phenotypes with the NZP2037 cosmid library ................................................................. 199
7.2.5 Multiple factors may be involved in the NZP2037 symbiotic phenotypes ..... 200
7.3 Discussion ........................................................................................................ 205

8 Concluding remarks ............................................................................................ 210
8.1 Future directions .............................................................................................. 218

9 References ........................................................................................................... 220

10 Appendices ......................................................................................................... 253
    Appendix A ........................................................................................................ 254
    Appendix B ........................................................................................................ 258
List of Tables

Table 2.1: Bacterial strains used in this study ................................................................. 48
Table 2.2: Plasmids used in this study ............................................................................. 50
Table 2.3: Antibiotics used in this study .......................................................................... 53
Table 2.4: Primers used in this study ................................................................................ 59
Table 3.1: Summary of R7A EPS mutant strains colony and symbiotic phenotypes ........ 84
Table 4.1: Glycosyl linkages identified in R7A and R7A mutant EPS extract fractions .......... 117
Table 4.2: Glycosyl linkages identified in MAFF303099 and NZP2037 EPS fractions .......... 118
Table 5.1: IT and nodule occupancy of co-inoculated L. japonicus Gifu ................................ 135
Table 6.1: Site of mTn5 insertion in R7AΔexoU/ mTn5 isolates ......................................... 162
Table 6.2: Dispersal of R7AΔexoU cell aggregates by cellulase treatment ....................... 175
Table 7.1: Co-ordinates of exoB and exoU-encoding NZP2037 library cosmids ............... 197
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Determinate and indeterminate nodule structure.</td>
<td>8</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td><em>M. loti</em> R7A Nod factor structure.</td>
<td>12</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>Rhizobial polysaccharides.</td>
<td>15</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>Rhizobial EPS structures.</td>
<td>17</td>
</tr>
<tr>
<td>Figure 1.5</td>
<td>Organisation of EPS biosynthesis genes.</td>
<td>18</td>
</tr>
<tr>
<td>Figure 1.6</td>
<td>Succinoglycan biosynthesis pathway in <em>S. meliloti</em> strain SU47.</td>
<td>20</td>
</tr>
<tr>
<td>Figure 1.7</td>
<td>Regulation of EPS biosynthesis in <em>S. meliloti</em>.</td>
<td>23</td>
</tr>
<tr>
<td>Figure 1.8</td>
<td>Structure of rhizobial LPS.</td>
<td>28</td>
</tr>
<tr>
<td>Figure 1.9</td>
<td>Structure of rhizobial KPS.</td>
<td>31</td>
</tr>
<tr>
<td>Figure 1.10</td>
<td>Structure of <em>B. japonicum</em> CBG.</td>
<td>34</td>
</tr>
<tr>
<td>Figure 1.11</td>
<td>The plant innate immune system.</td>
<td>40</td>
</tr>
<tr>
<td>Figure 1.12</td>
<td><em>M. loti</em> exo gene cluster.</td>
<td>42</td>
</tr>
<tr>
<td>Figure 1.13</td>
<td>Symbiotic proficiency of R7A EPS mutants.</td>
<td>44</td>
</tr>
<tr>
<td>Figure 2.1</td>
<td>Schematic of the in-frame markerless deletion mutagenesis strategy.</td>
<td>68</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Determinate nodule types formed by R7A EPS mutants.</td>
<td>79</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Determinate nodulation by R7A EPS mutants.</td>
<td>80</td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>Colony characteristics of R7A EPS mutants.</td>
<td>82</td>
</tr>
<tr>
<td>Figure 3.4</td>
<td>Cell aggregation by R7A EPS mutants.</td>
<td>83</td>
</tr>
<tr>
<td>Figure 3.5</td>
<td>Construction and confirmation of R7AΔexsH.</td>
<td>87</td>
</tr>
<tr>
<td>Figure 3.6</td>
<td>Nodulation of <em>L. corniculatus</em> by R7AΔexsH.</td>
<td>88</td>
</tr>
<tr>
<td>Figure 3.7</td>
<td>FRT/ Flp system site-specific recombination.</td>
<td>89</td>
</tr>
<tr>
<td>Figure 3.8</td>
<td>Construction and confirmation of pSKKFRT.</td>
<td>91</td>
</tr>
<tr>
<td>Figure 3.9</td>
<td>PCR confirmation of exoK-FRT-nptII integration.</td>
<td>93</td>
</tr>
<tr>
<td>Figure 3.10</td>
<td>PCR investigation of exoK-FRT-nptII integration and removal of pIJ3200.</td>
<td>95</td>
</tr>
<tr>
<td>Figure 3.11</td>
<td>exo nod promoter region-gfp fusions.</td>
<td>98</td>
</tr>
<tr>
<td>Figure 3.12</td>
<td>nod and exo gene expression during growth on G/ RDM.</td>
<td>99</td>
</tr>
<tr>
<td>Figure 3.13</td>
<td>nod and exo gene expression on <em>L. japonicus</em>.</td>
<td>101</td>
</tr>
<tr>
<td>Figure 3.14</td>
<td>Induction of <em>LjNin-Gus</em>.</td>
<td>102</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>Elution profile of EPS extracts.</td>
<td>112</td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>Glycosyl composition of EPS extracts.</td>
<td>114</td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>Glycosyl composition of MAFF303099 and NZP2037 EPS extracts.</td>
<td>119</td>
</tr>
<tr>
<td>Figure 4.4</td>
<td>Proposed R7A EPS biosynthesis and comparison to <em>S. meliloti</em> EPS I and <em>Rhizobium</em> sp. NGR234 EPS.</td>
<td>123</td>
</tr>
<tr>
<td>Figure 5.1</td>
<td>Confirmation of R7AexoUPF and R7AexoBexoU.</td>
<td>128</td>
</tr>
</tbody>
</table>
Figure 5.2: Colony characteristics of R7AexoBexoU .......................................................... 129
Figure 5.3: Nodulation of L. corniculatus by R7AexoBexoU and R7AexoUPF ......................... 129
Figure 5.4: Silver-stained LPS isolated from R7A, R7AexoB and R7AexoU ........................... 130
Figure 5.5: Composition of R7A and R7AexoB LPS .............................................................. 131
Figure 5.6: R7AexoU infection focal points ............................................................................ 134
Figure 5.7: Nodulation of L. japonicus Gifu co-inoculated with R7AexoU and R7AΔnodA .. 134
Figure 5.8: ITs formed on R7AexoU/ R7AΔnodA co-inoculated plants ......................... 136
Figure 5.9: Dual nodule occupancy of R7AexoU/ R7AΔnodA co-inoculated plants ......... 136
Figure 5.10: R7AexoU IT formation in the presence of R7A LMW EPS ............................. 138
Figure 5.11: Effect of pre-inoculation with R7AexoU on symbiotically-proficient strains ... 140
Figure 5.12: Effect of R7AexoU pre-inoculation on R7AexoB nodulation on split-root plant. .................................................................................................................. 141
Figure 5.13: R7AexoU nodulation of L. japonicus MG-20 under differing root lighting growth conditions ........................................................................................................ 142
Figure 5.14: R7AexoU IT formation on L. japonicus MG-20 .................................................. 144
Figure 6.1: Confirmation of R7AΔexoU .................................................................................. 156
Figure 6.2: Nodulation screen for R7AΔexoU/ mTn5 suppressors ...................................... 157
Figure 6.3: Nodulation of L. corniculatus by R7AΔexoU/ mTn5 isolates ............................ 159
Figure 6.4: Southern hybridisation of R7AΔexoU/ mTn5 isolates ....................................... 161
Figure 6.5: Genome context of the novel genes disrupted by mTn5 ................................. 164
Figure 6.6: Colony phenotypes of R7AΔexoU/ mTn5 isolates ........................................... 166
Figure 6.7: Southern hybridisation of R7A/ pSKPF4457 and R7AΔexoU/ pSKPF4457 clones. .................................................................................................................. 168
Figure 6.8: Genome spanning region of mll2385-encoding R7A library cosmids pSK2385 ... 170
Figure 6.9: Isolation of R7AΔexoU/ 2385 complementing cosmids and colony morphologies of complemented R7AΔexoU/ 2385 .................................................................................. 171
Figure 6.10: Restriction digests of R7AΔexoU/ 2385 complementing cosmids .................... 172
Figure 6.11: R7AΔexoU/ 2385 complementing cosmids overlapping region ....................... 173
Figure 6.12: Silver-stained LPS isolated from R7A and EPS mutant strains ....................... 174
Figure 6.13: Dispersal of R7AΔexoU cell aggregates upon cellulase treatment .................. 176
Figure 6.14: Cellulose biosynthesis operons identified in rhizobia .................................... 177
Figure 6.15: M. loti MAFF303099 putative cellulose biosynthesis genes ......................... 178
Figure 6.16: Confirmation of R7AΔcelA ................................................................................. 179
Figure 6.17: Confirmation of R7AΔcelAexoU ...................................................................... 180
Figure 6.18: Colony phenotypes of R7AΔcelA and R7AΔcelAexoU ...................................... 181
Figure 7.1: Confirmation of NZP2037ΔexoB and NZP2037ΔexoU ...................................... 191
Figure 7.2: Colony morphologies of NZP2037ΔexoB and NZP2037ΔexoU.......................... 192
Figure 7.3: Symbiotic proficiency of NZP2037ΔexoB and NZP2037ΔexoU.......................... 193
Figure 7.4: Restriction digest of cosmid DNA from random NZP2037 library clones............ 195
Figure 7.5: Restriction profiles of exoB- and exoU-encoding NZP2037 library cosmids........ 197
Figure 7.6: Abnormal colony morphology of R7A and R7AexoU harbouring NZP2037 exoU- encoding cosmids................................................................. 198
Figure 7.7: Abnormal nodules formed by R7A and R7AexoB with NZP2037 nodU.............. 203
Figure 7.8: Nodulation of L. pedunculatus by R7A and R7AexoB harbouring NZP2037 nodU. .................................................................................................................................................................................. 204
Figure 8.1: Model for R7A EPS acting as a signal to suppress the induction of PTI............ 212
Figure 8.2: Proposed models accounting for the symbiotic proficiency of R7AexoB........... 214
Figure 8.3: Proposed models accounting for the symbiotic impairment of R7AexoU........... 215
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>kdo</td>
<td>3-deoxy-D-manno-2-octulosonic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>Ara</td>
<td>arabinose</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local assignment search tool</td>
</tr>
<tr>
<td>bv.</td>
<td>biovar</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
</tr>
<tr>
<td>CCRC</td>
<td>complex carbohydrate research center</td>
</tr>
<tr>
<td>c-di-GMP</td>
<td>cyclic diguanylic acid</td>
</tr>
<tr>
<td>CBG</td>
<td>cyclic-β-glucan</td>
</tr>
<tr>
<td>dpi</td>
<td>days post-inoculation</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DXO</td>
<td>double crossover</td>
</tr>
<tr>
<td>EPS</td>
<td>exopolysaccharide</td>
</tr>
<tr>
<td>FBP</td>
<td>flavolan-binding polysaccharide</td>
</tr>
<tr>
<td>Gal</td>
<td>galactose</td>
</tr>
<tr>
<td>GC-MS</td>
<td>gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>Glc</td>
<td>glucose</td>
</tr>
<tr>
<td>GlcA</td>
<td>glucuronic acid</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>g</td>
<td>gravitational force</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HMW</td>
<td>high-molecular-weight</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>hpi</td>
<td>hours post-inoculation</td>
</tr>
<tr>
<td>IT</td>
<td>infection thread</td>
</tr>
<tr>
<td>IDM</td>
<td>insertion duplication mutagenesis</td>
</tr>
<tr>
<td>KPS</td>
<td>K-antigen/ capsular polysaccharide</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>L</td>
<td>litre</td>
</tr>
<tr>
<td>LMW</td>
<td>low-molecular-weight</td>
</tr>
<tr>
<td>LysM</td>
<td>lysin motifs</td>
</tr>
<tr>
<td>Mb</td>
<td>megabase</td>
</tr>
<tr>
<td>MAMP</td>
<td>microbe-associated molecular pattern</td>
</tr>
<tr>
<td>µM</td>
<td>micromolar</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>mL</td>
<td>millilitre</td>
</tr>
<tr>
<td>mm</td>
<td>millimetre</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cut-off</td>
</tr>
<tr>
<td>nm</td>
<td>nanometre</td>
</tr>
<tr>
<td>Nod</td>
<td>nodulation</td>
</tr>
<tr>
<td>PnptII</td>
<td>nptII promoter</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PTI</td>
<td>PAMP-triggered immunity</td>
</tr>
<tr>
<td>PMAA</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PAMP</td>
<td>partially-methylated alditol acetates</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern-recognition receptor</td>
</tr>
<tr>
<td>p</td>
<td>plasmid designation</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PS</td>
<td>polysaccharide</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RibA</td>
<td>riburonic acid</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SXO</td>
<td>single crossover</td>
</tr>
<tr>
<td>SSC</td>
<td>sodium chloride sodium citric acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>sp.</td>
<td>species</td>
</tr>
<tr>
<td>TILLING</td>
<td>targeting induced local lesions in genomes</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TMS</td>
<td>trimethylsilyl</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>TAE</td>
<td>tris-acetate</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>UDP</td>
<td>uridine diphosphate</td>
</tr>
<tr>
<td>V</td>
<td>volt</td>
</tr>
<tr>
<td>v/ v</td>
<td>volume to volume ratio</td>
</tr>
<tr>
<td>w/ v</td>
<td>weight to volume ratio</td>
</tr>
<tr>
<td>Xyl</td>
<td>xylose</td>
</tr>
</tbody>
</table>
1 Introduction
1.1 Introduction

The rhizobium-legume symbiosis is a mutualistic interaction between soil bacteria belonging to the α- and β-proteobacteria, collectively referred to as rhizobia (Masson-Boivin et al., 2009), and plants of the *Leguminosae* family, commonly known as legumes. The outcome of this symbiotic relationship is the formation of legume root nodules within which the rhizobia, following differentiation into bacteroids, fix biologically inaccessible atmospheric di-nitrogen (N$_2$) into the biologically available form, ammonia (NH$_3$). The fixation occurs through the reaction $N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$, which is catalysed by the oxygen-labile rhizobial enzyme nitrogenase.

The establishment of a successful symbiotic interaction between rhizobia and legumes requires complex molecular communication between the two partners in order to determine compatibility and co-ordinate symbiotic developmental responses (Cooper, 2007; Gage, 2004; Oldroyd & Downie, 2008; Perret et al., 2000). Primary signalling molecules, flavonoids produced by the host legume and Nod factors produced in response by compatible rhizobia, have been identified and, along with their receptors, continue to be characterised. It has long been recognised that further secondary signalling events are required during the symbiotic process to ensure a successful outcome. Exopolysaccharide (EPS) production is ubiquitous amongst rhizobia and has frequently been implicated as one such secondary signalling molecule. However, due to the production of a multitude of polysaccharides by rhizobia, with at times intertwined biosynthetic pathways, defining the requirement/ function of EPS in symbiosis has proved challenging. Further adding to the ambiguity surrounding the requirement for EPS in nodulation is the general consensus that EPS is only required for symbiosis between rhizobia and host legumes that form indeterminate nodules and that there is no such requirement for determinate nodule-forming hosts (Borthakur et al., 1988; Cheng & Walker, 1998a; Gage, 2004; Gonzalez et al., 1996b; Hotter & Scott, 1991; Kim et al., 1989; Ko & Gayda, 1990; Leigh et al., 1985; Pellock et al., 2000; van Workum et al., 1998).

This study investigates the requirement and function of EPS in the determinate nodule-forming *Mesorhizobium-Lotus* symbiosis. This Introduction provides background information on the *Mesorhizobium-Lotus* symbiosis, beginning with descriptions of the rhizobia and legumes investigated in this study. An overview of
the symbiotic process is then provided, followed by details of the primary signalling molecules. The various rhizobial polysaccharides implicated as being involved in the establishment of symbiosis are then described with particular focus given to EPS. As this PhD study is a continuation of MSc research (Kelly, 2007), a summary of relevant results obtained from that study is provided. Due to the continuation of the research topic, sections of this introduction (particularly sections detailing rhizobial polysaccharides) are updated versions of those presented previously (Kelly, 2007). Finally, the major aims of the experiments performed in this study are presented.

1.2 *Mesorhizobium loti*

The rhizobia investigated in this study belong to the species *Mesorhizobium loti*, with its members largely characterised by their ability to form nodules on *Lotus* plant species. Originally rhizobia isolated from *Lotus* plants were separated into fast- and slow-growing strains (Pankhurst, 1977), with the fast-growing strains having previously been termed *Rhizobium loti* (Jarvis et al., 1982). Following the removal of *R. meliloti* and *R. fredii* into a new genus, *Sinorhizobium* (de Lajudie et al., 1994), a revision of the remaining members of the *Rhizobium* genus in 1997 further separated the group’s members giving rise to the *Mesorhizobium* genus, with ‘meso’ indicating the species grow at a slower rate than the other fast-growing rhizobia (Jarvis et al., 1997). The *Mesorhizobium* genus comprises *M. loti*, *M. huakui*, *M. ciceri*, *M. tianshanense* and *M. mediterraneum*. To date the most well-characterised strains of *M. loti* include NZP2213, NZP2037, NZP2235, NZP2238, MAFF303099 and R7A. The symbiosis genes are encoded on the chromosome of *M. loti* strains in clusters known as symbiosis islands (Pankhurst et al., 1986; Sullivan & Ronson, 1998). This distinguishes them from other rhizobia such as *S. meliloti*, *Rhizobium* sp. strain NGR234 and *R. leguminosarum* in which the symbiosis genes are located on large plasmids (Freiberg et al., 1997). Although the symbiosis genes of *M. loti* strains are encoded on the chromosome, the transfer of symbiosis genes to non-symbiotic *M. loti* strains has been demonstrated in the case of *M. loti* R7A (Sullivan et al., 1995; Sullivan & Ronson, 1998).

This study largely focuses on *M. loti* strain R7A, with strains MAFF303099 and NZP2037 also used. R7A is a field reisolate of *M. loti* strain IMCP3153 that contains a 502-kb symbiosis island (Sullivan & Ronson, 1998). The symbiosis island encodes 414
genes including all of the genes required for Nod factor synthesis, nitrogen fixation and transfer of the island (Sullivan et al., 2002). Transfer of the island occurs via conjugation involving a rolling-circle process. The transferred island integrates into the chromosome of the recipient cell at the strain’s sole phenylalalanine tRNA gene (Sullivan & Ronson, 1998). Integration of the island is dependent on a P4-type integrase encoded by intS, located 198 bp downstream of the phe-tRNA gene, which acts on an attachment site (attS) of the circular form of the island and a chromosomal attachment site (attB). Integration of the island reconstructs the entire phe-tRNA gene at one end (arbitrarily termed the left end) and a 17-bp repeat of the three-prime end of the phe-tRNA gene at the right end of the integrated island (Sullivan & Ronson, 1998; Sullivan et al., 2002).

MAFF303099 is a closely-related strain to R7A that has had its entire genome sequenced. The genome consists of a ~7 Mb chromosome including a symbiosis island of 610 kb and two plasmids of ~352 kb and ~208 kb (Kaneko et al., 2000). Comparison of the symbiosis islands of R7A and MAFF303099 revealed that they share a conserved backbone of about 250 kb that is disrupted by strain-specific insertions and deletions (Sullivan et al., 2002).

NZP2037 was originally isolated from L. divaricatus (Jarvis et al., 1982) and displays some symbiotic characteristics that distinguish it from other M. loti strains. NZP2037 has a broad host range and is able to form both determinate and indeterminate nodules depending on the host plant. It is able to form nitrogen-fixing nodules on L. pedunculatus whereas other M. loti strains form only uninfected nodule primordia (Hotter & Scott, 1991; Pankhurst et al., 1979).

1.3 Lotus legumes
The Lotus genus of legumes consists of over 200 species that generally grow in temperate regions, although there are also tropical and subtropical species (Handberg & Stougaard, 1992). The Lotus genus is extremely diverse and species may exhibit differing appearances depending on the environmental conditions. They can grow in a range of soil conditions including relatively infertile soils. The plants may be either annual or perennial with branching tap roots and compound leaves.
*L. japonicus* is a perennial temperate species that forms determinate nodules in association with *M. loti* and has been designated as a ‘model legume’ due to characteristics that make it suitable for genomic research including a short life cycle (2-3 months), self-fertility, a diploid genome (2n=12) of relatively small size (472.1 Mb). It is also susceptible to transformation by *Agrobacterium* (Handberg & Stougaard, 1992). *L. japonicus* sequence has been determined for 315 Mb representing 67% of the genome (Sato et al., 2008) and is available on the Kazusa website ([http://www.kazusa.or.jp/lotus/](http://www.kazusa.or.jp/lotus/)). Various DNA, recombinant inbred lines and TILLING mutant libraries are available for investigations of *L. japonicus*, as reviewed by (Sato & Tabata, 2006; Udvardi et al., 2005). In addition, the endogenous retrotransposon *Lotus* retrotransposon 1 (LORE1) (Madsen et al., 2005) has been used to generate transposon mutant libraries of *L. japonicus* that are available for investigating the phenotype of plants disrupted in particular genes (Fukai et al., 2012; Urbanski et al., 2012).

Other *Lotus* species used in this study include *L. corniculatus* and *L. pedunculatus*. *L. corniculatus* (birdsfoot trefoil) is of agricultural importance and is used for pastures and hay. Both *L. corniculatus* and *L. pedunculatus* contain condensed tannins which have been shown to reduce bloating problems in ruminants (Aerts et al., 1999).

### 1.4 The symbiotic infection process

This section provides an overview of the symbiotic process which results in the formation of nitrogen-fixing nodules. Attachment of rhizobia to the root hairs of host legumes is considered the first step in the symbiotic infection process and various rhizobial and legume components have been implicated as involved in the attachment process (Rodriguez-Navarro et al., 2007). It has been proposed that attachment occurs initially via weak attachment mediated by the rhizobial rhicadhesin protein (Smit et al., 1987) and possibly interaction between rhizobial polysaccharides and plant lectins (Hirsch, 1999). A glucomannan polysaccharide produced by *R. leguminosarum* bv. *viciae* that exhibits high binding affinity for pea and vetch lectins was found to be located only at the bacterial pole and may be involved in attachment to the root surface (Laus et al., 2006). A mutant strain disrupted in production of the glucomannan polysaccharide was strongly outcompeted by the wild-type strain in mixed inoculation nodulation assays.
An arabinoglycan protein from pea root exudate has recently been demonstrated to affect polar attachment of *R. leguminosarum* bv. *viciae* through an interaction that requires the production of EPS by the rhizobia (Xie *et al.*, 2012b). A second stronger attachment between rhizobia and root hair tips through the production of rhizobial cellulose fibrils has been reported (Smit *et al.*, 1986; Smit *et al.*, 1987) but has not been confirmed to be important for nodulation. It is believed that the close association between compatible partners allows for localised concentration of Nod factor to signal nodule development (Downie, 2010; Hirsch, 1999).

A proportion of attached rhizobial cells may become enclosed in a curled root hair tip forming an infection focal point often referred to as a ‘shepherd’s crook’ due to its appearance, with the curling of the root hairs being a response by the plant to Nod factor recognition (Esseling *et al.*, 2003; Heidstra *et al.*, 1994). Rhizobia then enter the legume through the formation of an infection thread (IT). IT formation is initiated at the root hair tip by the degradation of root hair cell walls at the infection focal point. Localised degradation of the plant cell wall has recently been linked to a pectate lysase gene in *L. japonicus* that is induced in response to Nod factor recognition (Xie *et al.*, 2012a). *L. japonicus* containing mutations in the pectate lysase gene produce uninfected nodules with the infection process halted at the infection focal point. New, inwardly-directed cell walls are deposited forming a tubular structure composed of a plant matrix that extends the length of the root hair through which rhizobia gain entry to the developing nodule (Brewin, 2004; Napoli & Hubbell, 1975; Ridge & Rolfe, 1985). Rhizobia divide at the growing IT tip forming a column of cells down the length of the root hair. Rhizobia released from the IT tip into the nodule primordia are encapsulated in a plant-derived membrane (termed the peribacteroid membrane) in an endocytosis-like process to form what are known as symbiosomes. Rhizobia within symbiosomes differentiate into bacteroids at which stage the nitrogenase enzyme becomes active and nitrogen fixation begins.
1.4.1 Nodule structure

Two fundamentally different types of nodules, indeterminate and determinate, form on legumes in association with rhizobia (Figure 1.1). The type of nodule that arises from a symbiotic interaction is determined by the host legume species. Some rhizobia are capable of forming both nodule types in association with different host legumes (Newcomb, 1981). For example *M. loti* NZP2037 forms indeterminate nodules on *Leucaena leucocephala* and determinate nodules on *L. pedunculatus* (Hotter & Scott, 1991).

Indeterminate nodules are elongated in shape and have a persistent meristem that continually grows after initial cell divisions occur in the inner cortex. ITs of indeterminate nodules fail to reach the meristematic cells, but continually invade surrounding plant cells. As a result of this, indeterminate nodules display a developmental gradient (Frayssé et al., 2003; Gage, 2004). Legumes that form indeterminate nodules include *Medicago sativa, M. truncatula, Pisum sativum, Vicia speares, Trifolium* sp. and *L. leucocephala*.

Determinate nodules are spherical in shape and lack a persistent meristem. Rhizobia spread within the nodule mainly through the division of already infected outer cortical cells. Due to this, no obvious developmental gradient exists, with most cells present at about the same developmental stage (Frayssé et al., 2003; Gage, 2004). Legumes that form determinate nodules include *Glycine max, V. faba, L. corniculatus* and *L. japonicus*.

The formation of two different nodule types on legumes by rhizobia is an important factor when investigating the role of EPS in nodulation. The majority of studies suggest that EPS is required for indeterminate nodule formation only and that no such requirement exists for the formation of determinate nodules (Becker et al., 1993c; Finan et al., 1985; Hotter & Scott, 1991; Kim et al., 1989; Leigh et al., 1985).
Figure 1.1: Determinate and indeterminate nodule structure.

A) Determinate nodule: meristematic activity is initiated in the outer cortex and is temporary. Continued division of infected cells is responsible for nodule development (NF - nitrogen fixing zone, S - senescent zone). B) Indeterminate nodule: meristematic activity is initiated in the inner root cortex and is persistent. Nodule development is due to continual invasion of newly-divided cells by rhizobia exiting ITs, resulting in a developmental gradient within the nodule (I - meristem, II - infection zone, III - nitrogen-fixing zone, IV - senescent zone). NC - nodule cortex, NE - nodule endodermis, NP - nodule parenchyma and VB - vascular bundle. Adapted from

http://commons.wikimedia.org/wiki/File:Determinate_Nodule_Zones_Diagram.svg

and

1.5 Molecular communication

The molecular communication required for the establishment of a successful symbiotic association between compatible symbiotic partners ensures specificity and lessens the chances of ineffective interactions that could be detrimental to both the rhizobia and the legume. This section outlines the major signalling and receptor molecules involved.

1.5.1 Legume signals that induce rhizobial nod gene expression

The symbiotic process is initiated in the rhizosphere where compounds secreted from the roots of legumes induce responses from compatible rhizobia. Flavonoids (2-phenyl-1,4-benzopyrone derivatives) are plant secondary metabolites that are considered the major signalling compounds secreted by legumes. Perception of certain flavonoids by compatible rhizobia results in the production of Nod factors which leads to the onset of nodule organogenesis (Downie, 1998; Spaink, 2000).

Specific substitutions on the benzene rings results in a large variety of flavonoid forms including flavonols, flavones, flavonones and isoflavonoids. Flavonoid production is not unique to legumes and different legumes produce various cocktails of flavonoids. Furthermore, the flavonoids produced by a particular legume may vary depending on the age and physiological state of a legume (Long, 2001). The variations in flavonoids produced allow for rhizobia to identify compatible hosts (Hirsch et al., 2001). For example, the isoflavones diadzein and genistein produced by soybean (*Glycine max*) are perceived as a positive signal by the symbiotically compatible *Bradyrhizobium japonicum* and *Rhizobium* sp. NGR234 to induce the symbiotic process but act as anti-inducers to incompatible rhizobia such as *S. meliloti* and *R. leguminosarum* (Cooper, 2007). Although a wide variety of flavonoids produced by *Lotus* have been identified, none of those tested have been found to induce nod gene expression in *M. loti* (Lopez-Lara et al., 1995; Steele et al., 1999). Other legume-derived inducers of nod genes include betaines such as stachydrine and trigonelline produced by *Medicago* species that act to co-induce some nod genes in *S. meliloti* (Goldmann et al., 1994; Phillips et al., 1992). Flavonoids are continually secreted by legumes in small quantities, although the level is increased in response to rhizobia or Nod factors (Broughton et al., 2000).
The flavonoid signal is perceived by rhizobia through interaction with the constitutively expressed membrane-associated NodD. NodD is a LysR-type regulatory protein that binds to a consensus sequence within the promoter region of inducible nod genes, termed the nod-box, causing a bend in the DNA at the binding site (Fisher & Long, 1993). It is known that both NodD and a flavonoid inducer are required to activate transcription of the nod genes, although the mechanism of the interaction is not fully understood (Schlaman, 1998). It has been proposed that the presence of an appropriate flavonoid causes sharpening in the bend of the DNA where NodD is bound allowing RNA polymerase to transcribe the gene (Chen et al., 2005).

Rhizobia encode between 1 to 5 copies of nodD and it is thought that NodD may itself be a factor in the specificity of symbiotic interactions (Spaink et al., 1987). S. meliloti contains three copies of nodD and mutation of all three copies is required to cause a nodulation-deficient symbiotic phenotype with M. sativa. It has been suggested that multiple copies of NodD within the same strain may serve to recognise different flavonoids (Gyorgypal et al., 1991; Hartwig et al., 1990). There does not appear to be a clear correlation between the number of flavonoids recognised by a rhizobial species and the host-range of that strain. Rhizobium sp. NGR234 and R. leguminosarum bv. viciae both recognise a wide-range of flavonoids yet whilst Rhizobium sp. NGR234 exhibits a broad host range, R. leguminosarum bv. viciae has a narrow host-range (Cooper, 2007).

In addition to the actions of NodD, further regulatory mechanisms of nod gene expression have been identified. B. japonicum encodes a two-component regulatory system, nodVW, that perceives flavonoids and in response induces nod gene expression (Loh et al., 1997; Sanjuan et al., 1994). In S. meliloti expression of nodD3 is positively controlled by another LysR-type regulator, SyrM, which itself is positively controlled by NodD2 and NodD3 (Barnett et al., 1996; Kondorosi et al., 1991a). Furthermore, a repressor of nod gene expression, NolR, has been identified in some S. meliloti strains that binds to the promoter regions of nodD1 and nodD2 and functions to regulate the expression of all three nodD homologues (Kondorosi et al., 1991b).
The *nod* genes transcribed through the action of flavonoids, NodD and associated regulatory proteins are responsible for the production of the major rhizobia signal molecules, lipochito-oligosaccharides commonly known as Nod factors.

### 1.5.2 Nod factor structure and synthesis

All Nod factors produced by rhizobia investigated to date consist of an oligosaccharide β-1,4-linked *N*-acetyl-D-glucosamine backbone with a fatty acyl group attached to the non-reducing end of the backbone (Figure 1.2). The only exception to this general structure is a minority Nod factor species produced by *S. fredii* USDA191 that includes a glucose residue in the backbone (BecFerte *et al.*, 1996).

Despite this common backbone, rhizobia produce a wide variety of Nod factors through various species-specific modifications. Nod factor structures differ through variations in the number of *N*-acetyl-D-glucosamine residues in the backbone, the length and saturation of the fatty acyl group, various substituents at the non-reducing and/or reducing end of the backbone as well as occasionally substituent groups located on non-terminal *N*-acetyl-D-glucosamine residues.

As mentioned, *nod* genes are responsible for the synthesis of Nod factor. The *nodABC* genes are found in all rhizobia and are required for assembly of the *N*-acetyl-D-glucosamine backbone through the action of a chitin-oligosaccharide synthase (NodC), a chitin-oligosaccharide de-acetylase (NodB) and an acyl transferase (NodA) (Atkinson *et al.*, 1994; Spaink *et al.*, 1994). Mutations in any of these genes generally result in a nodulation-deficient symbiotic phenotype, although an *M. loti* R7A *nodB* mutant was found to be symbiotically effective on three host *Lotus* species; however, the mutant was severely compromised in competition assays (Rodpothong *et al.*, 2009). It is through the addition of various substituent groups to the Nod factor backbone that specificity is formed. Addition of these substituent groups is carried out by ‘accessory’ *nod, noe* or *nol* genes to complete the Nod factor structure. *M. loti* Nod factor harbours an acetyl-fucosyl substituent at the reducing terminal residue. NodZ is responsible for the addition of the fucosyl substituent and NolL adds the acetyl group to the fucosyl residue. *M. loti* mutants disrupted in either *nodZ* or *nolL* are inhibited in symbiosis with usual hosts *L. filicaulis* and *L. corniculatus*, forming uninfected nodule primordia, whilst their symbiotic proficiency on another host, *L. japonicus*, is only slightly delayed (Rodpothong *et al.*, 2009).
Figure 1.2: *M. loti* R7A Nod factor structure.

Structure of the R7A Nod factor, based on (Rodpothong et al., 2009). The sites of action of the various *nod*, *noe* and *nol* gene products are indicated. *nodA*, *nodB* and *nodC* genes are common amongst all rhizobia and are responsible for assembly of the *N*-acetyl-*D*-glucosamine backbone. R7A decorations to the backbone include the addition of a carbamoyl group by NolO, a methyl group by NodS and an acetyl-fucosyl residue (green) by NodZ and NolL. R- *cis*-vaccenic acid (C18:1) or stearic acid (C18:0).

Investigations have indicated that Nod factor influences host range, yet findings reveal no strict association. The Nod factors produced by *M. loti* and *R. etli* are the same, yet the host species differ with *M. loti* nodulating *Lotus* sp. and *R. etli* nodulating *Phaseolus* sp. (Cardenas et al., 1995). However *R. etli* forms early-senescing nodules on *L. japonicus*, indicating that *R. etli* may initially be perceived as compatible allowing for successful nodule infection prior to subsequent recognition as an incompatible partner resulting in a defence response leading to the death of infected nodule cells (Banba et al., 2001). Further adding to the complexity in
unravelling the role of Nod factor structure on host range is the production of multiple Nod factor species by individual rhizobia that vary depending on environmental conditions. *R. tropici* was found to produce 52 different Nod factor structures in acidic conditions compared to 29 at neutral pH with only 15 of the Nod factor structures produced in both conditions (Moron et al., 2005).

1.5.3 Nod factor receptors

Observations that Nod factors induce plant responses at concentrations as low as $10^{-9}$ to $10^{-12}$ M suggest they are likely perceived by plant receptors. Proposed Nod factor receptors have been identified in *P. sativum* (SYM10), *Medicago* sp. (NFP and LYK3) and *L. japonicus* (NFR1 and NFR5) (Amor et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003). The identified proteins are members of the receptor serine/threonine kinase family and have lysin motifs (LysM). It is proposed that the LysM domains are extracellular and upon binding Nod factor the intracellular kinase domain is autophosphorylated which induces a signal transduction cascade resulting in the development of root nodules (Radutoiu et al., 2003). *L. japonicus* plants carrying mutations in *nfr1* or *nfr5* fail to exhibit the usual symbiotic plant responses to inoculation with *M. loti* or purified Nod factor, demonstrating their involvement in recognition of Nod factor (Radutoiu et al., 2003). Nod factor receptors have been shown to influence the symbiotic host range as demonstrated by experiments in which the introduction of *nfr1* and *nfr5* into *M. truncatula* allowed for nodule development by the *L. japonicus* symbiont *M. loti* (Radutoiu et al., 2007).

The perception of compatible Nod factor by legumes is critical for early symbiotic events and results in a number of physiological responses in the host. Host responses induced by Nod factor perception include the formation and deformation of root hairs, alkalisation, membrane potential depolarisation, changes in ion fluxes, induction of early nodulin gene expression and the formation of nodule primordia as reviewed by (Gibson et al., 2008; Gough & Cullimore, 2011; Oldroyd et al., 2011). One of the earliest plant responses following perception of compatible Nod factor is an influx of calcium ($\text{Ca}^{2+}$), followed by a $\text{Ca}^{2+}$-spiking response (Ehrhardt et al., 1996) that activates signalling pathways.

Mycorrhizal fungi that establish a symbiotic interaction with various species of plants have been found to produce lipochito-oligosaccharides (termed Myc factors)
that are analogous to the Nod factors produced by rhizobia (Maillet et al., 2011). In the non-legume *Parasponia*, a LysM receptor-like kinase (PaNFP) that is closely related to the NFP and NFR5 of *M. truncatula* and *L. japonicus* respectively, is required for both nodulation and mycorrhization (Op den Camp et al., 2011). A common signalling pathway in legumes leading to plant responses that facilitate symbiosis with both rhizobia and mycorrhizal fungi is thought to exist (Gough & Cullimore, 2011). In *L. japonicus* the leucine-rich receptor kinase SYMRK perceives both rhizobial and mycorrhizal signals and is thought to be involved at the junction of the common pathway with the rhizobia-specific pathway (Endre et al., 2002; Stracke et al., 2002). The observation that *nfr1* and *nfr5* mutations do not inhibit mycorrhizal infection suggests that the Nod factor receptors are specific for rhizobial symbiosis of *L. japonicus* (Radutoiu et al., 2003).

1.6 Rhizobial polysaccharides

Rhizobia produce a range of polysaccharides (PS) either associated with the cell surface or secreted into the surrounding environment (Figure 1.3). Rhizobial PS have been implicated as performing important roles in nodulation based on the investigation of a wide range of rhizobial PS mutants. The PS identified as potentially having a role in symbiosis include exopolysaccharide (EPS), lipopolysaccharide (LPS), K-antigen (KPS), cyclic-β-glucan (CBG) and cellulose. Various roles of the PS during symbiosis have been proposed that include passive roles such as protecting the rhizobia from plant defence compounds and aiding attachment to root hairs. Active roles proposed for PS include functioning as signalling molecules to induce legume developmental changes and modulate plant defence responses. The following sections detail the structure, biosynthesis and proposed function during nodulation of each of these rhizobial PS molecules with particular focus given to EPS.
Figure 1.3: Rhizobial polysaccharides.

Graphic of the rhizobial cell surface displaying the range of polysaccharides that may be involved in associations with host legumes. OM - outer membrane, PS - periplasmic space, PG - peptidoglycan layer, PM - cytoplasmic membrane, EPS - exopolysaccharide, CG - cyclic-β-glucan, PL - phospholipid, MP - membrane protein, KPS - capsular polysaccharide (K-antigen), and LPS - lipopolysaccharide. Reproduced from (Rodriguez-Navarro et al., 2007) with permission.
1.6.1 Exopolysaccharide (EPS)

EPS is the term given to PS secreted by bacteria that have little or no cell association. Production of EPS is ubiquitous amongst rhizobia and is evident from mucoid colony morphologies when cells are cultured on media containing sugars. Although EPS production by rhizobia is ubiquitous, the composition of EPS produced by differing strains varies widely (Figure 1.4). These variations in EPS composition are achieved through differences in monosaccharide substituents, variations of backbone homogeneity (either heteropolymers or homopolymers), altered composition of repeating units in heteropolymers, length of the structure and also by the addition of decorations to the backbone. Strain-specific decorations include non-carbohydrate substituents such as O-acetyl, pyruvyl and succinyl groups. Further variation to EPS structure may be achieved through the addition of side-chains and negatively charged groups such as uronic acids.

EPS produced by *S. meliloti* strain 1021 is the most well-studied to date and is often referred to as succinoglycan due to the presence of a succinyl decoration. Succinoglycan is composed of repeating octasaccharide sub-units, each composed of 7 glucose and 1 galactose harbouring succinyl, acetyl and pyruvyl groups (Figure 1.4) (Aman *et al.*, 1982; Glucksmann *et al.*, 1993b; Reinhold *et al.*, 1994). *S. meliloti* was found to have the ability to produce a secondary EPS termed EPS II (galactoglucan) composed of glucose and galactose at a 1:1 ratio decorated with pyruvyl and acetyl groups (Figure 1.4) (Her *et al.*, 1990). Although galactoglucan is able to function in place of succinoglycan in some mutant strains to facilitate successful symbiosis, it is not expressed at detectable levels by *S. meliloti* strain 1021 due to an insertion in the *expR* gene of the strain (Gage, 2004). Disruption of genes that affect succinoglycan production, such as *exp101* (Glazebrook & Walker, 1989) and *mucR* (Keller *et al.*, 1995), result in increased galactoglucan production.

Examples of differences in EPS production between rhizobia are evident in comparison of the structure of succinoglycan to EPS species produced by other rhizobia. Similar to succinoglycan, EPS produced by *R. leguminosarum* is an octasaccharide; however, it contains two glucuronic acid residues and is decorated with two pyruvyl and one acetyl substituent group (Figure 1.4) (Robertson *et al.*, 1981). The structure of *M. loti* EPS has yet to be determined.
Figure 1.4: Rhizobial EPS structures.
Examples of defined structures of EPS species produced by the indicated rhizobia, adapted from (Laus et al., 2005b).

1.6.1.1 EPS biosynthesis

Generally the genes involved in EPS biosynthesis are found in clusters on either the chromosome or indigenous plasmids of rhizobial strains. EPS biosynthesis by rhizobia requires enzymes for the production of nucleotide sugar precursors to form the backbone of the EPS molecule, glycosyltransferases to link the sugars, enzymes for the addition of strain-specific modifications and finally enzymes involved in translocation and polymerisation. In S. meliloti 21 genes encoding proteins to carry out the above-mentioned tasks have been identified. Most of the \textit{exo} genes responsible for succinoglycan production cluster on the megaplasmid pSymB (Finan \textit{et al.}, 2001), although some genes required for EPS production are coded for on the chromosome (Keller \textit{et al.}, 1995). The arrangement of the EPS biosynthesis genes of \textit{S. meliloti} and homologous regions identified in \textit{Rhizobium} sp. strain NGR234 and \textit{M.}
loti MAFF303099 are presented in Figure 1.5. The organisation of EPS biosynthesis genes appears reasonably well conserved between the three rhizobia, particularly with regard to exo genes involved in the earlier stages of EPS biosynthesis (Streit et al., 2004).

**Figure 1.5: Organisation of EPS biosynthesis genes.**

Organisation of EPS biosynthesis gene clusters identified in the indicated rhizobial strains. Green arrows represent exo genes and red arrows indicate exs genes involved in the biosynthesis of EPS. White arrows indicate genes not known to be involved in EPS production. Adapted from (Streit et al., 2004).

Figure 1.6 displays a schematic of EPS biosynthesis by *S. meliloti* strain SU47, indicating the stage at which the various exo gene products are involved. ExoC, ExoN and ExoB carry out the synthesis of the nucleotide sugar precursors (UDP-glucose and UDP-galactose) that will form the backbone of the succinoglycan molecule. A galactosyltransferase, ExoY, initiates the assembly of succinoglycan by adding the first UDP-galactose residue to a lipid carrier that requires the action of ExoF. A group of glucosyltransferases, ExoA, ExoL, ExoM, ExoO, ExoU and ExoW, are then required for the addition of UDP-glucose residues to form the backbone of the EPS molecule. Strain-specific acetyl, succinyl and pyruvyl groups are added through the action of ExoZ, ExoH and ExoV respectively. The function of the mentioned Exo enzymes has largely been assigned through the analysis of lipid-linked intermediates.
that accumulate in the media of various cultured exo mutants (Becker et al., 1993a; Becker et al., 1993c; Glucksmann et al., 1993a; Glucksmann et al., 1993b; Reed et al., 1991a; Reed et al., 1991b; Reed & Walker, 1991; York & Walker, 1998b). Following the synthesis of the EPS molecule, ExoP, ExoQ and ExoT are then required for the polymerisation and secretion of EPS. Finally, further processing of succinoglycan occurs outside the cell where extracellular glycanase enzymes ExoK and ExsH cleave high-molecular-weight (HMW) succinoglycan to yield low-molecular-weight (LMW) forms of succinoglycan (York & Walker, 1998b). A LMW trimeric form of succinoglycan has been implicated as the symbiotically-active form (Wang et al., 1999).
Figure 1.6: Succinoglycan biosynthesis pathway in *S. meliloti* strain SU47.

Stage 1 of EPS biosynthesis involves the generation of the nucleotide sugar precursors that form the EPS backbone. Stage 2 involves the step-wise addition of glycosyl residues through the action of glycosyltransferases and the addition of substituent groups. Stage 3 is translocation and polymerisation of the EPS subunits. Schematic adapted from (Becker et al., 2000).
1.6.1.2 Regulation of EPS biosynthesis

The regulation of EPS appears complex with various regulators at the level of transcription and post-translation identified (Figure 1.7). *exoS* and *exoR* were identified as involved in regulation of succinoglycan production by *S. meliloti* based on the over-production of EPS due to mutations in either gene (Doherty *et al.*, 1988). ExoR encodes a 268 amino-acid protein with no significant homology to known proteins (Reed *et al.*, 1991b). Studies have revealed that an *S. meliloti* *exoR* mutant produces increased levels of mRNA corresponding to various *exo* genes compared to wild-type *S. meliloti*, suggesting that ExoR negatively regulates EPS biosynthesis at the level of transcription (Reed *et al.*, 1991b). Interestingly the mutation of *exoR*, causing an increase in EPS production, reduced the nodulation efficiency of *S. meliloti* (Doherty *et al.*, 1988). The apparent nodulation deficiencies of the ExoR mutant may be due to pleiotropic effects that result in a reduced ability to colonise root hairs owing to the absence of flagella on the *exoR* mutant strain. The absence of flagella results from the repression of flagellum synthesis genes caused by the *exoR* mutation (Yao *et al.*, 2004). ExoS is a membrane-bound sensor histidine kinase that forms a two-component regulatory system with the response regulator ChvI. It has been proposed that in response to an as yet unidentified environmental signal, ExoS activates ChvI to induce the transcription of various *exo* genes (Cheng & Walker, 1998b). ExoR is located in the periplasm and interacts with ExoS to inhibit ExoS/ChvI signalling (Chen *et al.*, 2008; Wells *et al.*, 2007). Investigation of *S. meliloti* mutant strains indicate that the ExoR, ExoS and ChvI proteins are involved in regulatory networks that affect not only EPS and flagella production but also other cell surface components and carbon source utilisation (Belanger *et al.*, 2009; Chen *et al.*, 2008; Wang *et al.*, 2010). An additional three-component regulatory system consisting of EmmA, EmmB and EmmC shows functional similarity to the ExoR, ExoS and ChvI system and the two systems may act in parallel to regulate cell processes that include succinoglycan production and motility (Morris & Gonzalez, 2009).

ExoX is a negative regulator of EPS production, thought to act post-translationally. ExoX is 96 amino-acid inner-membrane attached protein identified in *S. meliloti* and *Rhizobium* sp. strain NGR234 through similarities with the previously characterised PsiA protein of *R. leguminosarum* bv. *phaseoli* (Borthakur & Johnston, 1987; Gray *et al.*, 1989).
The mutation of *exoX* causes overproduction of EPS in *S. meliloti*, however it was observed that the copy number of *exoX* compared to *exoY* affects the level of succinoglycan production. When the copy number of *exoX* is greater than that of *exoY* succinoglycan production is inhibited, whereas conversely if the copy number of *exoX* is less than that of *exoY* overproduction of succinoglycan is observed. If equal copy numbers of the genes are present normal levels of succinoglycan are produced (Zhan & Leigh, 1990). Because the expression of *exo* genes is unaffected by mutation of *exoX*, it is thought that regulation of EPS biosynthesis by the protein is at a post-translational level possibly mediated via interaction with ExoY (Zhan & Leigh, 1990).

SyrM and MucR are two positive regulators of succinoglycan production. SymR is a LysR-type transcriptional regulator with similarity to NodD which is responsible for the activation of *nod* gene expression (Barnett & Long, 1990). SymR is involved in regulating the ratio of HMW to LMW succinoglycan and is affected by the presence of nitrogen and plant flavonoids (Dusha et al., 1999). MucR influences the transcription of several *exo* genes by binding to palindromic sequences within the promoter regions. The positive stimulation of succinoglycan production by MucR is through the activation of transcription of biosynthesis genes and the repression of *exoX* transcription (Bertram-Drogatz et al., 1998). Succinoglycan production has also been found to be regulated by proteins involved in the phosphotransferase system that regulate succinate-mediated catabolite repression (Pinedo et al., 2008).

Regulation of EPS biosynthesis is further complicated in *S. meliloti* by the production of a secondary EPS, galactoglucan. Regulatory proteins identified as having a role in the regulation of galactoglucan include *expR* and *mucR* (Hoang et al., 2004; Keller et al., 1995; Lloret et al., 2002; Pellock et al., 2002; Ruberg et al., 1999). Furthermore, the ExpR and SinI/ SinR quorum-sensing systems of *S. meliloti* have been shown to regulate both succinoglycan and galactoglucan biosynthesis (Glenn et al., 2007; McIntosh et al., 2008; Pellock et al., 2002).
As with most of the rhizobial PS, the specific role EPS plays during nodulation remains unclear. Apparent functional redundancy amongst rhizobial polysaccharides impedes attributing specific roles in nodulation. A range of functions has been proposed for EPS including a role in bacteroid development, aiding attachment to root hairs, involvement in IT initiation and elongation and providing protection from plant defence compounds. Furthermore it has been suggested that EPS is a determinant of host-specificity and functions as a signalling molecule to induce cytoskeletal changes and/or modulate plant defence responses.

Some rhizobial mutant strains deficient in EPS production exhibit abnormal bacteroid formation, resulting in the development of ineffective nodules. Microscopic analysis of such nodules revealed fewer nodule cells infected compared to wild-type induced nodules with bacteroids appearing altered in their ultrastructure (Bialek et al., 1995; Parveen et al., 1996).
Although EPS has been implicated as having a role in the attachment of rhizobia to root hairs, it appears the role is passive. The covering of cellulose fibrils by EPS prevents cellulose-mediated agglutination allowing rhizobia to nodulate their host legume. Investigations of EPS and cellulose fibril mutant strains of *R. leguminosarum* demonstrated the requirement of EPS to prevent agglutination of cells and allow attachment to root hairs (Laus *et al.*, 2005a).

Identification of the stage of nodulation that EPS is required has been investigated using rhizobial EPS mutant strains constitutively expressing reporter genes encoding proteins such as green fluorescent protein (GFP) or β-galactosidase (LacZ) to allow visualisation of the EPS-deficient strains throughout the infection process (Cheng & Walker, 1998a; Laus *et al.*, 2004; Laus *et al.*, 2005b; Pellock *et al.*, 2000; Stuurman *et al.*, 2000). Generally such studies have revealed a requirement for EPS at the stage of IT initiation and elongation, with EPS-deficient strains forming fewer and swollen, aberrant ITs. Evidence for EPS functioning as a signalling molecule to promote IT formation rather than having a structural role is largely derived from studies in which EPS-deficient strains were ‘rescued’ from their symbiotic deficiencies via the addition of small quantities of purified EPS. For example, *S. meliloti* strains deficient in EPS production due to mutations in *exoa*, *exoB*, *exof* and *exoh* that were unable to form nitrogen-fixing nodules on *M. sativa* were able to form nitrogen-fixing nodules following the addition of purified LMW EPS derived from the parent strain (Battisti *et al.*, 1992; Urzainqui & Walker, 1992). Similarly, *Rhizobium* sp. strain NGR234 *exo* mutants unable to form nitrogen-fixing nodules on *L. leucocephala* could also be rescued via the addition of purified EPS derived from the parent strain (Djordjevic *et al.*, 1987). The inability of the addition of purified EPS to ‘rescue’ EPS mutant of rhizobial strains other than the strain from which the purified EPS was derived from also supports a signalling role for EPS that is species-specific and suggests EPS may be a determinant of host-specificity (Battisti *et al.*, 1992; Djordjevic *et al.*, 1987; Urzainqui & Walker, 1992). Further evidence supporting a signalling role for EPS is provided by the findings that an *exoK* mutant of *Rhizobium* sp. strain NGR234 that produces only HMW EPS is symbiotically inhibited on some hosts and that LMW EPS fractions of the parent strain are biologically active at low concentrations (Staehelin *et al.*, 2006).
EPS has also been proposed to function in the modulation of plant defence responses. For example, investigation of the small white ineffective nodules formed on *M. sativa* by an *exoY* mutant of *S. meliloti* revealed that the cortical cell walls were abnormally thick and contained increased amounts of phenolic compounds bound to the plant cell walls in comparison to nodules induced by wild-type *S. meliloti*. Although the EPS mutant appeared to evoke plant defence responses, it was observed that following prolonged incubation, the *exoY* mutant strain was occasionally able to overcome the defence response and form nitrogen-fixing nodules (Niehaus *et al.*, 1993). Evidence of plant defence responses elicited in response to EPS mutant strains has also been observed with *R. leguminosarum* and *B. japonicum*. Similar to the *S. meliloti* EPS mutants, enlarged plant cell walls and an increase in phenolic compounds were apparent in the small white ineffective nodules formed by the *R. leguminosarum* EPS mutants and *B. japonicum* *exoB* mutants, suggesting a severe plant defence response was mounted against the EPS-deficient strains (Parniske *et al.*, 1994; Wielbo *et al.*, 2004).

Support for EPS functioning in the modulation of plant defence responses is also provided by microarray studies of *M. truncatula* gene expression in response to inoculation with either wild-type *S. meliloti* or an EPS-deficient strain (*exoY* mutant) (Jones *et al.*, 2008). Roots inoculated with either strain were harvested three days post-inoculation and 5686 gene expression profiles were analysed with 1692 found to be differentially expressed. Of these, 390 genes exhibited at least a two-fold difference in expression (116 expressed more strongly following inoculation with wild-type *S. meliloti* and 274 expressed more in roots inoculated with the *exoY* mutant). The genes exhibiting increased expression in response to inoculation with wild-type *S. meliloti* represent various functional classes including ribosomal components and translation factors, cellular components involved in protein degradation and nodulins (proteins induced in host roots during nodule development). Genes of particular interest up-regulated in response to wild-type *S. meliloti* include two putative EPS receptors, TC104170 and a leucine-rich-repeat receptor TC103114. TC104170 was identified as a member of a multigene family of β-glucan receptors in *M. truncatula* (Leclercq *et al.*, 2008). The β-glucan receptors are found exclusively in legumes and are involved in plant defence responses following recognition of the hepta-β-glucoside elicitor found in the cell wall of the oomycete.
Phytophthora sojae that consists of a 1,6-β-Glc backbone with 1,3-β-Glc branches (Cote et al., 2000; Sharp et al., 1984).

Genes that showed increased expression following inoculation with the exoY mutant belonged mainly to pathogenesis/defence response and unknown functional classes. The observed increase in plant defence genes in response to inoculation with the exoY mutant suggests that the EPS-deficient strain may be inhibited in nodulation due to the triggering of plant defence responses that are perhaps normally suppressed through the signalling action of EPS (Jones et al., 2008).

Similar microarray studies of M. truncatula gene expression in response to wild-type S. meliloti were performed at various time-points post-inoculation. An initial increase in the expression of plant defence related genes was observed at 1 h post-inoculation; however, expression of these genes was suppressed by 6 h and remained low for at least three days (Lohar et al., 2006). Investigation of the L. japonicus transcriptome following inoculation with M. loti also revealed a down-regulation of defence related genes 24 h post-inoculation, indicating that it was recognised as symbiotically compatible (Hogslund et al., 2009).

As mentioned previously, the apparent requirement for EPS in the formation of indeterminate nodules but not determinate nodules has been widely reported. The investigation of M. loti NZP2037 EPS mutants by Hotter and Scott (1991) studied the requirement for EPS during nodulation in an isogenic system. M. loti NZP2037 has a broad host range and is able to form both indeterminate and determinate nodules depending on the host-legume species. Eight EPS mutants of M. loti NZP2037 generated by random Tn5 mutagenesis were investigated for their nodulation ability. The EPS-deficient strains were not affected in the nodulation of the determinate-nodule-forming legume L. pedunculatus, but formed ineffective nodules on the indeterminate-nodule-forming host L. leucocephala.

Overall investigations of rhizobial strains deficient in EPS have largely indicated that EPS is involved at the stage of IT initiation/elongation by possibly functioning as a signalling molecule to trigger plant developmental responses, allowing IT formation and modulating plant defence responses.
1.6.2 Lipopolysaccharide (LPS)

Unlike EPS that is secreted into the surrounding environment, LPS is attached to rhizobia and comprises a large percentage of the cell’s outer envelope (Campbell et al., 2003). The structure and function of rhizobial LPS is best characterised in the species R. leguminosarum and R. etli. Although the LPS produced by rhizobia is strain-specific, the general LPS structure consists of three components: a lipid A anchor that anchors LPS to the cell envelope, a core region of non-repeating oligosaccharide and an O-antigen that consists of repeating octasaccharide sub-units (Campbell et al., 2003; Russa et al., 1995). Rhizobia produce LPS in two forms, named on the basis of the colony morphology of cells producing them, rough LPS that consists of the lipid A anchor and core oligosaccharide and smooth LPS which has the O-antigen included (Carlson & Krishnaiah, 1992; Carrion et al., 1990). The lipid A portion of LPS displays the greatest variability between rhizobial strains, with each unique LPS species largely due to changes in carbohydrate composition of the lipid A component (Carlson et al., 1978). The structure of rhizobial LPS has been investigated to varying degrees in a range of rhizobia with the complete structure of R. etli LPS determined (Forsberg & Carlson, 1998) (Figure 1.8).

In comparison to the biosynthesis of EPS, details on the genes involved in LPS biosynthesis are sparse. Genes involved in LPS biosynthesis identified by mutant studies have been mapped to six genetic loci in R. leguminosarum and R. etli designated lps α, β, γ, δ, ε and 166 (Noel & Duelli, 2000). The function of the genes present in the 18-kb chromosomal lpsα genetic locus are not known with the involvement of the locus in production of the core and O-antigen components of LPS identified through mutation and complementation studies (Cava et al., 1990; Tao et al., 1992). The lpsβ locus is harboured on plasmids of R. leguminosarum and R. etli with two genes identified in the locus termed lpsβ1 and lpsβ2 that encode putative proteins with homology to glucosyl/ galactosyl transferases and known surface polysaccharide biosynthesis proteins such as WbpM of Pseudomonas aeruginosa (Garcia-de los Santos & Brom, 1997).
Three glycosyltransferases required for the synthesis of the LPS core termed lpcA, lpcB and lpcC were identified in R. leguminosarum that make up genetic locus lpsδ. lpcA and lpcB are located adjacent to each other on the chromosome with lpcC situated several kb downstream. LpcA codes for a galactosyltransferase, lpcB a distal Kdo-transferase and lpcC encodes a mannosyltransferase (Kadrmas et al., 1998). The lpeA, lpeB, lpeC and lpeD genes are proposed to function in the synthesis of the O-antigen component of LPS and compose the lpsc gene locus. Finally mutation of locus lps-166 caused production of reduced amounts of LPS that lacked quinovosamine (Noel et al., 2000). Further genes to those mentioned that may have an effect on LPS
production by rhizobia include genes involved in the synthesis of other polysaccharides. For example UDP-galactose synthesised by UDP-glucose 4-epimerase, the product of \textit{exoB}, is required for both EPS and LPS biosynthesis (Canter Cremers \textit{et al.}, 1990; Sanchez-Andujar \textit{et al.}, 1997).

The analysis of the symbiotic proficiency of a range of LPS mutants has resulted in the proposal of various roles in nodulation for rhizobial LPS. The functioning of LPS in IT formation was demonstrated through the application of LPS extracted and purified from \textit{R. leguminosarum} \textit{bv. trifolii} 0403 to the roots of white clover. The LPS was shown to bind to root hair tips and cross the root hair cell wall. In addition pre-inoculation treatment of roots with the extracted LPS at doses of 5 mg per plant promoted IT formation (Dazzo \textit{et al.}, 1991). Fraysse points out the possibility that the extracted soluble LPS utilised in the study by Dazzo \textit{et al.} may in fact represent KPS, once considered as LPS (Fraysse \textit{et al.}, 2005). Generally studies of LPS mutants indicate a role for LPS in later stages of symbiosis such as release from ITs and bacteroid development. Analysis of \textit{R. leguminosarum} \textit{bv. viciae} LPS mutants that formed small ineffective nodules showed that ITs were formed and the mutant bacteria released from the IT tips. However bacteroids failed to develop and the nodules suffered premature senescence, suggesting a role for LPS in bacteroid development (Priefer, 1989). Additional studies of LPS mutants of \textit{R. leguminosarum} indicated a block in the symbiotic process at the stage of bacterial release from ITs, with changes in the LPS mutants’ surface hydrophobicity implicated as a possible cause of failure of the LPS mutants to be successfully endocytosed by plant cells as they are released from IT tips (de Maagd \textit{et al.}, 1989; Kadarmas \textit{et al.}, 1998). Further evidence for LPS involvement during the later stages of nodulation is derived from investigations of \textit{B. japonicum} LPS mutants that formed ineffective nodules with abnormal structure that were mostly devoid of bacteria (Stacey \textit{et al.}, 1991).

Although in comparison to \textit{R. leguminosarum} few studies of the symbiotic properties of \textit{S. meliloti} LPS mutants have been performed, it would seem that they also are largely affected during the later stages of nodulation. Examination of an LPS mutant of \textit{S. meliloti} Rm2011 indicated that although both wild-type and mutant initiated ITs at the same time, nodule development was delayed on mutant-inoculated plants. Furthermore the LPS mutant was out-competed by the wild-type parent strain in co-
inoculation studies (Lagares et al., 1992). The involvement of LPS in host range
determination has also been suggested from studies in which LPS mutants of S.
meliloti effectively nodulated M. sativa but failed to form nitrogen-fixing nodules on
M. truncatula (Niehaus et al., 1998). Two M. loti LPS mutants harbouring mutations in
genes involved in O-antigen biosynthesis, lpsβ1 and lpsβ2, formed nitrogen-fixing
nodules but showed reduced competitiveness compared to the wild-type strain
(D’Antuono et al., 2005). Rhizobial LPS may play a role in modulating plant defence
responses with purified LPS components demonstrated to affect the production of
reactive oxygen species (ROS) in plants (Albus et al., 2001; Scheidle et al., 2005;
Tellstrom et al., 2007). S. meliloti LPS can suppress ROS production in host plants M.
sativa and M. truncatula whilst in contrast some LPS components can induce ROS in
the non-host Nicotiana tabacum (Scheidle et al., 2005).

To date investigations of LPS production by rhizobia have indicated the genes
responsible for biosynthesis of the molecule are spread throughout the genome and
are often involved in other cellular processes as well. Therefore attributing specific
symbiotic effects to LPS production is not easily achieved. However the investigation
of a range of rhizobial LPS mutants that exhibit symbiotic deficiencies generally
suggest LPS is involved in the later stages of nodule development.

1.6.3 K-antigens (KPS) / capsular polysaccharide

K-antigens (KPS), often referred to as capsular polysaccharide, are tightly associated
with the rhizobial outer membrane and are structurally similar to the group II K-
antigens of E. coli. Although rhizobial KPS are strain-specific, they are generally
acidic linear disaccharide repeats consisting of a 3-deoxy-D-manno-2-octulosonic acid
(kdo) or related sugar and a neutral hexose or uronic acid (Figure 1.9) (Le Quere et
al., 2006; Reuhs et al., 1993). Exceptions exist regarding the general structure of
rhizobial KPS such as the species produced by S. meliloti strain 1021 which is
composed exclusively of β-(2-7)-linked kdo molecules (Fraysse et al., 2005).
Three gene clusters identified in *S. meliloti* Rm41, termed *rkp*-1, *rkp*-2 and *rkp*-3, have been identified as encoding genes involved in KPS production by the strain. *rkp*-1 is located on the chromosome and encodes ten genes termed *rkpA*-J (Kiss et al., 1997; Petrovics et al., 1993). Analysis of the *rkpA*-J gene sequences indicated similarities with known fatty acid synthases and other proteins involved in the modification and transfer of lipid molecules. These similarities suggest that the *rkpA*-J genes may be involved in the production of a lipid carrier or anchor for the KPS molecule (Kiss et al., 1997). *rkp*-2 is also chromosomally located and contains two ORFs designated *lpsL* and *rkpK* that are transcribed in the same direction and separated by 150 bp (Kereszt et al., 1998). Investigation of the *rkp*-2 region revealed that both genes are required for normal LPS production by the strain, whereas only *rkpK* is needed for KPS production. Sequence analysis showed a high degree of similarity between *lpsL* and UDP-galacturonic acid epimerases of *Staphylococcus aureus* and *Streptococcus pneumoniae* and between *rkpK* and UDP-glucose dehydrogenases. The function of *rkpK* as a UDP-glucose dehydrogenase was supported by biochemical analysis of *rkpK* mutants compared to wild-type and complemented *rkpK* mutants (Kereszt et al., 1998). The *rkp*-3 region is located on the pSymB megaplasmid and encodes ten genes involved in the biosynthesis and modification of KPS as well as genes possibly involved in the transport of KPS (Kiss et al., 2001). *Rhizobium* sp. NGR234 KPS
structure was determined and the sequence of the rkp-3 region was compared to that of S. meliloti Rm41 (Le Quere et al., 2006). Most of the genes thought to be involved in KPS synthesis, rkpLMNOQ, were present in both strains as were the probable transport genes rkpRST. The presence of similar biosynthetic genes was not unexpected due to the similarity of the glycosyl composition of the KPS produced by the two strains. However differences in the organisation of the genes as well as the absence of rkpP in Rhizobium sp. NGR234 were observed. The rkp-3 region of S. meliloti strain 1021 shared similarities in the transport genes rkpRST; however no homologous genes to the KPS biosynthesis genes rkpLMNOPQ were identified (Kiss et al., 2001). This suggests that the genes of the rkp-3 region may be involved in determining the strain-specific composition of the KPS produced.

The function of KPS during the process of nodulation may simply be a passive role in protecting the rhizobia against abiotic factors and plant defence responses or KPS may in fact play an active role in promoting successful symbiosis. Resolving the importance and role of KPS in nodulation by rhizobia is made difficult due to its biosynthesis being linked to EPS and LPS biosynthesis.

Investigation of a Rhizobium sp. NGR234 rkpMNO mutant showed that the mutant was symbiotically deficient compared to the parent strain (Le Quere et al., 2006). Importantly this mutant was unaffected in LPS or EPS production because the mutation only affected the synthesis of pseudaminic acid that is an important part of KPS produced by the strain but not LPS or EPS. In addition, the importance of KPS in the formation of successful symbiosis between S. fredii and host legumes has been demonstrated (Parada et al., 2006). The symbiotic proficiency of strains harbouring mutations in rkpH and rkpG was investigated and compared to an exoA EPS mutant as well as an rkpH/ exoA double mutant. The results of plant assays indicated that the exoA mutant was unaffected whereas both KPS mutant strains and the EPS/ KPS double mutant were impaired in their symbiotic abilities. Interestingly S. meliloti KPS is capable of functionally replacing EPS to promote successful symbiosis with alfalfa. An exoB mutant that fails to produce any EPS and that also lacks the lpsZ gene required for KPS production is unable to nodulate alfalfa, but introduction of a functional copy of lpsZ allows the strain to nodulate. The inability of the strain to produce EPS but produce KPS and promote successful symbiosis suggests that EPS
and KPS play similar roles in *S. meliloti* during symbiosis and can functionally substitute for each other (Reuhs et al., 1995). The stage of nodulation at which KPS is involved has not been widely studied, but the requirement for KPS at the stage of IT elongation has been demonstrated with regard to the *S. meliloti*-alfalfa symbiosis (Pellock et al., 2000).

### 1.6.4 Cyclic-β-glucans (CBGs)

CBGs are produced by all rhizobia studied to date and their production is almost exclusively found in the *Rhizobiaceae* (Amemura, 1984; Bhagwat et al., 1992; Breedveld & Miller, 1994). Other organisms for which CBGs production has been reported also interact with a eukaryotic host, suggesting CBGs are important for the interaction (Amemura & Cabrera-Crespo, 1986; Bundle et al., 1988; Talaga et al., 1994; Talaga et al., 1996). CBGs are made up of glucose residues linked via β-glycosidic bonds (Figure 1.10). The number of glucose residues that make up the ring structure varies between species ranging from 17 in *R. leguminosarum* to 40 in *S. meliloti* (Benincasa et al., 1987; Koizumi et al., 1984). Further variations in CBGs produced by rhizobia are achieved through the addition of anionic substituent groups such as phosphoglycerol and succinyl groups (Miller et al., 1988).

Two genes, *ndvA* and *ndvB*, were identified in *S. meliloti* as responsible for the production of CBGs based on their similarity to the previously identified CBG synthesis genes of *Agrobacterium tumefaciens chvA* and *chvB* (Douglas et al., 1985; Dylan et al., 1986). Investigations of NdvA and NdvB of rhizobia and their counterparts ChvA and ChvB of *Agrobacterium* strains indicate that NdvA, a 67-kDa protein with homology to various bacterial ATP-binding transport proteins, functions to transport synthesised CBGs into the periplasm (Stanfield et al., 1988). The synthesis of CBGs from UDP-glucose is mediated by the 319-kDa NdvB cytoplasmic protein (Ielpi et al., 1990). A two-component regulation system consisting of FeuP/FeuQ that is responsive to extracellular osmotic conditions was identified in *S. meliloti* that regulates the expression of the CBG export gene *ndvA* (Griffitts et al., 2008).
The requirement for CBGs in the establishment of successful symbiosis by rhizobia has been demonstrated with *S. meliloti* strains harbouring mutations in *ndvA/ndvB* that form ineffective white nodules on *M. sativa* (Dylan *et al.*, 1986). However, a small percentage of CBG mutants are able to form nitrogen-fixing nodules at rates much lower than wild-type *S. meliloti* (Dylan *et al.*, 1990). Second-site pseudorevertants isolated by Dylan *et al.* based on their restored motility and attachment ability displayed only slight restoration of IT initiation and symbiotic abilities. The observation that the pseudorevertants had not recovered the ability to produce CBGs suggested that *S. meliloti* probably has excess capabilities for attachment and IT initiation and that CBGs likely function later in the symbiotic process, perhaps at the stage of IT elongation (Dylan *et al.*, 1990).

*M. loti* produces both neutral and anionic fractions of CBG through the incorporation of glycerophosphoryl and succinyl residues (Kawaharada *et al.*, 2008). Investigations
of a CBG mutant of *M. loti* that formed small white ineffective nodules on *L. tenuis* revealed that the CBG mutant formed very few ITs (D'Antuono *et al.*, 2005). Similar symbiotic deficiencies were reported when an *M. loti* mutant disrupted in a putative cell envelope protein that resulted in the production of reduced amounts of CBG was used to inoculate *L. japonicus* (Kawaharada *et al.*, 2007).

Many functions for CBGs during nodulation have been proposed. Observations that CBGs are produced at higher levels by cultures grown in low osmolarity media indicate that they may be involved in osmoprotection (Breedveld *et al.*, 1990; Dylan *et al.*, 1990). A role for CBGs as osmoprotectants is supported by the accumulation of CBGs in the periplasm. Alternative roles for CBGs include a role in aiding attachment of rhizobia to root hairs, as indicated by the severely reduced ability of *ndvA/ndvB* *S. meliloti* mutants to attach to the roots of *M. sativa* seedlings (Dylan *et al.*, 1990). Further support for the involvement of CBGs in mediating attachment of rhizobia to roots is afforded by the findings that CBGs of *R. leguminosarum* have the capacity to bind to pea lectins (Planque & Kijne, 1977). Finally modulation of plant defences through the action of CBGs has been proposed due to findings that *B. japonicum* CBGs may prevent phytoalexin production in soybean (Mithofer *et al.*, 1996; Mithofer *et al.*, 2001).

The presence of CBGs exclusively in organisms associated with a eukaryotic host suggests that CBGs have an important role to play in the host-microbe interaction. Although the majority of studies investigating CBGs production by rhizobia indicate a passive osmoprotectant role, further roles cannot be ruled out.

### 1.6.5 Cellulose

Once thought to be a plant-specific compound, cellulose has now been identified in algae, fungi and bacteria and it is one of the most abundant biological polymers produced in nature (Ross *et al.*, 1991). Cellulose has a seemingly simple structure, consisting solely of 1,4-β-linked glucose with multiple linear chains arranged in parallel which form intra- and inter-chain hydrogen bonds that vary the crystalline structure (O'Sullivan, 1997). Although identical in chemical composition, cellulose varies in the degree of polymerisation and crystallinity depending on the producing organism.
Cellulose production by bacteria has been most well-studied in *Gluconacetobacter xylinus* (formerly known as *Acetobacter xylinus*) with an operon *bcsABCD* responsible for its biosynthesis identified (Ross et al., 1987; Wong et al., 1990). The mechanism of cellulose biosynthesis by *G. xylinus* is not well understood; however, it is thought that the cellulose synthase (BcsA) and the bis-(3’,5’) cyclic diguanylic acid (c-di-GMP) binding protein (BcsB) are structurally associated forming a cellulose synthase complex in the cytoplasmic membrane (Lin et al., 1990). The binding of c-di-GMP by BcsB is thought to regulate the activity of BcsA and result in the production of cellulose from the substrate UDP-glucose (Romling, 2002). Although the function of *bcsC* and *bcsD* remain unknown, they are required for normal cellulose production *in vivo* and it is thought that they may be involved in the polymerisation/transport and/or the regulation of cellulose synthesis (Ross et al., 1991; Wong et al., 1990). The cellulose synthase is well conserved amongst bacteria in which cellulose production has been demonstrated (Romling, 2002). The remaining genes associated with cellulose production are less well conserved, perhaps an indication of differing biosynthetic and regulatory mechanisms.

Cellulose production has been demonstrated in the plant-associated bacteria *Agrobacterium tumefaciens* and *Rhizobium leguminosarum* bv. *trifolii* (Amikam & Benziman, 1989; Ausmees et al., 1999). Two operons, *celABC* and *celDE* were identified in *A. tumefaciens* as involved in cellulose biosynthesis (Matthysse et al., 1995b). Only the cellulose synthase (*celA*) and an endoglucanase (*celC*) share homology to cellulose biosynthesis genes identified in *G. xylinus*. A mechanism for cellulose production involving lipid-linked intermediates through the actions of the CelD and CelE has been proposed for *A. tumefaciens* (Matthysse et al., 1995a). This mechanism is distinctly different than the ‘direct’ synthesis of cellulose by a cellulose synthase complex in the mechanism thought to occur in *G. xylinus*.

Six genes required for the biosynthesis of cellulose in *R. leguminosarum* bv. *trifolii* were identified through transposon mutagenesis (Ausmees et al., 1999). The identified genes share homology to those of *A. tumefaciens* with similar operon structure. Two of the genes identified, *celR1* and *celR2*, are thought to encode a two-component regulatory system involved in the regulation of cellulose biosynthesis.
Several roles for bacterial cellulose production have been suggested. For *G. xylinus* some experimental evidence suggests that the obligate aerobic bacteria produces cellulose to provide a solid floating matrix which allows embedded cells to remain in close association with the atmosphere on the surface in liquid environments (Cook & Colvin, 1980). However, in nature *G. xylinus* is usually found on decaying fruit, a solid substrate, which led to alternative roles for cellulose production being investigated (Williams & Cannon, 1989). These included providing protection from UV light and preventing potential competitors from gaining access to nutrient sources.

For *A. tumefaciens*, the production of cellulose is largely reported to be involved in aiding attachment of the bacteria to host plant cells (Matthysse *et al.*, 1981). Cellulose-deficient mutants of *A. tumefaciens* were shown to be comparatively loosely attached to carrot cells compared to cellulose-producing controls. However, cellulose is clearly not the only bacterial component involved in attachment as cellulose-deficient strains are still able to attach and cause infection (Matthysse, 1983), although the mutants exhibit reduced virulence to varying degrees depending on gene disrupted (Minnemeyer *et al.*, 1991). The reduced virulence is presumably related to the less effective attachment of the cellulose-deficient strains.

Similar to *A. tumefaciens*, the major role of cellulose production by *R. leguminosarum* is reported to be involvement in the attachment to host plant cells (Ausmees *et al.*, 1999; Laus *et al.*, 2005a; Smit *et al.*, 1986). Attachment of *R. leguminosarum* to root hair tips is proposed to occur in a two-step process. The first is loose attachment involving the cell surface protein rhicadhesin followed by a stronger secondary attachment step mediated by cellulose fibrils that results in the formation of a bacterial ‘cap’ on the root-hair tip (Smit *et al.*, 1987). Cellulose over-producing strains were shown to form larger caps on root-hair tips whilst cellulose-deficient strains did not form caps at all. Despite the involvement of cellulose in attachment, cellulose production is not required for successful nodulation as demonstrated by the symbiotic proficiency of cellulose-deficient strains (Ausmees *et al.*, 1999; Smit *et al.*, 1987; Williams *et al.*, 2008). Although generally not affected in symbiosis, an EPS-deficient *R. leguminosarum* mutant was found to be inhibited in the nodulation of *V. sativa* due to cellulose-mediated agglutination of the bacteria within curled root-hairs that prevented entry
into the IT (Laus et al., 2005a). IT entry was partially restored in an EPS- and cellulose-deficient mutant strain leading the authors to propose that in the wild-type situation, EPS prevents bacterial agglutination by masking the cellulose fibrils.

To date cellulose production has not been reported in *M. loti*.

1.7 The involvement of plant pathogen surface polysaccharides in evasion of innate immunity

Although the rhizobium-legume symbiosis is a mutualistic interaction, the infection process bears parallels with plant-pathogen interactions. In both instances the success of the outcome requires avoiding recognition or suppressing host defence responses. Investigations into the role surface polysaccharides of plant pathogens play in modulating host defence responses may inform on their role in the rhizobium-legume symbiosis.

The innate immunity of plants is essential in their defence against infecting microbes. Microbe-associated or pathogen-associated molecular patterns (MAMPs/ PAMPs) are recognised by plant transmembrane pattern-recognition receptors (PRR) which leads to basal plant defence responses known as PAMP-triggered immunity (PTI) (Boller & Felix, 2009; Chisholm et al., 2006; Jones & Dangl, 2006; Schwessinger & Ronald, 2012) (Figure 1.11). For example, a synthetic 22 amino-acid peptide (flg22), that was designed based on a conserved bacterial flagellin domain, strongly induced *Arabidopsis thaliana* plant defence responses following its recognition by a leucine rich repeat-receptor FLS2 (Chinchilla et al., 2006). Pathogenic and symbiotic bacteria have evolved effector proteins that are delivered to the host plant cells, often through the action of type III secretion systems. Effector proteins interfere with the signalling cascade following PAMP recognition and inhibit induction of PTI. In response to these effectors, plants have acquired resistance (R) proteins that recognise effectors or the action of the effector proteins, leading to effector-triggered immunity (ETI) (Jones & Dangl, 2006) (Figure 1.11).

Surface polysaccharides of plant pathogens have been demonstrated to function in both the activation and suppression of PTI (Silipo et al., 2010). LPS of various bacteria including several plant pathogens stimulates a burst of nitric oxide production in *Arabidopsis thaliana* (Zeidler et al., 2004) and *Xanthomonas campestris* LPS was found to
induce the transcription of *A. thaliana* defence genes *PR1* and *PR2* (Silipo *et al.*, 2005). Interestingly, a non-pathogenic strain of *X. campestris* produces LPS with an altered lipid A region that did not induce *A. thaliana* defence genes, suggesting that *X. campestris* may be able to modify its LPS to avoid eliciting PTI (Silipo *et al.*, 2008).

An *ndvB* mutant of *X. campestris* deficient in the production of CBG was found to be compromised in the infection of *Nicotina benthamiana*, with increased callose deposition and expression of the *PR1* defence gene observed in plants inoculated with the strain. Addition of purified CBG to either the same leaf or a different leaf to that inoculated with the *ndvB* mutant restored virulence, indicating that CBG induced systemic suppression of defence responses (Rigano *et al.*, 2007).

EPS of plant pathogens have generally been associated with suppression of plant defence responses with the mechanism of action proposed to be through the chelation of Ca$^{2+}$ (Aslam *et al.*, 2008). It was noted that the EPS of pathogen species are polyanionic due to the incorporation of uronic acid residues and substituent groups such as succinate and pyruvate giving them the potential to bind or chelate cations. Calcium functions as a messenger in MAMP-perception and a Ca$^{2+}$ ion influx to the cytosol from the apoplast is required for PTI (Lecourieux *et al.*, 2006). Purified EPS from plant, animal and symbiotic bacteria was shown be able to bind Ca$^{2+}$ and suppress the Ca$^{2+}$ ion influx in *Arabidopsis* cells that was induced by MAMPs, thereby supressing PTI (Aslam *et al.*, 2008).
Figure 1.11: The plant innate immune system.

A) PAMPs are recognised by plant PRRs, resulting in a downstream signalling cascade that leads to PTI. B) Pathogenic and symbiotic bacteria have acquired effectors (purple stars) that suppress PTI, resulting in effector-triggered susceptibility. C) Plants have acquired R proteins that recognize these bacterial effectors, resulting in a secondary immune response ETI. Reproduced from (Pieterse et al., 2009) with permission.
1.8 Summary of MSc research

This section summarises relevant results obtained during previous research into the requirement for EPS in the *Mesorhizobium-Lotus* symbiosis (Kelly, 2007). This includes the identification of R7A EPS biosynthesis genes and the symbiotic phenotypes of R7A EPS mutant strains.

1.8.1 R7A EPS biosynthesis

Although the R7A genome had not been sequenced, a closely-related strain, *M. loti* MAFF303099, has been completely sequenced (Kaneko et al., 2000) and the annotated sequence is publicly available on the Rhizobase website (http://genome.kazusa.or.jp/rhizobase).

Sequencing of a ~30-kb *exo* cosmid, pSK11, isolated from R7A revealed that the two *M. loti* strains share identical *exo* gene arrangement in the region with a high degree of nucleotide identity (98%). Genes involved in all stages of EPS production are located in the *exo* cluster region on the genome. This includes genes involved in the regulation, biosynthesis of nucleotide sugar precursors, synthesis and decoration of EPS subunits as well as transport and polymerisation-related genes (Figure 1.12). Whilst the majority of EPS-related genes are clustered, others are located elsewhere on the chromosome.

Comparison of the EPS genes identified in R7A/ MAFF303099 to those of rhizobia in which the EPS structure has been determined allows for insight into the potential composition of R7A EPS. *S. meliloti*, *Rhizobium* sp. strain NGR234 and *M. loti* MAFF303099 share the EPS biosynthesis genes required up to and including the step catalysed by ExoU, the formation of the hexasaccharide \([\text{Gal-}-(\text{Glc})_5]\), but not subsequent steps (Streit et al., 2004). The absence of pyruvyltransferase (*exoV*) and succinyltransferase (*exoH*) homologues in MAFF303099 suggests it is unlikely that EPS produced by R7A / MAFF303099 would contain these substituents. Although not located in the *exo* cluster, several acetyltransferases (*exoZ*) homologues were identified throughout the genome indicating that the EPS may be acetylated. The identification of a predicted UDP-glucose dehydrogenase (*mlr5265*) and a gluconate dehydrogenase (*mlr5266*) amongst the cluster of *exo* genes suggested that the EPS may contain uronic acid groups. No homologues of the *S. meliloti* secondary EPS
biosynthesis genes were identified, indicating that the *M. loti* strains likely produce a solitary EPS species.

**Figure 1.12: M. loti exo gene cluster.**

The cluster of *exo* genes identified in MAFF303099 was found to be present in identical arrangement in R7A with 98% nucleotide identity. The ends of the R7A cosmid pSK11 are indicated by arrows and the MAFF303099 genome co-ordinates of the region are displayed.
1.8.2 Symbiotic phenotypes of R7A EPS mutants

A range of R7A EPS mutant strains were constructed with the genes targeted for mutation predicted to be involved at various stages of the EPS biosynthetic pathway.

The ability of the EPS mutant strains to form nitrogen-fixing nodules on the determinate-nodule-forming host *L. corniculatus* was examined (Figure 1.13). The nodulation proficiency differed depending on the mutant. *L. corniculatus* inoculated with R7AexoB or R7AΔexoA exhibited nodule formation similar to control R7A-inoculated plants. In contrast, strains R7AexoU, R7AexoO and R7Amlr5265 formed white nodule primordia and, only following prolonged incubation, the occasional larger pink/brown effective nodule. R7AexoK and the R7Amlr5266 mutant had an intermediate phenotype between the R7AexoB and R7AexoU phenotypes. Shoot growth of plants inoculated with nodulation-impaired *exo* mutants was stunted and the leaves were yellowing compared to R7A-inoculated plants, indicating nitrogen starvation. The stunted growth of plants inoculated with the EPS mutants is highlighted by the reduced weight of the plant shoots at the conclusion of the nodulation assay (Figure 1.13).

Investigation of strains constitutively expressing *lacZ* revealed that the symbiotically inhibited EPS mutants were defective at the stage of IT formation.
Figure 1.13: Symbiotic proficiency of R7A EPS mutants.

A) Total number of nitrogen-fixing nodules formed on 15 plants of *L. corniculatus* inoculated with the indicated R7A strains. B) Average plant shoot weights 6-weeks post-inoculation with the indicated strains. The standard errors of the mean are indicated.
1.9 The aim of this study

The overall aim of this PhD project was to investigate a possible signalling role for EPS in the determinate-nodule-forming *Mesorhizobium-Lotus* symbiosis. This was investigated through the characterisation of previously isolated and newly constructed R7A EPS mutant strains. Colony phenotypes on various media were analysed to investigate potential correlations between observed colony properties and symbiotic proficiencies. EPS mutant strains that exhibit contrasting symbiotic phenotypes (R7AexoB and R7AexoU) were thoroughly examined. Determining differences between the two strains may reveal insights into the potential function of EPS during nodulation.

The structure of *M. loti* EPS has not been reported. It was explored in this study through the chemical analysis of wild-type and mutant strain extracts. A major aim of the chemical analysis was to determine the structure of wild-type R7A EPS to reveal if genes other than those identified may be involved in EPS biosynthesis and also to allow comparison to EPS mutant extracts that may indicate the production of alternative polysaccharides or truncated forms of the wild-type EPS.

In order to gain insight into potential additional factors involved in the symbiotic deficiencies of strain R7AexoU, transposon mutagenesis was performed to investigate if strains carrying secondary mutations that suppressed the R7AexoU symbiotic phenotype could be isolated.

*M. loti* NZP2037 exhibits symbiotic proficiencies that distinguish it from other *M. loti*, including its unique ability to successfully nodulate *L. pedunculatus*. Various mutation and complementation approaches were employed in this study to investigate if EPS produced by the strain was involved in its symbiotic phenotypes.
2 Materials and methods
2.1 **Bacterial strains and plasmids**

*Escherichia coli* and *Mesorhizobium loti* strains used in this study are described in Table 2.1. Plasmids used in this study are described in Table 2.2.

2.2 **Media and growth conditions**

*E. coli* strains were cultured at 37°C. Media used for the growth of *E. coli* strains were Luria Bertani (LB) (Miller, 1972) agar and broth, and Tryptone yeast (TY) (Beringer, 1974) agar and broth. *M. loti* strains were cultured at 28°C. Media used for the growth of *M. loti* strains were TY broth or rhizobium defined media (RDM) (Ronson et al., 1987) broth and agar plates, usually supplemented with 0.4% glucose as the sole carbon source (G/ RDM). RDM with 5% sucrose as the sole carbon source was utilized in the creation of markerless deletion mutants. Congo red agar used in this study was yeast-extract mannitol (YM) agar (Vincent, 1970) or TY agar containing 0.005% [w/ v] Congo red dye (C6277, Sigma). Calcofluor medium was TY or G/ RDM with the addition of 0.02% [w/ v] Fluorescent brightener-28 (F3543, Sigma). Media recipes are given in Appendix A. Antibiotics were added to media as required (Table 2.3).

2.3 **Storage of bacterial strains**

Bacterial strains used in this study were stored at -70°C in Nalgene cryogenic vials (Nalgene, USA). Cultures were prepared for storage by adding 70 µL of dimethylsulfoxide (DMSO) to 800 µL of stationary-phase cultures that were grown in an appropriate medium.

2.4 **Enzymes and chemicals**

Enzymes were purchased from Roche Diagnostics and New England Biolabs. Antibiotics and chemicals were purchased from Sigma. Antibiotics were dissolved in Milli-Q water and filter sterilized through a 0.45 µm syringe filter at appropriate concentrations, except Tc and Rf which were dissolved in 100% methanol. All chemicals were analytical grade with solutions made up in distilled water unless otherwise stated. Recipes for solutions and buffers are provided in Appendix B.
Table 2.1: Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or relevant characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HB101</td>
<td><em>leuB thi xyl mtl ara Δ(gpt-proA) lacY hsdS recA galK supE mcrB, Str</em></td>
<td>(Boyer &amp; Roulland-Dussoix, 1969)</td>
</tr>
<tr>
<td>S17-1/ λpir</td>
<td>RP4:2 tra region, <em>pir</em>, host for <em>pir</em>-dependent plasmids</td>
<td>(Herrero <em>et al.</em>, 1990)</td>
</tr>
<tr>
<td>EPI300</td>
<td><em>F mcrA Δ(mrr-hsdRMS-mcrBC)</em>, Φ80dlacZ ΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK rpsL nupG trfA tonA, Str*</td>
<td>Epicentre</td>
</tr>
<tr>
<td><em>Mesorhizobium loti</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R7A</td>
<td>Field reisolate of ICMP3153 (NZP2238; Lc265Da) originally isolated in Ireland</td>
<td>(Sullivan <em>et al.</em>, 2002)</td>
</tr>
<tr>
<td>NZP2037</td>
<td>Nod ‘Fix’ on <em>Lotus pedunculatus</em>; isolated in New Zealand from <em>L. divaricatus</em></td>
<td>(Jarvis <em>et al.</em>, 1982)</td>
</tr>
<tr>
<td>MAFF303099</td>
<td>Isolated in Japan from <em>L. japonicus</em></td>
<td>(Kaneko <em>et al.</em>, 2000)</td>
</tr>
<tr>
<td>CJ6</td>
<td>Non-symbiotic <em>M. loti</em> strain, Det’</td>
<td>(Sullivan <em>et al.</em>, 1996)</td>
</tr>
<tr>
<td>R7AexoB</td>
<td>R7A <em>exoB::mTn5, Nm</em></td>
<td>(Hubber, 2005)</td>
</tr>
<tr>
<td>R7AexoU</td>
<td>R7A <em>exoU::mTn5, Nm</em></td>
<td>(Hubber, 2005)</td>
</tr>
<tr>
<td>R7AexoU3'</td>
<td>R7A <em>exoU::mTn5, insertion 83 bp from 3’ end of the gene, Nm</em></td>
<td>(Hubber, 2005)</td>
</tr>
<tr>
<td>R7AexoO</td>
<td>R7A <em>exoO::pFUS2, Gm</em></td>
<td>(Kelly, 2007)</td>
</tr>
<tr>
<td>R7AexoK</td>
<td>R7A <em>exoK::pFUS2, Gm</em></td>
<td>(Kelly, 2007)</td>
</tr>
<tr>
<td>R7A5265</td>
<td>R7A <em>mlr5265::pFUS2, Gm</em></td>
<td>(Kelly, 2007)</td>
</tr>
<tr>
<td>R7A5266</td>
<td>R7A <em>mlr5266::pFUS2, Gm</em></td>
<td>(Kelly, 2007)</td>
</tr>
<tr>
<td>R7AΔexoA</td>
<td>R7A <em>exoA</em>, in-frame markerless deletion</td>
<td>(Kelly, 2007)</td>
</tr>
<tr>
<td>R7AΔndvB</td>
<td>R7A <em>ndvB</em>, in-frame markerless deletion</td>
<td>(Morgan, 2007)</td>
</tr>
<tr>
<td>R7AΔexsH</td>
<td>R7A <em>exsH</em>, in-frame markerless deletion</td>
<td>This study</td>
</tr>
<tr>
<td>R7AΔexoU</td>
<td>R7A <em>exoU</em>, in-frame markerless deletion</td>
<td>This study</td>
</tr>
<tr>
<td>R7AexoBexoU</td>
<td>R7AexoB containing an <em>exoU::pFUS2</em> insertion, Nm, Gm</td>
<td>This study</td>
</tr>
<tr>
<td>R7AexoUPF</td>
<td>R7A <em>exoU::pFUS2, Gm</em></td>
<td>This study</td>
</tr>
<tr>
<td>R7AΔexoU/ 2244</td>
<td>R7AΔexoU containing a <em>mlr2244::mTn5</em> insertion, Nm</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Strain</strong></td>
<td>Description</td>
<td>Source</td>
</tr>
<tr>
<td>----------------------------</td>
<td>------------------------------------------------------------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>R7AΔexoU/ 2385</td>
<td>R7AΔexoU containing a <em>mll2385::mTn5</em> insertion, Nm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>R7AΔexoU/ 4457</td>
<td>R7AΔexoU containing a <em>mlr4457::mTn5</em> insertion, Nm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>R7AΔexoU/ 5197</td>
<td>R7AΔexoU containing a <em>mll5197::mTn5</em> insertion, Nm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>R7AΔexoU/ 5271</td>
<td>R7AΔexoU containing an <em>exoL::mTn5</em> insertion, Nm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>R7AΔcelA</td>
<td>R7A celA (<em>mll7873</em>), in-frame markerless deletion</td>
<td>This study</td>
</tr>
<tr>
<td>R7AΔcelAexoU</td>
<td>R7AΔcelA containing an <em>exoU::pFUS2</em> insertion, Gm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>R7AΔnodA</td>
<td>R7A nodA, in-frame markerless deletion</td>
<td>(Rodpothong <em>et al.</em>, 2009)</td>
</tr>
<tr>
<td>NZP2037ΔexoB</td>
<td>NZP2037 <em>exoB</em>, in-frame markerless deletion</td>
<td>This study</td>
</tr>
<tr>
<td>NZP2037ΔexoU</td>
<td>NZP2037 <em>exoU</em>, in-frame markerless deletion</td>
<td>This study</td>
</tr>
</tbody>
</table>
### Table 2.2: Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBH474</td>
<td>Expresses Flp recombinase, SacB, Gm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(House et al., 2004)</td>
</tr>
<tr>
<td>pBLUESCRIPT II SK</td>
<td>Cloning vector, oriV&lt;sup&gt;ColE1&lt;/sup&gt;, lacZ&lt;sub&gt;a&lt;/sub&gt;, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pCRS487</td>
<td>pUT::mTn5-GNm, Ap&lt;sup&gt;R&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Reeve et al., 1999)</td>
</tr>
<tr>
<td>pDsRed-Express-N1</td>
<td>Reporter vector, encodes dsred, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Clonetech</td>
</tr>
<tr>
<td>pFAJ1700</td>
<td>Stable RK2-derived cloning vector, Ap&lt;sup&gt;R&lt;/sup&gt;, Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Dombrecht et al., 2001)</td>
</tr>
<tr>
<td>pFAJ1708</td>
<td>Stable RK2-derived cloning vector containing the promoter of the nptII gene in the multiple cloning site, Ap&lt;sup&gt;R&lt;/sup&gt;, Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Dombrecht et al., 2001)</td>
</tr>
<tr>
<td>pFUS2</td>
<td>Suicide vector, oriV&lt;sup&gt;ColE1&lt;/sup&gt;, oriT, Gm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Antoine et al., 2000)</td>
</tr>
<tr>
<td>pIJ3200</td>
<td>pLAFR1 derivative containing the polylinker of Bluescript M13, with λ, cos sites, oriV, oriT, IncP1, Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Liu et al., 1990)</td>
</tr>
<tr>
<td>pIJPAR</td>
<td>pIJ3200 derivative containing the parDE genes, Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Miller et al., 2007)</td>
</tr>
<tr>
<td>pJQ200SK</td>
<td>pACYC derivative, oriV&lt;sup&gt;p15A&lt;/sup&gt;, Gm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Quandt &amp; Hynes, 1993)</td>
</tr>
<tr>
<td>pMH1701</td>
<td>Source of B. subtilis sacB gene, Tc&lt;sup&gt;R&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Hynes et al., 1989)</td>
</tr>
<tr>
<td>pPH1J1</td>
<td>IncP plasmid, Gm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Hirsch, 1979)</td>
</tr>
<tr>
<td>pPR3</td>
<td>pPROBE-KT containing the nptII promoter amplified from pFAJ1708, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Rodpothong et al., 2009)</td>
</tr>
<tr>
<td>pPROBE-GT</td>
<td>pVS1/ p15A replicon containing promoterless gfp, Gm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Miller et al., 2000)</td>
</tr>
<tr>
<td>pPROBE-KT</td>
<td>pVS1/ p15A replicon containing promoterless gfp, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Miller et al., 2000)</td>
</tr>
<tr>
<td>pPS854</td>
<td>Cloning vector containing FRT cassette, Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Hoang et al., 1998)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Helper plasmid, derivative of RK2, oriV&lt;sup&gt;ColE1&lt;/sup&gt;, Mob&lt;sup&gt;+&lt;/sup&gt;, Tra&lt;sup&gt;+&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Figurski &amp; Helinski, 1979)</td>
</tr>
<tr>
<td>pRS239</td>
<td>PUC19-Cm containing a SmaI nptII-encoding fragment from pUC4KIXX, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Studholme, 1995)</td>
</tr>
<tr>
<td>pSK105B</td>
<td>NZP2037 library cosmid encoding exoU, Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>Vector</td>
<td>Description</td>
<td>Reference</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>pSK139F</td>
<td>NZP2037 library cosmid encoding <strong>exoU</strong>, Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pSK2385</td>
<td>pLAFR1 R7A library cosmid containing <strong>mll2385</strong>, Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pSK39-1</td>
<td>R7A library cosmid that complements Calcofluor fluorescence of R7AΔexoU/2385, Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pSK39-2</td>
<td>R7A library cosmid that complements Calcofluor fluorescence of R7AΔexoU/2385, Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pSK39-3</td>
<td>R7A library cosmid that complements Calcofluor fluorescence of R7AΔexoU/2385, Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pSK57C</td>
<td>NZP2037 library cosmid encoding <strong>exoU</strong>, Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pSK84-1</td>
<td>R7A library cosmid that complements Calcofluor fluorescence of R7AΔexoU/2385, Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pSK84-3</td>
<td>R7A library cosmid that complements Calcofluor fluorescence of R7AΔexoU/2385, Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pSK84-5</td>
<td>R7A library cosmid that complements Calcofluor fluorescence of R7AΔexoU/2385, Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pSKASG</td>
<td>pFAJ1700 containing an <strong>exoL</strong>-<em>exoA</em> complementation product, Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pSKB1</td>
<td>NZP2037 library cosmid encoding <strong>exoB</strong>, Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pSKB4</td>
<td>NZP2037 library cosmid encoding <strong>exoB</strong>, Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pSKB6</td>
<td>NZP2037 library cosmid encoding <strong>nodU</strong>, Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pSKCELA</td>
<td>pJQ200SK::<strong>celA</strong> in-frame markerless deletion mutagenesis construct, Gm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pSKDSRED</td>
<td>pFAJ1708 containing <strong>dsred.14</strong>, Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pSKEXSH</td>
<td>pJQ200SK::<strong>exsH</strong> in-frame markerless deletion mutagenesis construct, Gm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pSKF5</td>
<td>NZP2037 library cosmid encoding <strong>nodU</strong>, Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pSKFRT</td>
<td>pPS854 carrying <strong>nptII</strong> flanked by terminator sequence and FRT sites, Km&lt;sup&gt;r&lt;/sup&gt;, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pSKFRT2</td>
<td>pIJ3200 carrying a FRT-<em>nptII</em> cassette (<strong>nptII</strong>)</td>
<td>This study</td>
</tr>
</tbody>
</table>
flanked by terminator sequence and FRT sites), Km\(^r\), Tc\(^r\)

- **pSKGFP**: pFAJ1700 containing *gfp* expressed from the *nptII* promoter, Tc\(^r\) - This study
- **pSKJJSAC**: pJ3200 containing a BamH1 *sacB* encoding fragment from pMH1701, SacB, Tc\(^r\) - This study
- **pSKKFRT**: pJQ200SK carrying an *exoK-FRT-nptII* mutagenesis cassette, Gm\(^r\), Km\(^r\) - This study
- **pSKKFRT2**: pJ3200 carrying an *exoK-FRT-nptII* mutagenesis cassette, Tc\(^r\), Km\(^r\) - This study
- **pSKKSG1**: pFAJ1700 containing an *exoK* single-gene complementation product with 154-bp promoter region, Tc\(^r\) - This study
- **pSKKSG2**: pFAJ1700 containing an *exoK* single-gene complementation product with 387-bp promoter region, Tc\(^r\) - This study
- **pSKM2B**: pJQ200SK::NZP2037*exoB* in-frame markerless deletion mutagenesis construct, Gm\(^r\) - This study
- **pSKM2U**: pJQ200SK::NZP2037*exoU* in-frame markerless deletion mutagenesis construct, Gm\(^r\) - This study
- **pSKMU**: pJQ200SK::*exoU* in-frame markerless deletion mutagenesis construct, Gm\(^r\) - This study
- **pSKNU-GT**: pPROBE-GT containing an NZP2037 *nodU* encoding genomic fragment, Gm\(^r\) - This study
- **pSKNU-KT**: pPROBE-KT containing an NZP2037 *nodU* encoding genomic fragment, Km\(^r\) - This study
- **pSKPAGFP**: pFAJ1700 containing *gfp* expressed from the *nodA* promoter, Tc\(^r\) - This study
- **pSKPBGFP**: pFAJ1700 containing *gfp* expressed from the *nodB* promoter, Tc\(^r\) - This study
- **pSKPF2244**: pFUS2::mlr2244 IDM mutagenesis construct, Gm\(^r\) - This study
- **pSKPF2385**: pFUS2::mlr2385 IDM mutagenesis construct, Gm\(^r\) - This study
<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Abbreviation</th>
<th>Concentration (µg/mL)</th>
<th>E. coli</th>
<th>M. loti</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>Ap</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Cm</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Gm</td>
<td>25</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>Km</td>
<td>50</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Neomycin</td>
<td>Nm</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>Rf</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Tc</td>
<td>15</td>
<td>2</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.3: Antibiotics used in this study
2.5 Spectroscopy
Optical densities of cultures were determined using a Jenway 6300 spectrophotometer (Jenway Instruments). Absorbance readings and concentrations of DNA were measured using a Nanodrop ND-100 Spectrophotometer (Nanodrop Technologies, USA).

2.6 DNA isolation and manipulation
2.6.1 Plasmid DNA extraction
2.6.1.1 Alkaline lysis method
Plasmid DNA was extracted using a method based on (Feliciello & Chinali, 1993). Overnight cultures (3 mL) grown in LB broths at 37°C were harvested by centrifugation. Pelleted cells were resuspended in 250 µL Buffer P1 (QIAGEN) before being lysed by the addition of 250 µL lysing solution (0.2 M NaOH, 1% [w/ v] SDS). Neutralising solution (350 µL of 3 M KAc, 5% [w/ v] formic acid), was then added and mixed by inversion. The bacterial lysate was then centrifuged at 14,926 g for 5 min and 800 µL of the supernatant transferred to a new tube where the DNA was precipitated by the addition of 560 µL of isopropanol. The samples were centrifuged again at 14,926 g for 10 min after which the supernatant was discarded and the DNA pellets were washed in 1 mL of 70% ethanol. DNA pellets were air-dried and resuspended in 50 µL of filter-sterile Milli-Q water.

2.6.1.2 Commercial plasmid isolation kits
High-quality plasmid DNA was isolated using commercial kits. High-copy-number plasmids were isolated using the QIAprep Spin Miniprep kit (QIAGEN cat# 27106) or the Qiagen Midi kit (QIAGEN cat# 12143). For low-copy-number plasmids the Qiagen Maxi kit (QIAGEN cat# 12145) was used. Plasmid extractions were performed according to the provided manufacturer protocols.

2.6.2 Genomic DNA isolation
2.6.2.1 Ultra-quick genomic DNA preparations
Genomic DNA of Mesorhizobium sp. strains was isolated by modification of the ultra-quick genomic DNA preparation method described by (Gonzalez-y-Merchand et al., 1996). TY cultures (5 mL) were grown for 48 h at 28°C with shaking. Cultures (3 mL) was harvested by centrifugation with the bacterial pellets then resuspended in 500 µL lysis buffer (4 M guanidinium thiocyanate, 1 mM 2-mercaptoethanol, 10 mM
EDTA, 0.1% [w/ v] Tween-80). The lysate was then snap-frozen in a dry ice/ ethanol bath before incubation at 65°C for 10 min. The snap-freezing/ heating process was repeated twice followed by chilling the tubes on ice for 5 min. Lysate was then extracted once in chloroform, once in phenol/ chloroform, then once more in chloroform. Genomic DNA was then precipitated with 100% ethanol, washed in 70% ethanol then resuspended in 50 µL of filter-sterile Milli-Q water.

2.6.2.2 PrepMan™ Ultra genomic DNA preparations
Crude genomic DNA for use in PCR was extracted from bacterial pellets harvested from 1-ml broth cultures, using 200 µL of PrepMan™ Ultra reagent (Applied Biosystems). Pellets resuspended in the reagent were boiled for 10 min, ice-cooled for 2 min then centrifuged at 14,926 g for 3 min. One µL of supernatant was used in PCR reactions.

2.6.3 Colony-crack DNA isolation
Clones stored in microtitre plates were patched onto LB plates containing appropriate antibiotics using a 48-well stamper and grown overnight at 37°C. Sterile toothpicks were used to transfer fresh colony growth into microtitre plate wells containing 25 µL of cracking solution (50 mM NaOH, 0.5% SDS, 5 mM EDTA, 10% glycerol, 0.01% bromocresol green). Samples were heated to 65°C for 30 min. Following cracking, samples were loaded into the wells of 1% agarose gels and electrophoresed at 60 V for 30 min with only the bottom half of the gel submerged in 1x TAE buffer. Gels were then fully submerged in buffer and electrophoresed at 90 V for 1 h. The DNA was then transferred from the gels to membranes and used in Southern hybridisations as described in Section 2.6.6.

2.6.4 Restriction enzyme digestion
DNA was digested with restriction enzymes and the appropriate buffer for at least 1 h at 37°C.

2.6.5 Agarose gel electrophoresis
DNA samples were mixed with one-tenth volume bromophenol blue tracking dye (1 mL Milli-Q water, 1 mL glycerol, 1 mL bromophenol blue [10 mg/mL]) prior to loading onto gels made up of 1% agarose dissolved in 1x Tris-acetate (TAE) buffer (40 mM Tris [pH 8], 20 mM acetic acid, 1 mM EDTA [pH 8]) containing 1 µg/mL
ethidium bromide. Gels were electrophoresed at 90 V for approximately 1 h, except for gels used for Southern blotting which were electrophoresed at 22 V overnight. DNA was visualised and captured on an El Logic 200 Gel Documentation system (Kodak Ltd.)

2.6.6 Southern hybridisation

2.6.6.1 Downward capillary blotting

Transfer of genomic DNA after electrophoresis from 1% agarose gels to Hybond-N’ membranes (Amersham BioScience) was achieved by immersing the gels for 15 min in 0.2 M HCl, rinsing twice in distilled water prior to submersing in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 15 min, followed by a final 15 min soak in neutralisation solution (1.5 M NaCl, 0.5 M Tris [pH 8]). The downward capillary system involved the construction of a stack of the following: a 7 cm high stack of paper towels formed the base of the stack, on top of this 4 dry and 1 wet (soaked in 20x SSC) pieces of Whatman filter paper were placed. The membrane (pre-soaked for 5 min in distilled water, followed by 5 min in 20x SSC) was then placed on the stack and the treated agarose gel that had the wells cut off was placed on top of the membrane, ensuring no air bubbles formed. Finally 3 more pieces of pre-soaked Whatman paper, 2 of which were cut with an extension that was placed in a reservoir of 20x SSC, were placed on the top of the stack. The stack was left for ~3 h for transfer of the DNA to occur. The DNA was then fixed to the membrane by soaking the membrane in 0.4 M NaOH for 20 min. Finally the membrane was rinsed in 2x SSC and air-dried.

2.6.6.2 Hybridisation of probes to membrane-bound DNA

Membrane prehybridisation was carried out in a Hybaid bottle containing 16.5 mL (0.125 mL/cm² of membrane) of AlkPhos Direct™ hybridisation buffer (GE Healthcare) that was incubated at 55°C in a rotating Hybaid hybridisation oven (Sci. Tech. NZ Ltd) for 15-30 min.

Appropriate probe DNA (purified PCR product or plasmid DNA) was diluted to 10 ng/µL in water supplied with the AlkPhos Direct™ labelling system (GE Healthcare cat# RPN3680). To 10 µL of diluted probe DNA, 1 µL of a 1/50 dilution of HindIII-digested λ phage DNA was added in a 0.5 mL microfuge tube and boiled for 5 min. After cooling on ice for 5 min the following reagents from the AlkPhos Direct™
labelling system kit were then added to the tube: 10 µL of reaction buffer, 2 µL labelling reagent and finally 10 µL of working-concentration crosslinker (1/5 dilution made up in kit-supplied water). Gentle mixing was performed following the addition of each reagent after which the tube was spun briefly, before being placed at 37°C for 30 min.

After the prehybridisation period, the prepared probe was added to the Hybaid bottle containing the membrane with the bottle then returned to the hybridisation oven for overnight incubation at 55°C.

2.6.6.3 Membrane washing
Following hybridisation, membranes were washed for 10 min at 55°C in primary wash buffer (2 M urea, 0.1% [w/v] SDS, 50 mM sodium phosphate pH 7.0, 150 mM NaCl, 1 mM MgCl₂, and 0.2% [w/v] blocking reagent). Membranes were then washed twice for 5 min at room temperature in secondary wash buffer (200 mM Tris-base, 400 mM NaCl, and 2 mM MgCl₂).

2.6.6.4 Signal generation/detection
Secondary wash buffer was drained from the membrane and the membrane placed DNA-side up onto Gladwrap. CDP-Star detection reagent (GE Healthcare cat# RPN3682) (30-40 µL/cm² of membrane) was pipetted onto the membrane and left for 5 min. Excess detection reagent was drained off and the membrane was sealed in plastic and exposed to X-Ray film (Cronex, Dupont) for 2-24 h in the presence of Lightening-plus intensifying screens.

2.6.6.5 Membrane stripping
Probes were stripped from membranes using 0.5% [w/v] SDS. The 0.5% SDS solution was boiled then poured over the membranes and left shaking for 30 min. The process was repeated before stripped membranes were rinsed in 2x SSC, air-dried and stored at room temperature.

2.6.7 Polymerase chain reaction (PCR)
PCR was used for multiple purposes including amplification of DNA for mutant construction, amplification of DNA for strain confirmation and amplification of DNA for sequencing. The sequences of PCR primers used in this study are listed in Table
2.4. Primers were ordered from Invitrogen (Life Technologies). All PCR cycling was performed using a Hybaid PCR express thermal cycler (Hybaid Ltd).

2.6.7.1 Standard PCR protocol
PCR reactions used the Phusion High-Fidelity PCR system (Finnzymes). Standard 100-µL PCR reactions typically contained 20 µL 5x Phusion HF buffer, 3 µL DMSO, 2 µL of supplied dNTPs (10 mM), 1 µL of each primer (final concentration 0.5 µM), 1 µL of template, filter-sterile Milli-Q water to make the volume 100 µL, and finally 0.5 µL of Phusion DNA polymerase enzyme.

PCR products were purified using the High Pure PCR Purification kit (Roche cat# 1732688) or extracted from agarose gel and purified using the QIAEX-II gel extraction kit (QIAGEN cat# 20021). Both purification methods were performed according to the manufacturer’s instructions.

2.6.7.2 Phusion PCR programme:
1 cycle: 98°C (1 min)

25-30 cycles: 98°C (10 s)  57°C* (10-30 s)  72°C (15-30 s/ 1 kb product)

1 cycle: 98°C (10 s)  57°C* (10-30 s)  72°C (5 min)

*Temperature varied depending on primers.
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5'to 3'</th>
<th>Used for</th>
</tr>
</thead>
<tbody>
<tr>
<td>1708DsRedL</td>
<td>ATATATGGATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>Reporter</td>
</tr>
<tr>
<td>1708DsRedR</td>
<td>AAATTTGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>Reporter</td>
</tr>
<tr>
<td>2385pFUS2L</td>
<td>ATATATAGCTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>IDM</td>
</tr>
<tr>
<td>2385pFUS2R</td>
<td>ATATATAGCTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>IDM</td>
</tr>
<tr>
<td>AgfpL</td>
<td>AAATTTGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>Fusion</td>
</tr>
<tr>
<td>AgfpLR</td>
<td>GGTTTTGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>Fusion</td>
</tr>
<tr>
<td>BgfpL</td>
<td>AAATTTGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>Fusion</td>
</tr>
<tr>
<td>BgfpLR</td>
<td>AAATTTGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>Fusion</td>
</tr>
<tr>
<td>celAcheckL</td>
<td>ATATATAGCTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>MD</td>
</tr>
<tr>
<td>celAcheckR</td>
<td>ATATATAGCTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>MD</td>
</tr>
<tr>
<td>celALL</td>
<td>AAATTTGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>MD</td>
</tr>
<tr>
<td>celALR</td>
<td>AAATTTGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>MD</td>
</tr>
<tr>
<td>celARR</td>
<td>AAATTTGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>MD</td>
</tr>
<tr>
<td>exoBcheckL</td>
<td>GGGGCTTGGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>MD</td>
</tr>
<tr>
<td>exoBcheckR</td>
<td>GGGGCTTGGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>MD</td>
</tr>
<tr>
<td>exoBLL</td>
<td>GGGGCTTGGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>MD</td>
</tr>
<tr>
<td>exoBLR</td>
<td>GGGGCTTGGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>MD</td>
</tr>
<tr>
<td>exoBRL</td>
<td>GGGGCTTGGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>MD</td>
</tr>
<tr>
<td>exoBRR</td>
<td>GGGGCTTGGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>MD</td>
</tr>
<tr>
<td>exoKFRTL</td>
<td>GGGGCTTGGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>FRTMD</td>
</tr>
<tr>
<td>exoKFRTLL</td>
<td>GGGGCTTGGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>FRTMD</td>
</tr>
<tr>
<td>exoKFRTLR</td>
<td>GGGGCTTGGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>FRTMD</td>
</tr>
<tr>
<td>exoKFTRTR</td>
<td>GGGGCTTGGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>FRTMD</td>
</tr>
<tr>
<td>exoKSGL1</td>
<td>GGGGCTTGGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>SGC</td>
</tr>
<tr>
<td>exoKSGR</td>
<td>GGGGCTTGGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>SGC</td>
</tr>
<tr>
<td>exoLASGL</td>
<td>GGGGCTTGGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>SGC, Fusion</td>
</tr>
<tr>
<td>exoLASGR</td>
<td>GGGGCTTGGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>SGC</td>
</tr>
<tr>
<td>exoUcheckL</td>
<td>GGGGCTTGGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>MD</td>
</tr>
<tr>
<td>exoUcheckR</td>
<td>GGGGCTTGGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>MD</td>
</tr>
<tr>
<td>exoULL</td>
<td>GGGGCTTGGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>MD</td>
</tr>
<tr>
<td>exoULR</td>
<td>GGGGCTTGGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>MD</td>
</tr>
<tr>
<td>exoURL</td>
<td>GGGGCTTGGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>MD</td>
</tr>
<tr>
<td>exoURR</td>
<td>GGGGCTTGGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>MD</td>
</tr>
<tr>
<td>exoUSGL</td>
<td>GGGGCTTGGGAATTTTTCTGACTAGCTAAGGAGAGAAAGATGGCCTCTCTGGAGATCG</td>
<td>SGC, Fusion</td>
</tr>
</tbody>
</table>
exoUSGR  ATATATAAGCTTTTGGATGAACGGAG  SGC
exsHcheckL TCAAGCCTTTAGGGTCCT  MD
exsHcheckR GGTCCAGTCATTTCCACATT  MD
exsHLL  AAATTTGGATCTTTTATGCTACATCTG  MD
exsHLR  ATGTAGTGATCTTCATCTGAGTTAGATGCTAC  MD
exsHRL  GGGATGACATCTATTACCTCGAGATGAGATCGACTACAT  MD
exsHRR  ATATATGGATCCAGATCATGCCTGCCGTCAT  MD
KconfirmL TCAAGAGCTTCGATAAGTCG  P
KconfirmR TTGACGTACCAGCGCAAACT  P
KFRTlocatL GCTTTTGCAAGTCAATCCAA  P
KFRTlocatR CGATCGAATGATGGT  P
lacZ  GCTATTACGCCAGCTGGCGA  S
LgfpLR  AGTTCTTCTCCTTTACTCATGAAAAACTCGACCGCTGTGAGG  Fusion
LgfpRL  CAACACCGGTGCAAGTTTTTCATGTAAGGAGAAGAACT  Fusion
M13 Forward  CCCAGTCACGACGTTGTAAAACG  S
M13 Reverse  AGCGGATAACAATTTACACAGG  S
nodUL  TTTAGCTACAGAAGCTATCC  P
nodUR  CAAGTGAGATTCCGAGTTG  P
nptIII  AAATTTCTAGACAGGCGTCGCCCAAGCCTCA  FRT
nptIII1  AAATTTCTAGACAGTCCGCCCAAGCCTCA  Reporter
nptIIIR  AAATTTCTAGACAGTCCGCCCAAGCCTCA  FRT
nptIIROL  GTGACCTACGTGCTACATCG  P
nptIIROL  TTCTATGCCTTTCTTGTACGA  P
NWnodUL  TCGCATATCGACCAGTTC  P, S
NWnodUR  GGGCTATCTACATTTGGATATT  P, S
pLAFRF  GGCATTCAGCGACTCATGG  S
pLAFRR  CGAGCTGGCAAAGTACTAA  S
PnptIlgfpR  ATATATAAGCTTGAATGCTGCCCAATTATTTCA  Fusion
PnptIlgfpR1  ATATATAAGCTTGAATGCTGCCCAATTATTTCA  Reporter
T3  ATTACCCCTACATTTAAGGA  S, P
T7  TAATAGCGACTCACTATAGG  S, P
TAC-105F  GCCATCTAGGCCGCCCTATG  S
UgfpLR  AGCTTCTCTCCTTTACTCATGTGCTGACAGG  Fusion
UgfpRL  CCTGTGCAAGAGCTAACCCATGAGTAAAGGAGAAGAACT  Fusion
UpFUS2L  AAATTTAAGCTTGTGCAAGCAGCGCCCTAC  IDM
UpFUS2R  TATATAGGAATCCCTCAGAAGACGACCGTCA  IDM

* MD, used in construction of in-frame markerless deletion mutants. FRT, used in FRT construct assembly. FRTMD, used in the construction of an in-frame markerless deletion mutant utilising the FRT/Flp recombination system. P, used in PCR to amplify products to be sequenced to confirm recombination events. S, used for sequencing. SGC, used for single-gene complementation constructs. Fusion, used for the construction of promoter region-\textit{gfp} fusion constructs. Reporter, used in the assembly of reporter gene constructs. IDM, used in the construction of insertion duplication mutants.
2.6.8 **DNA sequencing**

Plasmid clones and PCR products were sequenced using the primers described in Table 2.4. Relevant primers were mixed with template DNA and filter-sterile Milli-Q water, and the mixture sent to the Allan Wilson Centre Genome Service (Massey University, Palmerston North, NZ) for sequencing.

2.6.9 **Computer analysis**

DNA sequence was viewed and edited using 4peaks software (Mekentosj.com). Further manipulation of DNA sequences was performed using the Lasergene suite of software (DNASTar). National Center for Biotechnology Information (NCBI) databases were searched for similar nucleotide or amino-acid sequences using Blast N, X and P (Altschul et al., 1997). Comparisons of sequence data to rhizobia were carried out using the Blast functions of the Rhizobase website (http://genome.kazusa.or.jp/ rhizobase).

2.6.10 **Cloning of DNA fragments**

2.6.10.1 **Preparation of insert and plasmid DNA**

Plasmid and insert DNA was digested with the desired restriction enzyme(s). Following digestion, plasmids were dephosphorylated by the addition of 2 µL of alkaline phosphatase (Roche cat# 713 023) and incubated at 37°C for 30 min. Plasmid DNA was then gel-extracted and purified using the QIAEX-II gel extraction kit (QIAGEN cat# 20021) according to the manufacturer’s instructions. Digested inserts were purified either using the High Pure PCR Purification kit (Roche cat# 1732688) or using the QIAEX-II gel extraction kit (QIAGEN cat# 20021).

2.6.10.2 **End-filling of restriction sites with Klenow**

Digested DNA with 5’ overhangs was end-filled with Klenow to form blunt ends. To ethanol-precipitated DNA that was resuspended in 35 µL of sterile Milli-Q water, 8 µL of dNTP mix (Roche cat# 11581295001), 5 µL of buffer A (Roche cat# 1417959) and 2 µL of Klenow (Roche cat# 1008404) were added and incubated at 37°C for 30 min. End-filled products were then purified using the High Pure PCR Purification kit (Roche cat# 1732688).
2.6.10.3 Ligations
Appropriate volumes of prepared plasmid and insert DNA were mixed with 2.5 µL of 10x ligation buffer and 1.5 µL of T4 DNA ligase (Roche cat# 481220) in a total volume of 20 µL, and incubated overnight at 12°C. Ligated DNA was ethanol-precipitated (50 µL 100% ethanol, 2 µL of 3 M NaAc and 1 µL of Pellet paint® co-precipitant [Novagen]), washed with 70% ethanol and then resuspended in 5 µL of filter-sterile Milli-Q water.

2.6.11 Preparation of electrocompetent cells

2.6.11.1 Escherichia coli
Electrocompetent cell stocks of *E. coli* EPI300 and S17-1/λpir were prepared using a protocol adapted from (Sheng *et al.*, 1995). Strains were grown to stationary phase in 5-ml LB broths and 0.5 mL of this culture was used to inoculate 500-ml broths that were incubated at 37°C with shaking at 200 rpm. Cultures were harvested at OD<sub>600</sub> 0.6-0.8 by centrifugation at 4°C for 10 min at 5930 g. Following two washes in 500 mL chilled 10% [w/v] glycerol, cells were washed in 30 mL of chilled 10% glycerol, pelleted, then resuspended in 1 mL of 10% glycerol. Aliquots (40 µL) were snap-frozen in a dry ice/ethanol bath and stored at -80°C.

2.6.11.2 Mesorhizobium loti
Electrocompetent *M. loti* cells were prepared by a method developed by Kazuhiko Saeki (personal communication). Five-mL TY broth cultures of strains were grown to stationary phase with 1 mL then used to inoculate 200-mL TY broths which were incubated at 28°C with shaking. Cells were harvested at OD<sub>600</sub> 0.1-0.3 by centrifugation at 4°C for 10 min at 5930 g. Pelleted cells were washed in 200 mL chilled 10% glycerol, centrifuged, washed in 100 mL 10% glycerol, centrifuged, washed in 4 mL 10% glycerol, centrifuged, then resuspended in 200 µL 10% glycerol. Aliquots (40 µL) were snap-frozen in a dry ice/ethanol bath and stored at -80°C.

2.6.12 Electrotransformation
Electrocompetent cells (40 µL) were thawed on ice. DNA (1-2 µL) was added to the thawed cells which were then transferred to a pre-chilled 1 mm gapped electroporation cuvette (Biorad). Cells were then transformed at 1800 V using an *E. coli* TransPorator electroporation system (BTX). Immediately following shocking, *E. coli* cells were suspended in 1 mL of LB broth and incubated at 37°C with shaking for
45 min. *Mesorhizobium* cells were suspended in 1 mL of TY broth and incubated at 28°C with shaking for 4 h. Following incubation, aliquots were spread onto media containing appropriate antibiotics.

### 2.6.13 Preparation of an *M. loti* NZP2037 cosmid library

An NZP2037 cosmid library was constructed using a protocol based on previously described methods (Chua *et al.*, 1985; Downie *et al.*, 1983; Hombrecher *et al.*, 1984).

#### 2.6.13.1 Preparation of NZP2037 insert DNA

Initially, small-scale trial Sau3A restriction digests of NZP2037 genomic DNA were performed to establish the appropriate amount of enzyme and digest time required to generate a partial digest in which the majority of the fragments were ~25 kb in size. A large-scale digest of NZP2037 genomic DNA was then performed using the identified conditions (1/50 dilution of Sau3A for 2 min at 37°C). Partially-digested DNA was then loaded into the wells of a 1% Seaplaque agarose gel and electrophoresed at 22 V overnight. A gel band representing DNA of ~25 kb was excised and purified using the QIAEX-II gel extraction kit (QIAGEN cat# 20021) according to the manufacturer’s instructions. The extracted NZ2037 genomic DNA was then resuspended in filter-sterile Milli-Q water.

#### 2.6.13.2 Preparation of plasmid DNA

The plasmid pIJPAR was prepared as described in Section 2.6.10.1. Following digestion with BamHI the plasmid was purified using the QIAEX-II gel extraction kit (QIAGEN cat# 20021) according to the manufacturer’s instructions.

#### 2.6.13.3 Ligation

A ligation mix containing a 2:1 ratio of insert to plasmid DNA in a final volume of 20 µL was incubated at 12°C overnight. Prior to addition of the ligation mix to the packaging extract, the T4 DNA ligase was heat-killed by incubating at 65°C for 15 min.

#### 2.6.13.4 *In vitro* packaging of NZP2037 cosmid DNA into λ phage coat particles and transduction into *E. coli*

An overnight seeder broth culture of *E. coli* HB101 was used to inoculate a 50-mL LB broth containing 0.2% maltose and 10 mM MgSO₄. The broth was grown at 37°C until the culture reached an OD₆₀₀ of 0.7 at which point cells were harvested from 10 mL of
the culture by centrifugation (10 min at 5930 g, 4°C). The cell pellet was resuspended in 5 mL of LB broth containing 0.2% maltose and 10 mM MgSO₄ and stored on ice until required.

Packagene® extract (Packagene® Lambda DNA Packaging System cat# K3154, Promega Corp, WI, USA) was thawed on ice and 10 µL of the ligation mix (Section 2.6.13.3) was added to it. Following incubation of the mix for 3 h at 22°C, 445 µL of phage buffer (20 mM Tris-HCl [pH7.4], 100 mM NaCl, 10 mM MgSO₄) and 25 µL of chloroform were added.

After allowing the chloroform to settle to the bottom of the tube, 500 µL of the packaging mix was added to 1 mL of the concentrated HB101 cells. The mixture was incubated at 37°C without shaking to allow for phage absorption. The mixture was then added to 3 mL of LB broth and incubated with shaking at 37°C for 1 h after which 50-µL aliquots were plated onto LB containing Tc agar plates that were then incubated at 37°C overnight.

2.6.13.5 NZP2037 cosmid library storage

The NZP2037 cosmid library was stored as individual clones as well as pools of colonies. Individual colonies (total of 1700) were transferred into microtitre plate wells containing 200 µL of LB storage media (36 mM K₂HPO₄, 13.2 mM KH₂PO₄, 1.7 mM sodium citrate, 0.4 M MgSO₄ and 6.8 mM (NH₄)₂SO₄) containing Tc. Following overnight growth at 37°C, the library microtitre plates were stored at -80°C. A total of 3000 NZP2037 library clones were stored as pools with each pool representing ~175 clones. Colonies were resuspended from plates in 1 mL of storage media and used to inoculate 5-mL storage media broths containing Tc that were grown overnight at 37°C. Aliquots (800 µL) of the overnight pool broths were dispensed into Nalgene cryogenic vials (Nalgene, Rochester, NY, USA) and stored at -80°C.

2.6.14 Transfer of plasmids by conjugation

2.6.14.1 Biparental and triparental spot-matings

Plasmids containing an RK6 origin of replication were transferred from E. coli strain S17-1/λpir into M. loti R7A through biparental spot-matings. Triparental spot-matings incorporated the helper plasmid pRK2013 to aid in the transfer of non-self-transmissible plasmids from E. coli strains HB101 and EPI3000. For spot-matings E.
coli and *M. loti* strains were grown to stationary phase in TY broth. Aliquots of each culture (30 µL) were dispensed together as a spot onto a TY agar plate and incubated at 28°C overnight. The resultant bacterial growth was then streaked onto selective media containing appropriate antibiotics.

### 2.6.14.2 Filter matings

Stationary-phase TY broth cultures were dispensed (1 mL each culture) onto the surface of a 0.45 µm filter (Type HA, 47 mm, Millipore Corporation, USA) and filtered using a vacuum filter apparatus (Sterifil Aseptic System, Millipore). Filters were transferred to a TY agar plate and incubated at 28°C overnight. Filter growth was resuspended in sterile water using a spreader and dilutions of the suspension were plated onto media containing appropriate antibiotics.

### 2.7 Mutagenesis

#### 2.7.1 Insertion duplication mutagenesis (IDM)

IDM mutants were constructed using the suicide plasmid pFUS2 (Antoine *et al.*, 2000). Target gene sequence (~350 bp) was PCR-amplified with restriction sites incorporated, generally BamHI and HindIII. Purified PCR products were ligated into appropriately-digested pFUS2 and the mutant constructs were then electroporated into *E. coli* S17-1/λpir cells. Cloned fragments were confirmed by sequencing using a lacZ primer (Table 2.4). Spot-matings were carried out between confirmed S17-1/λpir clones and *M. loti* strains. Single-colony purifications of matings to isolate the desired mutant strains were performed by passaging on G/RDM containing Gm. Southern hybridisations were performed on DNA extracted from putative mutant clones to confirm that a single copy of the plasmid had integrated into the genome at the desired location.

#### 2.7.2 In-frame markerless deletion mutagenesis

In-frame markerless deletion mutants were constructed through allelic replacement of the wild-type gene with a mutated form containing an in-frame markerless internal deletion through homologous recombination, as depicted in Figure 2.1.

#### 2.7.2.1 Overlap extension PCR

An overlap extension PCR approach was employed to create in-frame markerless deletion mutant fragments. Primer sets xLL/ xLR and xRL/ xRR were used to PCR-
amplify left and right arms of ~1 kb which encoded respective flanking regions either side of the target gene along with ~100 bp of 5’ (left arm) or 3’ (right arm) gene sequence. Primer design incorporated restriction enzyme sites at the outermost ends of the left and right arm PCR products and 20 bp of overlapping sequence between the internal regions of the two arms.

The overlap extension PCR process required two separate PCR reactions. The first amplified the left and right arms separately using genomic DNA as the template. A second PCR reaction was then performed using the left and right arm PCR products as the template DNA and the outermost left-arm (xLL) and right-arm (xRR) primers. An ~2-kb product made up of the two arms joined by in-frame overlapping sequence was amplified.

The overlap extension PCR product was digested with the appropriate enzyme and cloned into similarly digested pJQ200SK (Quandt & Hynes, 1993). Electrocompetent E. coli S17-1/λpir cells transformed with the mutant construct were selected on LB containing Gm. Plasmid DNA extracted from Gm<sup>R</sup> clones were confirmed as containing the overlap extension PCR product by restriction digestion. Sequencing of the insert in a representative clone confirmed the presence of the desired mutant fragment of the correct sequence.

2.7.2.2 Isolation of in-frame markerless deletion mutants

Appropriate M. loti strains were spot-mated with a confirmed S17-1/λpir clone and transconjugants that had received and integrated the mutant construct through recombination between the left or right arms with the homologous genomic DNA, to form single-crossover (SXO) clones, were selected on G/ RDM containing Gm plates. Following single-colony purification, genomic DNA recovered from several SXO clones was used as template DNA in PCR with primers that amplify across the joining site of the left and right arms (xcheckL/ xcheckR). Two PCR products should be amplified if the first-crossover event was successful, a ~350 bp product from the integrated mutant construct and a larger product from the wild-type chromosomal copy of the gene.

To complete the markerless deletion process, a second crossover event is required to remove the integrated pJQ200SK vector and form double-crossover (DXO) clones.
The second-crossover event can occur either through reversal of the first-crossover, resulting in the clone reverting to wild-type, or through recombination between the mutant fragment and targeted genomic DNA sequence resulting in the in-frame markerless deletion. Selection for the second-crossover event was obtained by plating SXO clones onto sucrose/ RDM (S/ RDM) as pJQ200SK contains $sacB$, the product of which is lethal to the cell when expressed in the presence of sucrose. Following plating on S/ RDM, DXO clones were patch-plated to check for Gm$^S$ indicating the loss of pJQ200SK.

Genomic DNA from Gm$^S$ DXO clones was used as template for PCR with primers xcheckL/ xcheckR. If the second-crossover event resulted in the markerless deletion, only the ~350-bp product would be amplified. However, if the second-crossover resulted in the clone reverting to wild-type, only the larger product would be amplified.
Figure 2.1: Schematic of the in-frame markerless deletion mutagenesis strategy.
2.7.3 Transposon mutagenesis

R7AΔexoU was randomly mutated with the transposon mTn5-Nm (Reeve et al., 1999). *E. coli* containing pCRS487, which carries mTn5-Nm, was introduced into R7AΔexoU through filter-matings (Section 2.6.14.2). Following overnight incubation at 28°C on TY plates, filter growth was resuspended in 2 mL sterile water using a spreader. Serial 10-fold dilutions of the suspension were made down to 10⁻⁵ and 100 µL aliquots of the dilutions were plated onto G/ RDM containing Nm and Rf.

2.8 Cellulase assay

A cellulase assay was performed based on a previously described protocol (Kawano et al., 2011). Cells pelleted from 48 h G/ RDM broth cultures were resuspended in 1.5 mL sterile Milli-Q water. One mL of the suspensions was transferred to cuvettes and after resting for 10 min (to allow aggregates to settle), the OD₆₀₀ was measured. Cellulase purified from *Trichoderma reesei* ATCC26921 (Sigma cat# C8546) was added (12.5 U) to the suspensions and incubated with gentle shaking at 28°C for 1 h. Following 10 min to allow settling, the OD₆₀₀ of the samples post-treatment with cellulase was measured.

2.9 Hydrophobicity assay

Cell surface hydrophobicity was investigated based on a previously described method (Rosenberg et al., 1980). TY and G/ RDM broth cultures were grown for either 24 h (early-log phase) or 48 h (stationary phase) prior to harvesting cells from 3 mL of culture by centrifugation. Cell pellets were washed twice in PUM buffer pH7.1 (17.0 g K₂HPO₄, 7.26 g KH₂PO₄, 1.8 g urea, 0.2 g MgSO₄·7H₂O made up to 1 L in distilled water) and the OD₆₀₀ of the washed suspension then measured. In a glass test tube, 200 µL of the solvent hexadecane was added to 1.2 mL of washed cell suspensions. Following 10 min incubation at 30°C, test tubes were mixed for 2 min then left to settle for 15 min before the OD₆₀₀ of the aqueous fraction was again measured. If cells exhibit hydrophobic properties, the OD₆₀₀ value following the addition of hexadecane would be expected to drop as the cells would have migrated from the aqueous to solvent phase.

2.10 Plant studies

The symbiotic proficiency of various wild-type and mutant *Mesorhizobium* strains on a range of *Lotus* species was assessed through plant studies.
2.10.1 Seedling preparation

Seeds were surface-sterilised to prevent contamination influencing the results of plant studies. Surface sterilisation was achieved by first scarifying the seed coat through immersion in concentrated sulfuric acid for 6-10 min, followed by washing in five changes of sterile water. Seeds were then shaken in a bleach solution (10% [w/ v] commercial bleach and 0.03% [w/ v] Tween-80) for 20 min, followed by washing in five changes of sterile water. Surface-sterilised seeds were then transferred aseptically onto 0.8% agar and left to germinate inverted in the dark at 24°C for 24-48 h. Following germination, seeds were placed into sterile plant growth plates (10 cm x 10 cm) containing a slant of Jensen’s seedling agar (Vincent, 1970). For seedlings that were examined by microscopy, autoclaved lens tissue was placed on top of the agar slant so that the seedling root would grow on top of the tissue rather than within the agar. Seedlings were left to grow for 48 h prior to inoculation.

2.10.2 Split-root seedling preparation

Seeds were surface-sterilised and germinated as described in Section 2.10.1. Radicle tips were pinched with sterile tweezers to encourage lateral root development and transferred to plant growth plates. Seedlings that developed lateral roots of similar size were then transferred to new plant growth plates that had the centre section of the agar slant aseptically removed to separate the two growing lateral roots so that each could be individually inoculated.

2.10.3 Plant inoculation

For standard plant inoculations, growth from fresh G/ RDM plate cultures was resuspended in 5 mL sterile water. Each seedling root was inoculated with 50 µL of the appropriate suspension. Various controls including contamination checks of inoculum suspensions, inoculation of plants with wild-type strains and uninoculated plants were included in each plant experiment.

For co-inoculation plant assays, G/ RDM plate growth was again resuspended in 5 mL sterile water and the OD₆₀₀ was measured. Suspensions were adjusted to an OD₆₀₀ of 0.1. Viable cell counts were performed by plating dilutions of adjusted suspensions to ensure that equal numbers of cells were present. Seedlings were inoculated with 50 µL of each of the adjusted suspensions.
Plants were also inoculated with filter-mating transconjugants in complementation and R7AΔexoU transposon mutagenesis studies. Colony growth from mating or mutagenesis plates containing selective antibiotics was resuspended in sterile water. Cells were pelleted by centrifugation and washed twice in sterile water prior to plant inoculation with 50 µL each of the final resuspension.

2.10.4 Plant growth conditions

Plants were grown in a room with a controlled environment. Relative humidity was kept at 70% with the temperature maintained at 22-25°C during the day and 14°C at night on a 16 h day / 8 h night cycle. During experiments plant plates were re-arranged within the growth room to ensure their relative positioning to the overhead lamps had a negligible effect.

In normal plant growth conditions used, the roots of the plants were exposed to the overhead light. For experiments in which plant roots were kept in the dark, the plant plates were slotted into a blackened-out container that resulted in only the shoots being exposed to the light.

2.10.5 Plant nodulation observations

Various host plants inoculated with Mesorhizobium were observed periodically for nodule formation over 4-7 week nodulation assays. The symbiotic effectiveness of strains was determined through comparing the growth response of plants inoculated with the different strains to plants inoculated with wild-type positive controls and uninoculated negative control plants. Plants that formed effective (nitrogen-fixing) nodules grew well and appeared healthy with green foliage. In contrast, plants that formed no nodules or small white ineffective bumps exhibited stunted growth and yellowing foliage.

2.10.6 Isolation of rhizobia from nodules

A random selection of nodules was removed from plants at the conclusion of nodulation assays. Nodules were surface-sterilised by firstly shaking for 1 min in 70% ethanol, followed by shaking in a 1:1 mix of 95% ethanol and hydrogen peroxide (H₂O₂) for 1 min. The ethanol/ H₂O₂ mix was then removed and the nodules were washed in four changes of sterile water. Individual nodules were then crushed in 50 µL sterile water using sterile sticks. Resultant milky exudate (30 µL) was streaked
onto G/ RDM plates that were incubated at 28°C. Phenotypic characteristics and antibiotic sensitivities of colonies that grew were then investigated to determine if the nodule isolates represented the strain used to inoculate the plant.

2.10.7 Infection thread and nodule occupancy assays
Visualisation of fluorescently-labelled *M. loti* strains during the infection process on various host plants was achieved by examining plants using epifluorescent and confocal microscopy. Roots were removed from seedlings 7-21 dpi and placed in filter-sterile Milli-Q water on microscope slides. Roots were examined with an Olympus microscope (model BX51TRF) with fluorescence illuminator (model BX-RFA). GFP-expressing cells were visualised using a fluorescence mirror unit (model U-MWIB3) consisting of a 460-495 nm bandpass exciter, a 505 nm longpass dichroic mirror and a 510 nm longpass emitter. DsRed-expressing cells were visualised using fluorescence mirror unit (model U-MWIG3) consisting of a 530-550 nm bandpass exciter, a 570 nm longpass dichroic mirror and a 575 nm longpass emitter. Fluorescent images were captured with an Olympus digital camera (model DP70).

To determine nodule occupancy on plants co-inoculated with GFP and DsRed expressing strains, epifluorescent and confocal microscopy were used. Nodules were harvested 4-6 weeks post-inoculation and either viewed whole or as sections, formed by slicing nodules with a sterile razor blade and suspending in filter-sterile Milli-Q water on microscope slides. Epifluorescent microscopy details are given above. For confocal microscopy, nodules were viewed using a Zeiss LSM 510 upright confocal microscope, with excitation at 488 nm (GFP) and 568 nm (DsRed), and a BP505–530 emission filter for GFP and a LP585 filter for DsRed. Images were recorded with LSM image browser software.

2.10.8 Visualisation of *LjNin* expression in *LjNin-Gus* transgenic *L. japonicus* Gifu
*L. japonicus* Gifu seeds carrying an *LjNin-Gus* reporter gene were provided by Dr Niels Sandal, Aarhus University, Denmark. Seedlings were prepared as described in Section 2.10.1. Plant roots were removed from growth plates 10 dpi and incubated in staining buffer (50 mM sodium phosphate pH 7.0, 10 mM EDTA pH 8.0, 0.3 M mannitol pH 7.0 and 1 mM 5-Bromo-4-chloro-3-indoly1-β-D-glucuronic acid) at 37°C for 12 h.
2.11 Chemical analysis of *M. loti* EPS extracts

2.11.1 EPS extraction

G/ RDM broths (5 mL) of *M. loti* strains grown at 28°C for 48 h were used to seed 800-mL G/ RDM broths in 2-L baffled flasks that were grown at 28°C with shaking for 72 h. Cells were pelleted by centrifugation at 5930 g for 20 min at 4°C with the supernatant recovered and concentrated down to a volume of ~100 mL by rotary evaporation. Concentrated supernatant was again centrifuged at 5930 g for 20 min at 4°C to remove any remaining contaminants. Three volumes (300 mL) of absolute ethanol were added to the supernatant and left to stand at 4°C for 4 h. Precipitated EPS was then recovered by centrifugation at 5930 g for 20 min at 4°C and resuspended in 20-30 mL Milli-Q water. The suspension was transferred to dialysis tubing (6000 MWCO) that had been prepared by autoclaving in a 2% sodium bicarbonate solution and rinsed in Milli-Q water. The EPS extract in the dialysis tubing was then dialysed against several changes of Milli-Q water over a 24 h period. Extract was then transferred to Falcon tubes in 15-mL aliquots, snap-frozen in a dry ice/ ethanol bath and lyophilised for 48 h.

2.11.2 Gel permeation chromatography

Size-fractionation of EPS extracts was achieved by Sephacryl S-400 gel permeation chromatography. Gel beads were prepared by initially washing in a solution of de-gassed filter-sterile Milli-Q water containing 0.2% sodium azide followed by three washes with de-gassed filter-sterile Milli-Q water. The clean gel slurry was then carefully poured into a glass BioRad Econo-Column (2.5 x 120 cm) and left to settle for several hours using gravity-fed de-gassed filter-sterile Milli-Q water for 24 h prior to sample loading.

Crude EPS extract (10 mg) was suspended in 1 mL of de-gassed filter-sterile Milli-Q water and placed in an ultrasound bath for 10 min. For each run the column was charged with 500 μL of the EPS solution. Elution from the column was monitored with a refractive index detector (RID-10A, Shimadzu) and plotted on chart paper. Fractions were collected with a Spectra Chrom Fraction Collector CF-2 at 15 min intervals and the major fractions were separated based on the plotted elution profile. At the conclusion of each run, fractions representing HMW or LMW EPS were
pooled separately and after concentration by rotary evaporation were lyophilised to recover the EPS.

2.11.3 Phenol-Sulfuric acid colourimetric assay
EPS samples (25 µg/ mL) along with a standard of glucose sample were prepared in filter-sterile Milli-Q water. To 25 µL of the prepared samples, 1 mL of concentrated Sulfuric acid and 100 uL of 5% phenol were added. Samples were then heated to 80°C for 10 min and observed for colour changes.

2.11.4 Trimethylsilyl derivatisation of EPS samples
EPS suspensions (2 mg/ mL) and sugar standards (1 mg/ mL) were prepared in filter-sterile Milli-Q water. To 300 µg of EPS solution or 10 µg of the sugar standards in glass tubes, 10 µg of the internal standard inositol was added. Samples were lyophilised for ~4 h.

To identify the glycosyl residues present in the samples, trimethylsilyl methyl ester (TMS) derivatives were prepared as described by (York et al., 1986). Twenty drops of 1M HCL in MeOH were added to the glass tubes which were then placed in an 80°C heating block for 18 h. HCL-MeOH was evaporated from samples under a stream of dry air and 8 drops of anhydrous MeOH were added then evaporated. Samples were then re-N-acetylated through the addition of 15 drops of MeOH, 7 drops of pyridine and 7 drops of Ac₂O followed by incubation at 100°C for 1 h. Following evaporation, 11 drops of Pierce Tri-Sil® reagent were added to samples that were then incubated at 80°C for 30 min. After evaporation, TMS-derivatised samples were suspended in 20 drops of hexane and filtered through glass wool twice before final resuspension in 100 µL of hexane.

The TMS derivatives were analysed on a Hewlett-Packard HP5890 gas chromatograph equipped with a mass selective detector 5970 using an Alltech AT-1 fused silica capillary column (30 m x 0.25 mm I.D). Helium was used as the carrier gas. Initial oven temperature was 80°C for 2 min then ramped to 160°C at 20°C/ min with a 2-min hold. This was followed by a ramp to 200°C at 2°C/ min followed by an increase to 250°C at 10°C/ min with an 11-min hold. The results were processed and interpreted using The Standard Chemstation software (Hewlett-Packard; ver. A.03.00).
2.11.5 EPS glycosyl-linkage analysis

2.11.5.1 Partially-methylated alditol acetates (PMAA)

EPS samples (300 µg) were resuspended in 125 µL filter-sterile Milli-Q water. To the samples, 125 µL of 4 M Trifluoroacetic acid (TFA) were added followed by incubation at 121°C for 2 h. Ten microliters of inositol (1 mg/mL) were added to EPS samples prior to evaporation. Samples were resuspended in 1 mL filter-sterile Milli-Q water then evaporated down twice. A reducing solution (10 mg/mL NaBD₄ made up in 1M NH₄OH) was prepared and 200 µL was added to each sample after which they were incubated at room temperature overnight. Samples were neutralised by the addition of 3 drops of glacial acetic acid then washed three times by adding 10 drops of MeOH-HOAc (9:1) and evaporating followed by a further three washes by adding 10 drops of MeOH and evaporating. Samples were then dried by placing in a desiccator for at least 3 h. Twenty drops of Ac₂O were added to samples that were then incubated at 80°C for 15 min. After evaporation a further 20 drops of Ac₂O and 10 drops of pyridine were added and samples incubated at 80°C for 30 min. Samples were then extracted five times in dichloromethane (DCM) before passing through a glass wool filter containing sodium sulfate. Following evaporation, samples were resuspended in 40 µL of DCM.

The PMAA derivatives were analysed on a Hewlett-Packard HP5890 gas chromatograph equipped with a mass selective detector 5970 MSD using Supelco-SP™-2330 Capillary GC Column (30 m x 0.25 mm I.D). Helium was used as the carrier gas. For the gas chromatography mass spectrometry (GC-MS) analysis of the permethylated sugars, the initial oven temperature was 140°C for 2 min then ramped to 170°C at 2°C/ min with 4-min hold followed by a ramp to 240°C at 5°C/ min with 10-min hold. Results were processed and interpreted using The Standard Chemstation software.

2.11.5.2 Mild-methyl esterification and carboxyl reduction of uronic acids

EPS samples (500 µg) were suspended in 1 mL anhydrous MeOH, evaporated down and then resuspended in 1 mL of 0.5 M HCL-MeOH and incubated at 80°C for 30 min. After evaporation, three cycles of adding 1 mL of dry MeOH followed by evaporation were performed. Samples were reduced by resuspending in 200 µL of NaBD₄ solution (1 mg/mL made up in water) and incubating at room temperature
for 2 h. Samples were neutralised by the drop-wise addition of glacial acetic acid then washed three times by adding 10 drops of methanol/ acetic acid solution (9/ 1 [v/ v]) and evaporating followed by a further three washes by adding 10 drops of MeOH and evaporating. Samples were resuspended in 1.5 mL Milli-Q water, transferred to dialysis tubing (100-500 Da MWCO) and dialysed against several changes of distilled water over a 48 h period. Carboxyl-reduced samples were recovered from the dialysis tubing, lyophilised for 48 h and then resuspended in 200 µL DMSO by stirring overnight. A NaOH slurry base suspension was prepared by adding 150 µL NaOH to 200 µL DMSO. Following mixing, the slurry was centrifuged and the DMSO supernatant removed with the pellet then resuspended in fresh DMSO. This washing procedure was performed five times before final resuspension of the pellet in 0.5 mL DMSO to form the clear slurry base. To the EPS samples, 200 µL of the slurry base was added and tubes were incubated with stirring at room temperature for 2 h. Methyl Iodide (MeI, 500 µL) was added and samples were stirred for 30 min prior to the addition of a further 200 µL of MeI and stirring for another 1.5 h. DCM (1 mL) was added to samples and stirred for 15 min before the drop-wise addition of 1 M sodium thiosulfate until no yellow colouring was observed. Samples were then extracted with filter-sterile Milli-Q water five times before performing the PMAA procedure outlined in Section 2.11.5.1. Samples were analysed by GC-MS as described in Section 2.11.5.1.
3 Characterisation of R7A EPS mutants
3.1 Introduction
Although the requirement for EPS in the establishment of symbiosis has largely been thought to be restricted to indeterminate-nodule-forming interactions only (Section 1.4.1), characterisation of R7A EPS mutant strains revealed that EPS may play an important role during determinate nodulation between M. loti R7A and various Lotus hosts (Hubber, 2005; Kelly, 2007).

This chapter describes further characterisation of R7A EPS mutant strains. Differences in colony phenotypes between the strains may indicate differences in EPS production and correlate with observed symbiotic proficiencies. Isolation of additional in-frame markerless deletion EPS mutants and further complementation of mutant strains was performed in order to attribute phenotypic properties to specific EPS biosynthesis genes.

If EPS is required at a certain stage of the symbiotic infection process, an increase in expression of EPS biosynthesis genes at that stage may be evident. To investigate this, various exo and nod gene promoter region-GFP fusion reporter constructs were developed to allow for visualisation of gene expression during the symbiotic process.

3.2 Results
3.2.1 Characterisation of previously isolated R7A EPS mutant strains
R7A EPS mutant strains harbouring mutations in the EPS biosynthesis genes exoA, exoK, exoO, mlr5265 and mlr5266 had previously been isolated (Kelly, 2007). The strains designated R7AexoO, R7AexoK, R7Amlr5265 and R7Amlr5266 were constructed by an IDM approach and strain R7AΔexoA represents an in-frame markerless deletion mutant. Additional R7A EPS mutants disrupted in exoB and exoU genes, designated here as R7AexoB, R7AexoU and R7AexoU3’, had been isolated from a transposon mutagenesis screen (Hubber, 2005). This collection of EPS mutants represents strains disrupted at various stages of EPS biosynthesis.

3.2.1.1 Symbiotic phenotype of R7A EPS mutants
To establish the platform for the research described in this thesis, the symbiotic ability of the collection of R7A EPS mutants was re-examined on L. corniculatus and L. japonicus Gifu (Figure 3.2). The nodulation proficiency differed depending on the mutant and host. Strains R7AexoB and R7AexoU3’ formed effective nodules on both
hosts at a similar rate to strain R7A. Nodules determined as effective were large, pink/brown-coloured and the appearance of plants that harboured them indicated that they were nitrogen-fixing (Figure 3.1). Similarly, R7AΔexoA-inoculated *L. corniculatus* plants exhibited nodule formation similar to R7A-inoculated plants. However, R7AΔexoA-inoculated *L. japonicus* Gifu plants exhibited a lengthy delay in the formation of effective nodules. Initially, white nodule primordia formed, some of which eventually developed into nodules. In contrast, strains R7AexoU, R7AexoO and R7Amlr5265 formed white nodule primordia and, only following prolonged incubation, the occasional effective nodule on both hosts. R7AexoK and the R7Amlr5266 mutant had an intermediate phenotype between the R7AexoB and R7AexoU phenotypes. Shoot growth of plants inoculated with nodulation-impaired *exo* mutants was stunted and the leaves were yellowing compared to R7A-inoculated plants, indicating nitrogen starvation (Figure 3.2).

![Figure 3.1: Determinate nodule types formed by R7A EPS mutants.](image)

Examples of A) Effective, nitrogen-fixing nodule and B) Ineffective, non-nitrogen-fixing nodule formed by R7A EPS mutants on *L. japonicus* Gifu.
Figure 3.2: Determinate nodulation by R7A EPS mutants.

Total number of nitrogen-fixing nodules formed on 10 plants of A) *L. japonicus* Gifu and B) *L. corniculatus* inoculated with the indicated strains. Results represent three independent nodulation assays with the standard error of the mean indicated. C) *L. japonicus* Gifu and D) *L. corniculatus* plant appearance 6-weeks post-inoculation.
3.2.1.2 Colony morphology of R7A EPS mutants

Following 4 days growth, most of the isolated R7A EPS mutants formed small (1 to 2 mm) non-mucoid colonies on G/ RDM agar, with the exceptions being R7Amlr5266 and R7AexoU3’ (Figure 3.3A and B). R7Amlr5266 formed slightly mucoid colonies (3 to 4 mm) that were distinguishable from the larger R7A colonies (5 to 6 mm). R7AexoU3’ is a transposon mutant that harbours the mTn5 insertion 83 bp from the 3’ end of the gene’s coding sequence. Initially the strain formed small non-mucoid colonies; however, after prolonged incubation the colonies exhibited slightly mucoid growth.

In order to determine whether the various mutants could be distinguished on the basis of cell surface properties, their colony phenotypes on media containing Congo red or Calcofluor were investigated. On YMB plates containing Congo red, strains R7AexoO, R7Amlr5265, R7AexoU and R7AexoU3’ formed red colony growth due to absorption of the dye. Strains R7AexoK and R7Amlr5266 showed slight-red colouring whereas R7AΔexoA, R7AexoB and R7A remained essentially colourless. On TY medium containing Congo red, all strains including R7A formed non-mucoid colonies that did not appear to uptake the dye (Figure 3.3C and D). The Calcofluor reactions of the strains differed on TY versus G/ RDM medium (Figure 3.3E and F). On TY, a gradation from no fluorescence (R7AexoB, R7AexoO R7AΔexoA) through very weak (R7Amlr5265 and R7AexoK) to moderate (R7AexoU3’, R7Amlr5266) or strong fluorescence (R7A, R7AexoU) was observed. In contrast, on G/ RDM only R7AexoU, R7AexoU3’ R7Amlr5265 and R7Amlr5266 showed significant fluorescence. Interestingly, a fluorescent halo was observed around R7AexoU and R7AexoU3’ colonies grown on G/ RDM plates containing Calcofluor. In summary, strains R7AΔexoA and R7AexoB behaved identically on the three media whereas the other mutants could be uniquely distinguished.
Figure 3.3: Colony characteristics of R7A EPS mutants.

TY broth cultures were grown for 48 h and then spotted (10 µL) onto the various media. A and B: Colony growth of the indicated strains on G/ RDM after A) 48 h and B) 7 days. C and D: Growth after 48 h on C) YMB and D) TY containing 0.005% Congo red. E and F: Colony fluorescence observed under UV light after 48 h growth on E) TY and F) G/ RDM containing 0.02% Calcofluor.
3.2.1.3 Cell aggregation property of R7A EPS mutant strains

When grown in G/ RDM broth it was apparent that some of the mutant strains formed cell aggregates. Strains that displayed the aggregating phenotype in broth culture formed ‘hard’ colonies on G/ RDM agar compared to the easily dispersed colonies formed by the non-aggregating strains. Similar to R7A, mutant strains R7AexoB, R7AΔexoA and R7Amlr5266 did not form any cell aggregates. R7AexoO and R7AexoK exhibited weak aggregation, R7A5265 and R7AexoU3’ displayed intermediate aggregation whilst R7AexoU showed a strong cell aggregation phenotype (Figure 3.4). To determine if the cell aggregation phenotype was related to cell surface hydrophobicity, the cell surface hydrophobicity of the strongly aggregating R7AexoU was compared to non-aggregating R7A and R7AexoB. Hydrophobicity assays were performed as described in Section 2.9 on both early-log and stationary growth phase TY and G/ RDM broth cultures. If cells exhibit hydrophobic properties the OD$_{600}$ value following the addition of hexadecane would be expected to drop as the hydrophobic cells partition from the aqueous to solvent phase. However the results of the hydrophobicity assay revealed no change in the OD$_{600}$ value of any of the stains examined following the addition of hexadecane regardless of the growth phase or culture broth.

A summary of the colony and symbiotic phenotypes of the R7A EPS mutants is provided in Table 3.1.

![Figure 3.4: Cell aggregation by R7A EPS mutants.](image)

Varying levels of the cell aggregation phenotype exhibited by the indicated strains following 48 h growth in G/ RDM broths. R7A exhibited no cell aggregation, R7AexoK weak aggregation, R7Amlr5265 intermediate aggregation and R7AexoU strong cell aggregation.
Table 3.1: Summary of R7A EPS mutant strains colony and symbiotic phenotypes.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Colony phenotype on various media</th>
<th>Cell aggregation&lt;sup&gt;4&lt;/sup&gt;</th>
<th>Symbiotic phenotype&lt;sup&gt;5&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G/ RDM&lt;sup&gt;1&lt;/sup&gt;</td>
<td>YMB Colourless</td>
<td>TY Congo red&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>R7A</td>
<td>5 – 6 mm</td>
<td>Colourless</td>
<td>Colourless</td>
</tr>
<tr>
<td>R7AexoB</td>
<td>1 – 2 mm</td>
<td>Colourless</td>
<td>Colourless</td>
</tr>
<tr>
<td>R7AexoU</td>
<td>1 – 2 mm</td>
<td>Red</td>
<td>Colourless</td>
</tr>
<tr>
<td>R7AexoU3'</td>
<td>2 – 3 mm</td>
<td>Red</td>
<td>Colourless</td>
</tr>
<tr>
<td>R7AexoO</td>
<td>1 – 2 mm</td>
<td>Red</td>
<td>Colourless</td>
</tr>
<tr>
<td>R7AexoK</td>
<td>1 – 2 mm</td>
<td>Slight-red</td>
<td>Colourless</td>
</tr>
<tr>
<td>R7Amlr5265</td>
<td>1 – 2 mm</td>
<td>Red</td>
<td>Colourless</td>
</tr>
<tr>
<td>R7Amlr5266</td>
<td>3 – 4 mm</td>
<td>Slight-red</td>
<td>Colourless</td>
</tr>
<tr>
<td>R7AΔexoA</td>
<td>1 – 2 mm</td>
<td>Colourless</td>
<td>Colourless</td>
</tr>
</tbody>
</table>

<sup>1</sup>Diameter of a single colony following 7 days growth.
<sup>2</sup>Colour of colony growth.
<sup>3</sup>Colony fluorescence when viewed under UV light.
<sup>4</sup>Degree of cell aggregation, with – indicating no aggregation and +++ indicating a strong cell aggregation phenotype.
<sup>5</sup>Ability of the strain to form nitrogen-fixing nodules over the course of 6-weeks, with – indicating no nodules developed through to +++ indicating nodulation comparable to R7A. Note the mutants that did not induce nitrogen-fixing nodules did induce white nodule primordia like those shown in Figure 3.1B.
3.2.2 Construction of an R7A exsH mutant

Reportedly, LMW fractions of EPS are the symbiotically active form of the polysaccharide (Battisti et al., 1992; Gonzalez et al., 1996a; Wang et al., 1999). Studies with S. meliloti have suggested that two extra-cellular glycanases (ExoK and ExsH) are largely responsible for the cleavage of HMW EPS to yield the LMW symbiotically-active forms (York & Walker, 1998b). In both S. meliloti and M. loti, exoK is clustered with the exo genes responsible for EPS biosynthesis. In S. meliloti, exsH is located with other exs genes involved in EPS production. M. loti lacks exs homologues other than the exsH (mll2585) gene which is clustered amongst genes that have no apparent involvement in EPS production. Nevertheless, the exsH gene products of M. loti and S. meliloti share 60% amino-acid identity, indicating that ExsH may be involved in HMW EPS cleavage in R7A.

3.2.2.1 R7AΔexsH mutagenesis construct

To examine the involvement of exsH in R7A HMW EPS cleavage an in-frame markerless deletion mutant was constructed. The in-frame markerless deletion strategy involved allelic replacement of the wild-type exsH gene with a mutated form containing an in-frame markerless internal deletion through homologous recombination as outlined in Section 2.7.2.

Primer sets exsHLL/ exsHLR and exsHRL/ exsHRR (Table 2.4) were used to PCR-amplify left and right arms of ~1 kb that coded for respective flanking regions either side of exsH along with ~100 bp of 5' (left arm) or 3' (right arm) exsH sequence. Primer design incorporated BamHI restriction enzyme sites at the outermost ends of the left- and right-arm PCR products and 20 bp of overlapping sequence at the inner end of the left and right arms.

Left and right arms were PCR amplified using R7A genomic DNA as the template. Products of the expected sizes 946 bp (left arm) and 978 bp (right arm) were obtained (Figure 3.5A). A second overlap extension PCR was then performed using the left- and right-arm PCR products as template DNA with the outermost left-arm (exsHLL) and right-arm (exsHRR) primers. A product of 1925 bp made up of the two arms joined by in-frame overlapping sequence was amplified (Figure 3.5B).
The overlap extension PCR product was digested with BamHI and cloned into similarly digested pJQ200SK to form pSKEXSH that contained the 1925-bp insert (Figure 3.5C). Sequencing confirmed the presence of the *exsH* mutant fragment of the correct sequence.

### 3.2.2.2 Isolation of R7AΔ*exsH*

R7A was spot-mated (Section 2.6.14.1) with a confirmed S17-1/λpir clone and transconjugants that had received and integrated pSKEXSH through recombination between the left or right arms with homologous genomic DNA to form single-crossover (SXO) clones were selected on G/RDM containing Gm plates. Following single-colony purification, genomic DNA recovered from several SXO clones was used as template DNA in PCR with the primer pair *exsH*checkL/*exsH*checkR (Table 2.4) that amplify across the joining site of the left and right arms. Two PCR products should be amplified if the first-crossover event was successful, a 368-bp product from the integrated pSKEXSH and a 1526-bp product from wild-type chromosomal *exsH*. Agarose gel electrophoresis of the PCR products indicated that the first crossover occurred as expected (Figure 3.5D).

Selection for a second-crossover event to form double-crossover (DXO) clones was obtained by plating SXO clones onto sucrose/RDM (S/RDM). Following plating on S/RDM, DXO clones were patch-plated to check for Gm⁵ indicating the loss of pJQ200SK.

Genomic DNA from Gm⁵ DXO clones was used as template for PCR with primers *exsH*checkL and *exsH*checkR. If the second-crossover event resulted in the markerless deletion, only the 368-bp product would be amplified. However, if the second-crossover resulted in the clone reverting to wild-type, only the 1526-bp product would be amplified. Agarose gel electrophoresis of the PCR products indicated that the desired second-crossover occurred in five of the DXO clones examined (Figure 3.5E). One of the clones in which only the 368-bp product was amplified was designated R7AΔ*exsH*. 
Figure 3.5: Construction and confirmation of R7AΔexsH.

Agarose gel electrophoresis of PCR products amplified during the construction and confirmation of R7AΔexsH. DNA size marker (M) is λ DNA digested with HindIII and ΦX174 DNA digested with HaeIII, with the size of the fragments indicated in kb. A) PCR amplification of left (lanes 1 and 2) and right (lanes 3 and 4) exsH flanking arms. B) Lanes 1 and 2, overlap extension PCR to join the left and right arms and form the 1925-bp in-frame mutant fragment. C) Lanes 1-5, Plasmid DNA extracted from S17-1/λpirl pSKEXSH clones was digested with BamHI to confirm the presence of the 1925-bp fragment. D) PCR of SXO clones (lanes 1-10) with primer pair exsHcheckL/exsHcheckR. E) PCR of DXO clones (lanes 1-10) with primer pair exsHcheckL/exsHcheckR. * Denotes clone designated as R7AΔexsH.
3.2.2.3 Characterisation of R7AΔexsH

R7AΔexsH formed mucoid colonies on G/ RDM, which was expected as the mutant should not be affected in HMW EPS production. In addition, R7AΔexsH-inoculated *L. corniculatus* plants formed nodules at a comparable rate to control R7A-inoculated plants (Figure 3.6).

![Figure 3.6: Nodulation of *L. corniculatus* by R7AΔexsH.](image)

Total number of nitrogen-fixing nodules formed on 10 *L. corniculatus* plants inoculated with the indicated strains.

3.2.3 Attempts at isolation of further in-frame markerless deletion R7A EPS mutants

R7A EPS mutant strains (R7AexoK, R7AexoO, R7Amlr5265 and R7Amlr5266) had been isolated using an IDM approach (Kelly, 2007). Phenotypes exhibited by these strains could not necessarily be attributed to the targeted gene disrupted because of possible polar effects of the IDM mutations on downstream genes. In an effort to ascribe colony and symbiotic phenotypes to specific EPS biosynthesis genes, attempts were made to construct in-frame markerless deletion mutations of these genes.

Previous attempts to construct markerless deletion mutations in *exoK*, *exoO*, *mlr5265* and *mlr5266* had been unsuccessful using the overlap extension PCR and suicide vector pJQ200SK approach outlined in Section 2.7.2 (Kelly, 2007). It was intriguing
that isolation of markerless deletion mutants proved difficult as IDM of the targeted genes had proved successful and it therefore seemed unlikely that the mutations would cause lethal effects.

### 3.2.4 Attempted isolation of R7AΔexoK using the FRT/Flp system

An alternative strategy was devised for the construction of in-frame markerless deletion mutants. *exoK* encodes an endo-1,3-1,4-beta-glycanase cleavage enzyme responsible for the cleavage of HMW EPS to form LMW EPS and is involved after EPS production, following its translocation and polymerisation in the periplasm. Based on this, an *exoK* mutant would be expected to be viable and form mucoid colonies, as has been reported with *exoK* mutants of other rhizobia (Staehelin et al., 2006; York & Walker, 1997). In addition, the IDM *exoK* mutant (R7AexoK) forms non-mucoid colonies, suggesting that the mutation is polar on downstream EPS biosynthesis genes (*mlr5265* initiates 50 bp downstream of the *exoK* stop codon). For these reasons, *exoK* was used as a test case for an in-frame markerless deletion strategy using the FRT/Flp system (Sadowski, 1995). The major benefit of the FRT/Flp system is that it allows for antibiotic selection pressure to be maintained during the mutagenesis process, through the incorporation of an antibiotic-resistance gene that can later be removed through site-specific recombination between flippase recognition target (FRT) sites by the flippase recombination enzyme (Flp) leaving only an in-frame FRT scar (Figure 3.7).

![FRT/Flp system site-specific recombination](image)

**Figure 3.7: FRT/Flp system site-specific recombination**

The flippase recombination enzyme (Flp) mediates site-specific recombination between flippase recognition target (FRT) sites to remove any genetic material encoded between them, such as an antibiotic resistance gene.
3.2.4.1 Construction of a FRT-neomycin resistance cassette plasmid

A FRT-\textit{nptII} cassette (Nm-resistance gene flanked by FRT sites) plasmid was constructed. The plasmids used are described in Table 2.2 and the primers in Table 2.4. \textit{nptII} was PCR-amplified from pRS239 DNA using primers \textit{nptIIL} and \textit{nptIIR} which incorporated XbaI and Asp718 restriction sites respectively. The \textit{nptII} PCR product was cloned into appropriately-digested pFAJ1700 to allow for the incorporation of terminator sequences either side of \textit{nptII}. \textit{nptII} flanked by the terminator sequences was then recovered as an EcoRI fragment and cloned into the FRT-site-containing vector pPS854 to form the desired FRT-\textit{nptII} cassette vector (pSKFRT) that was confirmed by sequencing.

To test the FRT-\textit{nptII} cassette, the fragment was cloned out of pSKFRT and into pIJ3200 as a HindIII fragment to form pSKFRT2 which was then introduced into R7A. The Flp recombinase vector pBH474 was then introduced into R7A/ pSKFRT by electroporation. Fifty transformant colonies were patch-plated and all were Nm\textsuperscript{5}, indicating the complete removal of the FRT-\textit{nptII} cassette by the Flp recombinase.

3.2.4.2 Assembly of an \textit{exoK}-FRT-\textit{nptII} mutagenesis construct

PCR primer sets \textit{exoKFRTLL}/ \textit{exoKFRTLR} and \textit{exoKFRTRL}/ \textit{exoKFTRRR} were used to amplify \textasciitilde1-kb left and right arms, representing flanking genomic regions of \textit{exoK} along with \textasciitilde100 bp of 5\textquoteleft or 3\textquoteleft coding sequence respectively. Primers \textit{exoKFRTLL} and \textit{exoKFRTLR} were designed so that the left arm had a SpeI restriction site on the outer end and an XhoI site at the inner end (\textit{exoK} 5\textquoteleft sequence end). The right-arm primers \textit{exoKFRTRL} and \textit{exoKFRTRR} were designed to incorporate a SpeI site at the outer end and a HindIII site at the inner end (\textit{exoK} 3\textquoteleft sequence end). A further primer pair (\textit{exoKFRTL} and \textit{exoKFRTR}) was designed to amplify the FRT-\textit{nptII} cassette with XhoI and HindIII restriction enzymes sites incorporated.

PCR was performed using R7A genomic DNA as template to amplify the left and right arms and pSKFRT plasmid DNA as template to amplify the FRT-\textit{nptII} cassette (Figure 3.8A). The three PCR products were digested with appropriate restriction enzymes and cloned into SpeI-digested pJQ200SK via 3-way ligations. DNA precipitated from the ligation was electroporated into \textit{E. coli} S17-1/ \textit{\lambda}pir cells and transformants were selected on LB containing Gm and Nm. Plasmid DNA extracted from four of the S17-1/ \textit{\lambda}pir clones was digested with SpeI to check for the presence of
the \( \sim 4.3 \) kb insert (Figure 3.8B). The insert DNA from one clone was then sequenced to ensure no errors had occurred during PCR and that the construct would result in an in-frame \( \text{exoK} \) deletion once the FRT-\( \text{nptII} \) cassette had been excised through the action of the Flp recombinase. The confirmed construct was designated pSKKFRT.

**Figure 3.8: Construction and confirmation of pSKKFRT.**

A) PCR amplification of \( \text{exoK} \) left (lane 1) and right (lane 2) arms and amplification of FRT-\( \text{nptII} \) (lane 3). B) Restriction analysis of four clones (lanes 1-4) to confirm the presence of the \( \sim 4.3 \) kb \( \text{exoK} \)-FRT-\( \text{nptII} \) 3-way ligation product. DNA size marker (M) is \( \lambda \) DNA digested with HindIII and \( \Phi X174 \) DNA digested with HaeIII, with the size of the fragments indicated in kb.

### 3.2.4.3 Attempted isolation of R7A\( \Delta \text{exoK} \)

pSKKFRT was introduced into R7A via biparental spot-matings (Section 2.6.14.1). Transconjugants were selected on G/ RDM containing Gm and Nm and single-colony purified. At this stage these SXO clones should contain pSKKFRT integrated into the chromosome through recombination between the left or right arms with homologous genomic DNA adjacent to \( \text{exoK} \). To select for a second-crossover event, resulting in the removal of pJQ200SK and replacement of the wild-type gene with the \( \text{exoK} \)-FRT-\( \text{nptII} \) mutant fragment, dilutions of SXO clones were plated on S/ RDM containing...
Nm. All sucrose-resistant colonies displayed mucoid colony growth. To confirm that pJQ200SK had been removed, DXO clones were patch-plated to check for Gm sensitivity. All of the sucrose-resistant colonies grew in presence of Gm, indicating that the second-crossover event to remove pJQ200SK had not occurred but rather the sucrose-resistant colonies that arose were likely due to mutations in the sacB gene alleviating the selection pressure.

3.2.4.4 Attempted isolation of R7AΔexoK by plasmid incompatibility

Further attempts at isolating an in-frame markerless deletion exoK mutant were made using a plasmid incompatibility approach. The exoK-FRT-nptII fragment was recovered from pSKKFRT by restriction digest with SpeI and cloned into pIJ3200 to form pSKKFRT2. Given the previous difficulties encountered isolating a markerless deletion exoK mutant, pSKKFRT2 was introduced into R7AΔexoA as well as R7A to examine if the in-frame exoK deletion mutation could be formed in an EPS-deficient background.

Spot-matings were performed between R7A/ pSKKFRT2 or R7AΔexoA/ pSKKFRT2 and S17-1/λpir/ pPH1J1 with transconjugants selected on G/ RDM containing Gm and Nm. Plasmids pIJ3200 and pPH1J1 belong to the same incompatibility group (IncP) and therefore cannot both be maintained in the same cell. The introduction of pPH1J1 and selection for its maintenance by including Gm in the media should force the removal of pIJ3200. Maintenance of Nm selection should select for ‘rescue’ of the nptII gene by double recombination between the left and right arm of the exoK-FRT-nptII fragment with homologous genomic DNA adjacent to exoK. The appearance of non-mucoid colonies suggested that exoK-FRT-nptII had indeed integrated, causing polar effects on downstream EPS biosynthesis genes. Genomic DNA recovered from clones was used as template DNA in PCR with primer sets KFRTlocatL/ nptIIROL and KFRTlocatR/ nptIIROL to confirm the integration (Figure 3.9). If the exoK-FRT-nptII fragment had integrated at the correct location on the chromosome, products of 1538 bp or 1952 bp should be amplified. For most of the isolates tested in either the R7A or R7AΔexoA backgrounds, products of the expected size were amplified.
Figure 3.9: PCR confirmation of exoK-FRT-nptII integration.

A) Schematic of the PCR performed to confirm exoK-FRT-nptII integration following the introduction of pPH1J1 with primer binding sites and expected product sizes indicated. B) Genomic DNA extracted from four R7A/pPH1J1 clones was used as template with primer sets KFRTlocatL/nptIIROR (Lanes 1, 3, 5 and 7) or KFRTlocatR/nptIIROL (Lanes 2, 4, 6 and 8). C) Genomic DNA from five R7AΔexoA/pPH1J1 clones was used as template with primer sets KFRTlocatL/nptIIROR (Lanes 1, 3, 5, 7 and 9) or KFRTlocatR/nptIIROL (Lanes 2, 4, 6, 8 and 10). DNA size marker (M) is λ DNA digested with HindIII and ΦX174 DNA digested with HaeIII, with the size of the fragments indicated in kb.

Before the Flp recombinase plasmid pBH474 (Gm\(^{R}\)) could be introduced to complete the in-frame mutagenesis by removing the nptII cassette, it was necessary to first remove pPH1J1 from the clones. An incompatible vector with sucrose selection properties was constructed to perform this task. A BamHI fragment encoding sacB was isolated from pMH1701 and cloned into pIJ3200 to form pSKIJSAC. Spot-matings were performed between R7A/pPH1J1 or R7AΔexoA/pPH1J1 clones confirmed by PCR as harbouring exoK-FRT-nptII integrated at the correct location and S17-1/λpirl/pSKIJSAC. Transconjugants were selected on G/RDM containing
Nm and Tc and patch-plating was performed to check for Gm\textsuperscript{s} indicating loss of pPH1J1. All transconjugants tested were Nm\textsuperscript{R}, Tc\textsuperscript{R} and Gm\textsuperscript{R}, suggesting that both pPH1J1 and pSKIJSAC remained in the cell.

Further PCR was performed to investigate the clones obtained following the introduction of pPH1J1 (Figure 3.10). Genomic DNA isolated from representative R7A/ pPH1J1 and R7AΔexoA/ pPH1J1 clones was used as template DNA. Template DNA derived from R7A and an R7A/ pSKKFRT2 clone prior to the introduction of pPH1J1 were included as controls. Five different primer pairs were used to investigate the presence of pSKKFRT2 and pPH1J1. T3/ nptIIROL and T7/ nptIIJOR primer sets amplify products of ~1600 bp and ~1300 bp that read from either side of the pIJ3200 MCS into the exoK-FRT-nptII fragment. KFRTlocatL/ nptIIJOR and KFRTlocatR/ nptIIROL amplify ~1500 bp and ~2000 bp products if the exoK-FRT-nptII fragment has integrated into the genome to replace exoK. The fifth set of primers KconfirmL/ KconfirmR amplify a ~350 bp internal region of exoK deleted in the mutant construct. As expected only a ~350 bp product was obtained from control R7A template DNA with the KconfirmL/ KconfirmR primer set. Template DNA isolated from an R7A/ pSKKFRT2 clone prior to the introduction of pPH1J1 gave products of the expected size with T3/ nptIIROL and T7/ nptIIJOR primer pairs but not with KFRTlocatL/ nptIIJOR and KFRTlocatR/ nptIIROL, indicating the presence of the construct but that it was not integrated into the genome. Products of expected sizes were amplified with all primer pairs from template DNA derived from R7A/ pPH1J1 and R7AΔexoA/ pPH1J1 clones. Together the PCR results suggest that the introduction of pPH1J1 resulted in the integration of exoK-FRT-nptII into the genome but that the wild-type copy of exoK and pIJ3200 were not successfully removed.
**Figure 3.10: PCR investigation of exoK-FRT-nptII integration and removal of pIJ3200.**

A) A product of ~350 bp is amplified by primers KconfirmL/KconfirmR from the wild-type copy of exoK. B) PCR primer sets T7/nptII ROR and T3/nptII ROL amplify products of ~1300 bp and ~1600 bp from R7A/pSKKFRT2 prior to pPH1J1 introduction as recombination between exoK-FRT-nptII and the wild-type exoK region has not yet occurred. C) Integration of exoK-FRT-nptII is indicated by products of ~1500 bp and ~2000 bp amplified with primer sets KFRTlocatL/nptII ROR and KFRTlocatR/nptII ROL respectively. D) and E): PCR products amplified from the indicated template DNA with primer sets T3/nptII ROL (lanes 1 and 6), T7/nptII ROR (lanes 2 and 7), KFRTlocatL/nptII ROR (lanes 3 and 8), KFRTlocatR/nptII ROL (lanes 4 and 9) and KconfirmL/KconfirmR (lanes 5 and 10). DNA size marker (M) is λ DNA digested with HindIII and ΦX174 DNA digested with HaeIII, with the size of the fragments indicated in kb.
3.2.5 Complementation of R7AexoU, R7AΔexoA and R7AexoK mutants

All of the IDM R7A EPS mutants had previously been complemented with pSK11 that contains most of the exo cluster (Kelly, 2007). Due to the problems encountered with the construction of in-frame markerless deletion mutants and the possibility of polar effects from the constructed mutants, further complementation was carried out on strains of particular interest, R7AexoU, R7AΔexoA and R7AexoK. R7AexoU exhibited severe nodulation deficiencies and therefore it was important to determine that the effects were attributable to the disruption of exoU alone. R7AΔexoA was constructed as an in-frame markerless deletion mutant and therefore should not exhibit any polar effects. However, due to the location of exoA, in a large operon with several exo genes immediately downstream, it was important to verify that this was the case. As discussed above R7AexoK exhibited a non-mucoid colony morphology indicating polar effects were caused by the IDM.

3.2.5.1 Assembly of single-gene complementation constructs

Single-gene complementation constructs using the native promoters were developed for R7AexoU and R7AexoK. Because exoA is the second gene in the exoLAMON(P) operon, a complementation construct that also included exoL was necessary.

For the exoU single-gene construct, a 1267-bp product that included 212 bp of upstream region and the exoU coding sequence with primer set exoUSGL/ exoUSGR was amplified by PCR. For the exoA complementing construct, a 2396-bp PCR product was amplified that contained 197 bp of upstream region and the exoL and exoA coding sequences with primer set exoLASGL/ exoLASGR. Two exoK-encoding PCR products with varying upstream regions were PCR-amplified. One PCR product of 1039 bp that included 154 bp of upstream region was amplified with the primer set exoKSGL1/ exoKSGR whilst the other PCR product of 1272 bp included a 387-bp upstream region and was amplified with primer set exoKSGL2/ exoKSGR. Primer details including various restriction sites incorporated are provided in Table 2.4.

The PCR products were digested with appropriate enzymes and cloned into pFAJ1700 to form the complementation constructs pSKUSG (exoU), pSKASG (exoL-A), pSKKSG1 (exoK, 154-bp promoter region) and pSKKSG2 (exoK, 387-bp promoter region). The insert in each of the complementation constructs was sequenced to ensure no errors had been introduced during PCR.
3.2.5.2 Mutant complementation

The complementation constructs were introduced into the appropriate mutant strains by biparental spot-matings (Section 2.6.14.1). R7AexoU/ pSKUSG and R7AΔexoA/ pSKASG formed mucoid colonies on G/RDM agar and nodulated *L. japonicus* at rates comparable to R7A (data not shown). R7AexoK/ pSKKSG1 and R7AexoK/ pSKKSG2 formed non-mucoid colonies indistinguishable from R7AexoK, confirming that the *exoK* mutation was indeed polar on downstream EPS biosynthesis genes.

3.2.6 EPS and Nod factor gene expression during symbiosis

The expression of EPS biosynthesis genes during symbiosis was investigated through the fusion of *exo* promoter regions to *gfp*. If EPS was required at a particular stage during the infection process, then an increase in the expression of *exo* genes might be visualised by increased GFP fluorescence. Control *nod* gene promoter region-*gfp* fusions were included in the study, as the genes are known to be strongly expressed during infection (Sharma & Signer, 1990).

3.2.6.1 Assembly of *exo/nod* promoter region-*gfp* fusion constructs

Constructs for detecting the expression of *exoU* and the *exolAMON*(P) operon were assembled by using overlap extension PCR to form a translational fusion between the *exo* promoter regions and *gfp* at the ATG start site. Analogous expression constructs were developed with the promoter regions of *nodA* and the *nodACIJ* operon.

Initially, the promoter regions and *gfp* were PCR-amplified as separate products using R7A genomic DNA and pSKGFP plasmid DNA respectively as template. Primer sets were designed with overlapping sequence on the primers at the 3’ end of the promoter regions and 5’ end of the *gfp* PCR product. Details of the primers used are provided in Table 2.4. Overlap extension PCR was then performed using the promoter region and *gfp* PCR products as template with the outermost primers to amplify fusion products ranging in size from ~800 bp to ~1300 bp depending on the sizes of the promoter regions (Figure 3.11). The promoter region-*gfp* fusion PCR products were digested with BamHI/ HindIII and cloned into similarly digested pFAJ1700 to form pSKPUGFP (*exoU* promoter region), pSKPLGFP (*exolAMON*(P) promoter region), pSKPAGFP (*nodA* promoter region) and pSKPBGFP (*nodB* promoter region).
Clones identified as harbouring the desired constructs by restriction analysis were confirmed by sequencing.

**Figure 3.11:** *exo/nod* promoter region-*gfp* fusions.

Schematic of the *exo nod* promoter region-*gfp* overlap extension PCR products with the primers used indicated. Primers xLR and xRL contained overlapping sequence to allow the individually PCR amplified promoter regions and *gfp* PCR products to be joined at the ATG start codon by overlap extension PCR.
3.2.6.2 Examination of *exo* and *nod* gene expression using epifluorescent microscopy

The expression constructs were introduced into R7A by electroporation. Colonies of the R7A/ promoter region-*gfp* strains grown on G/ RDM plates were resuspended in sterile water and examined by epifluorescent microscopy (Figure 3.12). For all of the R7A/ promoter region-*gfp* strains, slight GFP fluorescence was observed. The level of fluorescence was significantly less intense than that of the control strain R7A/ pSKGFP that expressed *gfp* constitutively.

![Figure 3.12: nod and exo gene expression during growth on G/RDM.](image)

Resuspended colonies from G/ RDM agar of R7A containing the various promoter region-*gfp* constructs were observed by epifluorescent microscopy. A) R7A/ pSKGFP is a control strain that expresses *gfp* from the constitutive nptII promoter. Slight GFP fluorescence observed with B) R7A/ pSKPUGFP (*exoU* promoter fused to *gfp*) and C) R7A/ pSKPAGFP (*nodA* promoter fused to *gfp*). Similar levels of fluorescence were observed with R7A/ pSKPLGFP (*exoL* promoter fused to *gfp*) and R7A/ pSKPBGFP (*nodB* promoter fused to *gfp*).

*L. japonicus* seedlings were inoculated with R7A containing the promoter region-*gfp* constructs to investigate any changes in fluorescence of the strains during symbiosis (Figure 3.13). Control plants were inoculated with R7A/ pSKGFP. At 14 dpi, plant roots were examined by epifluorescent microscopy. On plants inoculated with R7A harbouring the *nod* gene fusions, pSKPBGFP and pSKPAGFP, bright green bacteria were clearly visible attached to the root and within ITs. Furthermore, developing nodules on these plants exhibited green fluorescence. The intensity of bacterial
fluorescence on plants inoculated with R7A/ pSKPAGFP was similar to that of the control R7A/ pSKGFP, whereas R7A/ pSKPBGFP bacteria exhibited slightly less fluorescent intensity. In contrast, no fluorescent bacteria were visible at all on roots inoculated with R7A harbouring the exo gene fusions, pSKUGFP or pSKPLGFP, and the nodules on these plants were not fluorescent.

Plants were examined again 28 dpi with similar results to those observed at 14 dpi. The only notable difference was that the developed nodules on plants inoculated with R7A/ pSKPBGFP and R7A/ pSKPAGFP had greatly diminished in fluorescent intensity whilst nodules on control R7A/ pSKGFP-inoculated plants remained intensely fluorescent.

Expression of pSKPLGFP containing the exoLAMON(P) promoter region fusion was also examined in the R7AexoB, R7AexoO and R7Amlr5265 mutant backgrounds. All strains grown on G/ RDM plates showed slight fluorescence comparable to that observed in the R7A background. L. japonicus inoculated with the EPS mutants harbouring pSKPLGFP were examined 14 and 28 dpi. As in the R7A background, no fluorescent bacteria were visible on any of the inoculated plant roots.
Figure 3.13: *nod* and *exo* gene expression on *L. japonicus*.

A) and E) No fluorescent bacteria were observed on roots or within nodules of plants inoculated with R7A/ pSKPUGFP (*exoU* promoter fused to *gfp*). Similarly, no fluorescence was observed on roots inoculated with I) R7A/ pSKPLGFP (*exoL* promoter fused to *gfp*). Fluorescent green bacteria were observed on plant roots inoculated with B), F) R7A/ pSKPBGFP (*nodB* promoter fused to *gfp*) and C) R7A/ pSKPAGFP (*nodA* promoter fused to *gfp*) with fluorescent intensity comparable to D) R7A/ pSKGFP (*nptII* promoter fused to *gfp*). Nodule fluorescence was comparable between G) R7A/ pSKPAGFP and H) R7A/ pSKGFP at 14 dpi however, fluorescence had greatly diminished 28 dpi in nodules formed by J) R7A/ pSKPBGFP and K) R7A/ pSKPAGFP compared to those formed by L) R7A/ pSKGFP.
3.2.7 Expression of \textit{LjNin} in response to R7A and R7AexoU and purified EPS extract

The \textit{L. japonicus} nodule inception (\textit{LjNin}) gene encodes a putative transcriptional factor that is expressed in response to compatible rhizobia and is essential for IT formation and the initiation of nodule primordia (Schauser \textit{et al.}, 1999). Transgenic \textit{L. japonicus} plants carrying the \textit{LjNin} promoter fused to the GUS reporter show detectible expression in response to R7A as soon as 3 hpi, with substantial levels evident by 10 dpi (Rodpothong \textit{et al.}, 2009).

To examine if the R7AexoU mutant was able to induce similar levels of \textit{LjNin} expression compared to R7A, \textit{LjNin-Gus} transgenic seedlings were inoculated with either strain. Purified EPS extract from R7A and R7AexoU strains (Section 2.11.2) was also used to inoculate \textit{LjNin-Gus} plants to investigate if EPS alone could induce expression. Roots stained 10 dpi (Section 2.10.8) revealed that both R7A and R7AexoU strongly induced \textit{LjNin-Gus} expression at comparable levels. Conversely, roots inoculated with EPS extract from either R7A or R7AexoU did not induce any detectible \textit{LjNin-Gus} expression (Figure 3.14).

![Figure 3.14: Induction of \textit{LjNin-Gus}.](image)

Transgenic \textit{LjNin-Gus} plants inoculated with R7A, R7AexoU or EPS extract from either strain were stained 10 dpi to observe expression of the early nodulation gene.
3.3 Discussion
Research described in this chapter investigated the symbiotic proficiency and phenotypic properties of R7A EPS mutant strains disrupted at varying stages of the EPS biosynthesis pathway to detect differences in their production of EPS. The requirement for HMW versus LMW EPS was examined through the construction of strain R7AΔexsH and attempts were made to isolate a non-polar exoK mutant using in-frame markerless deletion strategies. In addition, the expression of exo and nod genes during the symbiotic process and the influence of R7A EPS on the induction of the *L. japonicus* early nodulin gene *LjNin* were investigated.

The symbiotic proficiency of the R7A EPS mutants on the determinate-nodule-forming hosts *L. corniculatus* and *L. japonicus* varied depending on the mutant and the host. In general the symbiotic ability appeared to relate to the stage of EPS biosynthesis disrupted in the particular mutant strain. Strains harbouring mutations in genes involved in the early stages of EPS biosynthesis (R7AexoB and R7AΔexoA) formed nodules at rates comparable to R7A (R7AΔexoA was less effective on *L. japonicus* Gifu). In contrast, strains disrupted in the mid/late-stages (R7AexoO, R7AexoU, R7AexoK, R7Amlr5265 and R7Amlr5266) were impaired, some severely, in their ability to form nodules. These mutants induced the formation of small uninfected nodule primordia that failed to fix nitrogen.

R7A EPS mutants exhibited varying colony morphologies depending on both the media on which they were grown and the particular EPS biosynthesis gene in which they were disrupted. The mucoid appearance of rhizobia is due to the production of HMW EPS which constitutes lengthy polymers of repeating EPS monomers (Skorupska *et al*., 2006). All but two of the R7A EPS mutant strains formed non-mucoid colonies on G/RDM due to deficiencies in HMW EPS production. The appearance of slightly mucoid colonies following prolonged incubation on G/RDM by R7AexoU3’ suggests that the strain may produce a functional ExoU enzyme with significantly reduced activity, due to the site of the mTn5 insertion. ExoU is a glycosyltransferase with conserved domains located in the N-terminal half of the protein and the mTn5 insertion in R7AexoU3’ is 83 bp from the 3’ end of the gene (Hubber, 2005). Although the function of *mlr5266* in EPS biosynthesis is unknown, it is predicted to be involved in the addition of uronic acid groups during the later
stages of EPS biosynthesis (Section 4.3) and may produce a near-complete EPS molecule that is amenable to polymerisation.

Variations in absorption of the dye Congo red and altered fluorescence under UV light on Calcofluor-containing media have long been utilised to identify deficiencies in EPS production by rhizobia (Becker et al., 1993c; Glucksmann et al., 1993a; Glucksmann et al., 1993b; Hotter & Scott, 1991; Hotter & Scott, 1997; Laus et al., 2004; Leigh et al., 1985; Long et al., 1988). Despite the extensive use of both indicators, the reasons for the reactions of EPS-deficient strains cultured on media containing them are not well understood. Congo red is thought to preferentially bind to cellulose, with deficiencies in EPS production allowing greater interaction between cellulose and Congo red (Zevenhuizen et al., 1986). On YMB-Congo red agar, R7A formed pale pink mucoid growth whilst the EPS mutants displayed varying degrees of Congo red absorption resulting in colony growth ranging from pale pink through to dark red. On TY-Congo red agar, R7A showed non-mucoid growth, suggesting very little or no EPS is produced on this medium, and all strains formed pale pink colonies. The observation that all strains appeared the same on TY-Congo red (little or no EPS produced) yet different on YMB-Congo red (EPS produced) indicates that Congo red absorption may relate to EPS production for R7A. Variations in the absorption of the Congo red dye by the EPS mutants may reflect the production of altered forms of EPS due to the differing stages of EPS biosynthesis at which they are disrupted. Strains disrupted in the early stages of EPS biosynthesis (e.g. R7AΔexoA and R7AexoB) formed pale pink colonies on YMB-Congo red whilst strains disrupted midway through EPS biosynthesis (e.g. R7AexoO and R7AexoU) formed dark red colonies. The Congo red binding could be either to the altered EPS produced by the mutant or to a different polysaccharide made accessible to the Congo red by the lack of HMW EPS or newly synthesised as a regulatory response to the lack of EPS.

In the case of S. meliloti, EPS-producing strains fluoresce when grown on Calcofluor media viewed under UV light whilst EPS-deficient strains do not (Gonzalez et al., 1996b). The reverse is true of most other rhizobia, where EPS production results in non-fluorescent growth whilst EPS mutant strains fluoresce. In such cases the fluorescence may be due to the binding of Calcofluor to exposed cell-surface polysaccharides such as cellulose (Harrington & Raper, 1968). On TY containing
Calcofluor, R7A fluoresced brightly whilst it appeared non-fluorescent on G/ RDM containing Calcofluor. This suggests that the fluorescence observed with R7A was not due to the binding of wild-type EPS with Calcofluor. Fluorescence of the mutant strains varied, with no consistent correlation observed between Congo red absorption and Calcofluor fluorescence. If the fluorescence was caused by the binding of Calcofluor to cell-surface polysaccharides, then all of the non-mucoid EPS mutants would be expected to fluoresce unless those that appear dark are affected not only in EPS production but also in other cell-surface polysaccharides. Alternatively, some of the R7A EPS mutants may produce a truncated form of the EPS molecule that was able to bind Calcofluor and as a result cause fluorescence. This suggestion is supported by the observation of a halo around R7AexoU and R7AexoU3’ colonies grown on Calcofluor-containing G/ RDM, indicating that Calcofluor was binding to a LMW molecule that diffused into the medium.

Although the reasons behind the varying colony phenotypes are not well-established, the results indicate that the EPS mutants differ not only from wild-type but also when compared to one another, demonstrating cell-surface variations amongst the mutant strains.

Another phenotype in which the EPS mutants varied was cell aggregation. Hydrophobicity assays revealed no differences between strongly-aggregating and non-aggregating strains, indicating other factors were responsible. Multiple bacterial components have been associated with bacterial cell aggregation including polysaccharides such as EPS, LPS, KPS and cellulose as well as cell membrane-protein interactions. Identifying the particular factors responsible has proved difficult due to the involvement of various factors and at times contradictory results reported, as noted by (Burdman et al., 2000). As not all of the non-mucoid EPS mutant strains exhibited the aggregation phenotype, it raises the possibility that changes to other cell-surface polysaccharides in the non-aggregating strains, or the production of truncated EPS by some of the mutant strains is responsible for the observed aggregation.

This chapter investigated a potential role in EPS processing in R7A by a second extracellular glycanase encoded by exsH. Although the exoK gene encoding the previously identified extracellular glycanase is clustered with EPS biosynthesis genes
and exsH is not, it was possible that both enzymes were involved in HMW EPS cleavage, as has been reported in S. meliloti (York & Walker, 1998a; York & Walker, 1998b). The constructed mutant (R7AΔexsH) exhibited colony and symbiotic phenotypes indistinguishable to those of R7A, suggesting ExsH is not involved in R7A EPS production. The possibility remains that R7AΔexsH produces lesser amounts of LMW EPS due to reduced cleavage of HMW fractions. However, as LMW EPS has been implicated as the symbiotically-active form of EPS, reduced symbiotic proficiency by R7AΔexsH would have been expected if it was indeed responsible for the cleavage of R7A HMW EPS (Battisti et al., 1992; Gonzalez et al., 1996a; Staehelin et al., 2006).

Substantial efforts were made in this study to isolate in-frame markerless deletion EPS mutants after previous attempts had proved successful for only some EPS biosynthesis genes (Kelly, 2007). The inability to isolate markerless deletion mutants indicated that the mutation of certain EPS biosynthesis genes may be lethal to the cell. Lethal effects of exoL, exoM, exoP, exoQ and exoT mutations in an S. meliloti succinoglycan over-producing (expR mutant) strain have been reported (Reuber et al., 1991) with the authors hypothesising that the accumulation of lipid-linked intermediates may be toxic. Investigations of succinoglycan production by S. meliloti have largely been performed using mutants isolated from transposon mutagenesis screens, for example (Glucksmann et al., 1993a; Glucksmann et al., 1993b; Leigh et al., 1985; Long et al., 1988), and the transposon insertions may cause polar effects.

The IDM exoK mutant (R7AexoK) exhibited non-mucoid colony growth, suggesting that the mutation caused polar effects on downstream EPS biosynthesis genes, and this gene was therefore used to test further mutagenesis strategies. The FRT/ Flp system is a site-specific recombination system from Saccharomyces cerevisiae that allows for antibiotic selection pressure to be maintained during recombination steps through the incorporation of an antibiotic-resistance gene that can be subsequently removed. The FRT/ Flp system has been successfully employed for mutant construction in various systems including the gram-negative bacterium Pseudomonas aeruginosa (Hoang et al., 1998). In this study a FRT construct was developed containing the nptII gene that encodes NmR for use in mutant construction. Unfortunately, isolation of the desired exoK mutant proved unsuccessful with clones
isolated exhibiting antibiotic resistances indicating that the selection pressure was alleviated due to mutations in the *sacB* gene of pJQ200SK. A further mutagenesis strategy relying on plasmid incompatibility to select for desired recombination events resulting in an in-frame *exoK* deletion mutant also proved unsuccessful, even in an EPS-deficient (R7AΔexoA) background. The inability to isolate markerless deletion mutants of *exoK* with these strategies suggests that there are strong selection pressures against such a mutation, possibly due to a lethal effect. However, given the predicted function of *exoK*, no apparent causes for lethality due to its mutation are obvious. Furthermore, a *Rhizobium* sp. NGR234 *exoK* mutant that was shown to be non-polar by complementation was successfully isolated (Staehelein *et al.*, 2006). Despite the ineffectiveness of the mutagenesis strategies in the isolation of an *exoK* mutant, the constructs developed in this study should prove useful in the generation of further *M. loti* mutants.

Due to the difficulties encountered in construction of in-frame markerless deletion mutants, complementation of various mutant strains was performed to determine if polar effects were occurring. Constructs were generated that contained the coding sequence of the target genes and their native promoter regions. For mutants R7AexoU and R7AΔexoA, the complementing constructs restored wild-type EPS production as evidenced by mucoid growth on G/RDM agar plates and restored symbiotic proficiency. The complementation results demonstrate that the mutations in R7AexoU and R7AΔexoA did not cause polar effects and that the observed phenotypes of the mutants was due solely to the disruption of *exoU* or *exoA* respectively. In contrast complementation of R7AexoK was unsuccessful, confirming that the mutation in the strain was polar on the downstream EPS biosynthesis genes that include *mlr5265* and *mlr5266*.

In an effort to establish if EPS biosynthesis is increased during a particular stage of the symbiotic infection process, GFP-expressing constructs were developed that would allow for detection of expression of *exoU* and the *exoLAMON(P)* operon. Similar control constructs were generated for two *nod* gene operons known to be strongly expressed during the infection process in indeterminate nodules (Ampe *et al.*, 2003; Capela *et al.*, 2006; Sharma & Signer, 1990). Similar studies of *nod* gene expression in a determinate-nodule-forming symbiosis have not been reported. As
expected, bright green bacterial cells were readily visualised attached to root hairs, within ITs and also within young nodules on plants inoculated with R7A containing the \textit{nod} gene promoter region-\textit{gfp} fusion constructs. The fluorescence within nodules had greatly diminished by 28 dpi indicating that \textit{nod} gene expression was repressed by rhizobia within nodules, consistent with previous studies (Karunakaran \textit{et al.}, 2009). In contrast, no fluorescent bacteria were observed on plants inoculated with R7A or \textit{exo} mutant strains containing the \textit{exo} promoter region-\textit{gfp} fusion constructs. The lack of observed fluorescence could not be attributed to problems associated with the promoter regions used in the \textit{exo} constructs as these same promoter regions were used in the construction of the single-gene complementation constructs with success. The result therefore suggests that the investigated EPS biosynthesis genes are not up-regulated upon interaction with a compatible host to levels detectible by an increase in GFP fluorescence. As discussed in Section 1.6.1.2, regulation of EPS biosynthesis genes is complex. Previous studies have also noted low levels of EPS biosynthesis gene expression (Becker \textit{et al.}, 1993b; Reuber \textit{et al.}, 1991). Given that \textit{nod} gene expression is regulated in response to host flavonoids whilst EPS is produced regardless of association with a host, it is perhaps not surprising that increased \textit{exo} gene expression was not observed.

Expression of the \textit{L. japonicus} early nodulin gene \textit{LjNin} is induced following perception of compatible rhizobia by the host plant (Rodpothong \textit{et al.}, 2009; Schauser \textit{et al.}, 1999). Perception requires the Nod factor receptors NFR1 and NFR5 and Nod factor (Madsen \textit{et al.}, 2003; Radutoiu \textit{et al.}, 2003). To determine if R7A EPS was also involved in the induction of \textit{LjNin}, transgenic \textit{L. japonicus} plants carrying an \textit{LjNin-Gus} fusion were inoculated with R7A and R7AexoU mutant strains as well as EPS extract. Both the wild-type and EPS-deficient strain induced comparable levels of \textit{LjNin} expression 10 dpi, indicating that EPS was not involved in its induction which was confirmed by the observation that inoculation with EPS extract alone did not result in any detectible expression. Furthermore, this result confirmed that the EPS mutant strains were proficient in the early stages of symbiosis involving root hair colonisation and Nod factor production, as also evidenced by their ability to cause root-hair curling and the induction of nodule primordia.
4 Chemical analysis of R7A EPS extracts
4.1 Introduction

All rhizobia produce EPS, however the structure varies amongst different species and at times between strains. The structure of EPS produced by several rhizobia has been reported (Section 1.6.1) but little is known about the composition and structure of EPS produced by \textit{M. loti}. This chapter describes chemical analysis of EPS extracts isolated from various wild-type \textit{M. loti} and R7A mutant strains.

Defining the structure of wild-type R7A EPS was the main aim of the work described in this chapter. Determination of the structure of R7A EPS would allow for comparisons to known EPS structures of other rhizobia. Furthermore, correlating the structure of R7A EPS to the identified EPS biosynthesis genes could reveal further potential genes involved in R7A EPS biosynthesis.

LMW EPS extracts isolated from R7A EPS mutants that exhibit contrasting symbiotic phenotypes (R7AexoB and R7AexoU) were also examined. Comparing the EPS extracts produced by the strains may reveal a connection between the contents of the extract and the observed colony and symbiotic properties. Two further R7A mutant strains R7AexoU3’ and R7A\textDelta ndvB were also included in the analysis. R7AexoU3’ exhibited phenotypes that suggested the strain may produce small quantities of wild-type EPS, due to the transposon insertion at the 3’ end of \textit{exoU} not completely inactivating the gene. R7A\textDelta ndvB contains an in-frame markerless deletion of the CGB synthase (\textit{ndvB}) gene and is therefore expected to be deficient in CBG production.

Apart from EPS extraction, which was performed at the University of Otago, the work reported in this chapter was largely carried out during a two month visit to Professor Russell Carlson’s laboratory at the Complex Carbohydrate Research Center (CCRC) at the University of Georgia in Athens, GA, USA. In addition to the experiments performed during that visit, further characterisation of wild-type R7A EPS including nuclear magnetic resonance (NMR) analysis was completed by Dr Artur Muszynski in Professor Russell Carlson’s laboratory, to the extent that a proposed structure for R7A EPS can be presented.

EPS extracts from \textit{M. loti} strains MAFF303099 and NZP2037 were also partially characterised. The completely sequenced MAFF303099 had been used as a model for
R7A EPS production due to the close relatedness of the strains and the identified similar \textit{exo} gene arrangement (Section 1.8.1). Based on genetic analysis, R7A and MAFF303099 would be expected to produce similar EPS. NZP2037 EPS was examined because the strain exhibits a broad host range compared to other \textit{M. loti} and EPS mutants of the strain exhibit contrasting symbiotic phenotypes to those of R7A (Chapter 7). Therefore, it was interesting to investigate the EPS produced by NZP2037 and to compare it to that of R7A and MAFF303099.

4.2 Results

4.2.1 EPS extraction

Details of EPS extraction from \textit{M. loti} cultures are provided in Section 2.11.1. Briefly, EPS was ethanol-precipitated from concentrated supernatant of stationary-phase G/RDM cultures. Following dialysis, the EPS extract was lyophilised to recover EPS samples.

\textit{M. loti} strains that exhibited mucoid colony growth on G/RDM plates yielded lyophilised EPS samples that had a cotton-like appearance distinct to the fine powdered EPS extract recovered from EPS mutant strains that formed non-mucoid colonies.

4.2.2 Gel permeation chromatography

EPS extracts from wild-type and mutant strains were size-fractionated by Sephacryl S-400 gel permeation chromatography. For each strain, lyophilised EPS was resuspended in de-gassed Milli-Q water and the suspension was used to charge a Sephacryl S-400 gel column prepared as described in Section 2.11.2. It was observed that the EPS extract from the R7AexoB and R7AexoU mutant strains contained a water-insoluble fraction. This insoluble fraction was not observed in suspensions prepared from wild-type R7A, NZP2037 or MAFF303099 EPS extract or from that of R7AΔndvB and R7AexoU3’ mutant strains.

Soluble EPS suspensions were loaded onto the gel column that was gravity fed with de-gassed Milli-Q water. Elution from the column was monitored with a refractive index detector and fractions were collected at 15-min intervals. Fractions were pooled based on the readings from the detector. Elution profiles indicated that R7A and the mutant strains R7AΔndvB and R7AexoU3’ produced both HMW and LMW
EPS fractions whilst EPS mutant strains R7AexoB and R7AexoU contained only a LMW fraction (Figure 4.1). For each strain, collection tubes representing HMW (if present) and LMW EPS fractions were pooled separately. The pooled fractions were concentrated by rotary evaporation and then lyophilised to yield HMW and/or LMW EPS fractions for further chemical analysis.

**Figure 4.1: Elution profile of EPS extracts.**

EPS extracts were size-fractionated by Sephacryl S-400 size exclusion chromatography. Collected eluent representing HMW and LMW EPS fractions are indicated.
4.2.2.1 Carbohydrate content of the insoluble fraction

R7AexoB and R7AexoU contained a water-insoluble material in their crude EPS extracts. To investigate if this insoluble fraction consisted of carbohydrate, a phenol-sulfuric acid colourimetric assay was performed as described in Section 2.11.3. R7AexoB and R7AexoU insoluble material along with a positive glucose control and a negative Milli-Q water control were analysed. The glucose control tube produced a dark yellow solution, indicating the presence of carbohydrate, whilst the negative control remained clear. Both the R7AexoB and R7AexoU tubes displayed a very pale yellow colouring, suggesting that carbohydrate may be a minor component of the insoluble fractions.

4.2.3 Glycosyl composition of EPS samples

To determine the glycosyl composition of the isolated EPS fractions, trimethylsilyl (TMS)-derivatised samples were prepared and analysed by gas chromatography-mass spectrometry (GC-MS) as describe in Section 2.11.4. EPS fractions were subjected to methanolysis then re-N-acetylated prior to TMS-derivatisation with TriSil® reagent. Following evaporation of TriSil®, TMS-methyl glycosides were dissolved in hexane and filtered through glass wool prior to GC-MS analysis.

Analysis of the obtained chromatograms indicated that R7A EPS consisted of glucose (Glc), galactose (Gal) and glucuronic acid (GlcA). Similar profiles were observed for R7AexoU3’ and R7AΔndvB (not shown) samples whilst R7AexoU and R7AexoB chromatograms indicated only the presence of Glc in the EPS mutant extracts (Figure 4.2).

The water-insoluble material isolated from crude EPS extracts of R7AexoB and R7AexoU was also examined by conversion to TMS-methyl glycosides and GC-MS. No peaks representative of carbohydrates were observed in the chromatograms, suggesting that polysaccharide was at most a very minor component of the insoluble material (data not shown).
Figure 4.2: Glycosyl composition of EPS extracts.

EPS samples were converted to TMS-methyl glycosides and the glycosyl composition was determined by GC-MS.
4.2.4 Glycosyl-linkage analysis

In order to determine the linkages between the glycosyl residues in the EPS fractions from R7A and various mutant strains, several approaches were employed.

4.2.4.1 Determination of neutral glycosyl linkages

Initially neutral sugar linkages were determined through GC-MS analysis of partially-methylated alditol acetates (PMAA) (Section 2.11.5.1). The process involved methylation of the intact EPS followed by hydrolysis in trifluoroacetic acid (TFA). The partially-methylated sugars were then reduced overnight in sodium borodeuteride solution made up in 1 M ammonium hydroxide to form partially-methylated alditols. Acetylation was then performed by the addition of an acetic anhydride and pyridine solution. The positions of the O-acetyl groups on the resultant PMAAs are representative of the linkage positions of the glycosyl residues on the intact EPS.

PMAA samples were analysed by GC-MS. Identification of the glycosyl linkages present in the EPS samples was achieved through comparison of retention times relative to internal standard inositol and characteristic electron impact mass spectral fragments of particular alditol acetates. The PMAA mass spectra obtained were compared to the mass spectra of the standard PMAAs available on the CCRC website (http://www.ccrc.uga.edu/specdb/ms/pmaa/pframe.html).

The glycosyl linkages identified for R7A HMW and LMW EPS fractions as well as for various mutant strains are displayed in Table 4.1. The linkage data indicate that R7A, R7AexoU3’ and R7AΔndvB all produce similar EPS with the exception being the lack of 2-Glc in the LMW fraction of R7AΔndvB extract. R7AΔndvB is disrupted in CBG production which in M. loti is a cyclic 2-Glc polysaccharide (Kawaharada et al., 2008).

R7AexoU LMW extract lacked the 3-Gal and 4,6-Glc glycosyl linkages observed in R7A extract. R7AexoB extract contained most of the linkages observed in R7A extract other than 3-Gal. Additional 3,4-Glc and 2,3-Glc linkages that were absent from the extracts of other strains examined were detected in R7AexoB extract. However, due to the low values, it is likely that these were present as a result of under-methylation of the sample (Artur Muszynski, personal communication).
**4.2.4.2 Determination of uronic acid linkages**

Because GlcA was detected in glycosyl composition analysis of EPS samples (Section 4.2.3), further advanced linkage analysis protocols were required to determine the linkages of these reactive residues. Uronic acid carboxyl groups form sodium salts and are degraded during normal PMAA protocols and were therefore not detected in PMAA linkage analysis (Section 4.2.4.1). Two independent methods were employed to protect the ‘reactive’ groups prior to PMAA analysis of R7A and R7AΔndvB EPS extracts. The first involved permethylation based on the Hakomori protocol, utilising SuperDeuteride and was performed by Dr Artur Muszynski. The second involved mild-methyl esterification and carboxyl reduction prior to PMAA analysis (Section 2.11.5.2) that I performed during my visit to the CCRC.

GC-MS analysis of the two differently pre-treated PMAA samples of R7A and R7AΔndvB HMW fractions yielded comparable results (Table 4.1). In addition to the Glc and Gal linkages previously identified, the EPS extracts contained variously-linked GlcA residues and an early eluting penturonic acid peak that was determined to be riburonic acid (RibA).
Table 4.1: Glycosyl linkages identified in R7A and R7A mutant EPS extract fractions.

<table>
<thead>
<tr>
<th>Strain</th>
<th>R7A</th>
<th>R7A</th>
<th>R7AexoU3'</th>
<th>R7AexoU3'</th>
<th>R7AexoB</th>
<th>R7AexoU</th>
<th>R7AΔndvB</th>
<th>R7A</th>
<th>R7A</th>
<th>R7AΔndvB</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPS fraction</td>
<td>HMW</td>
<td>LMW</td>
<td>HMW</td>
<td>LMW</td>
<td>HMW</td>
<td>LMW</td>
<td>HMW</td>
<td>LMW</td>
<td>HMW</td>
<td>HMW</td>
</tr>
<tr>
<td>Linkage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t-Glc</td>
<td>3</td>
<td>16</td>
<td>14</td>
<td>28</td>
<td>40</td>
<td>31</td>
<td>16</td>
<td>2</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>2-Glc</td>
<td>-</td>
<td>24</td>
<td>-</td>
<td>9</td>
<td>23</td>
<td>30</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4-Glc</td>
<td>50</td>
<td>38</td>
<td>43</td>
<td>44</td>
<td>13</td>
<td>23</td>
<td>44</td>
<td>36</td>
<td>44</td>
<td>41</td>
</tr>
<tr>
<td>6-Glc</td>
<td>16</td>
<td>16</td>
<td>12</td>
<td>11</td>
<td>12</td>
<td>11</td>
<td>21</td>
<td>20</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>4,6-Glc</td>
<td>14</td>
<td>5</td>
<td>25</td>
<td>4</td>
<td>7</td>
<td>-</td>
<td>11</td>
<td>18</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>3,4-Glc</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2,3-Glc</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>t-Gal</td>
<td>1.5</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3-Gal</td>
<td>15</td>
<td>-</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>t-RibA</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>3-GlcA</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>4-GlcA</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>t-GlcA</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

Partially methylated alditol acetates (PMAA) determined via pre-treatment of samples by indicated methods prior to PMAA analysis. The values of uronic acids indicated do not necessarily reflect stoichiometric quantities of the glycosyl residues due to their labile nature and the derivatisation techniques.

nd = not detectable by PMAA analysis, t = terminal residue. Yellow shaded area highlights uronic acid linkages detect via pre-treatment of samples by indicated methods prior to PMAA analysis.
4.2.5 Analysis of EPS extracts from *M. loti* strains MAFF303099 and NZP2037

Partial characterisation of EPS extracts from *M. loti* strains MAFF303099 and NZP2037 was performed to allow comparison with R7A. Sephacryl S-400 gel permeation chromatography indicated that both MAFF303099 and NZP2037 EPS extract consisted of HMW and LMW fractions as was observed with R7A (data not shown). To investigate the glycosyl composition of the EPS extracts, TMS-derivatised samples were prepared and analysed by GC-MS as described in Section 2.11.4. The chromatograms revealed that MAFF303099 and NZP2037 EPS extract consisted of Glc, Gal and GlcA residues and were similar to R7A EPS extract (Figure 4.3).

Glycosyl linkages present within MAFF303099 and NZP2037 HMW EPS fractions were determined through GC-MS of PMAA as described in Section 2.11.5.1 and are displayed in Table 4.2. The neutral sugar linkages identified were comparable to those found in R7A HMW fractions at similar ratios. Glycosyl composition analysis indicated that, as with R7A EPS, uronic acid residues were present in MAFF303099 and NZP2037 extracts. As discussed above, uronic acid linkages are not detectable by GC-MS of PMAA without prior treatment of the EPS samples and therefore were not detected in the glycosyl linkage analysis performed.

Table 4.2: Glycosyl linkages identified in MAFF303099 and NZP2037 EPS fractions.

<table>
<thead>
<tr>
<th>Strain</th>
<th>EPS fraction</th>
<th>Linkage</th>
<th>R7A</th>
<th>MAFF303099</th>
<th>NZP2037</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>HMW</td>
<td>HMW</td>
<td>HMW</td>
</tr>
<tr>
<td>t-Glc</td>
<td></td>
<td></td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>2-Glc</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4-Glc</td>
<td></td>
<td></td>
<td>50</td>
<td>42</td>
<td>38</td>
</tr>
<tr>
<td>6-Glc</td>
<td></td>
<td></td>
<td>16</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>4,6-Glc</td>
<td></td>
<td></td>
<td>14</td>
<td>17</td>
<td>21</td>
</tr>
<tr>
<td>t-Gal</td>
<td></td>
<td></td>
<td>1.5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3-Gal</td>
<td></td>
<td></td>
<td>15</td>
<td>11</td>
<td>13</td>
</tr>
</tbody>
</table>
Figure 4.3: Glycosyl composition of MAFF303099 and NZP2037 EPS extracts.

EPS samples were converted to TMS-methyl glycosides and the glycosyl composition was determined by GC-MS.
4.3 Discussion

EPS extracted from R7A and several R7A mutant strains was investigated. R7A, R7AΔndvB and R7AexoU3’ extract contained both HMW and LMW fractions of EPS. Colony and symbiotic phenotypes of R7AexoU3’ (Sections 3.2.1.1 and 3.2.1.2) suggested that the strain produced an ExoU enzyme with reduced functionality due to the location of a transposon insertion at the 3’ end of exoU. The presence of HMW EPS of similar composition to that of R7A in the extracts of R7AexoU3’ supports this proposal. Thus, the symbiotic effectiveness of R7AexoU3’ is likely due to the production of some wild-type EPS. Based on the strain’s colony morphology (Section 3.2.1.2), the amount of EPS produced is significantly reduced compared to R7A, indicating that small quantities of wild-type EPS are sufficient for nodulation, supporting a signalling role for R7A EPS.

EPS extract isolated from strain R7AΔndvB was included in chemical analysis as the strain is disrupted in CBG synthesis. Although predominantly localised in the periplasmic compartment, CBG is also found in the extracellular medium of rhizobial cultures (Breedveld & Miller, 1994). LMW EPS extracted from R7AΔndvB would therefore be free of CBG contamination. The only difference observed between R7AΔndvB and R7A EPS extracts was the complete absence of 2-Glc in R7AΔndvB LMW fractions. CBG produced by rhizobia have largely been found to consist solely of 2-Glc often with non-glycosidic substituents (Breedveld & Miller, 1994), the exception being Bradyrhizobium strains which have been reported to produce CBG that contain 1,3-Glc and 1,6-Glc residues (Choma & Komaniecka, 2011). Characterisation of M. loti determined that both the neutral and anionic CBG fractions produced consist solely of 2-Glc, with glycerophosphoryl and succinyl substituents present in the anionic fraction (Kawaharada et al., 2008). The absence of 2-Glc in R7AΔndvB LMW extract and its presence in all other LMW fractions examined indicates that the LMW fractions from all other strains contain ~25% CBG.

As expected based on their non-mucoid colony morphology, EPS extracts from both R7AexoB and R7AexoU consisted of only a LMW fraction. A water-insoluble component observed in the ethanol extracts of R7AexoB and R7AexoU was found by composition analysis not to be polysaccharide-related and therefore was most likely a contaminant from the EPS extraction process. The LMW fractions from R7AexoB
and R7AexoU differed from that of R7A. Both the mutant strains produced a LMW fraction that consisted solely of Glc with Gal and uronic acid groups not detected. Glycosyl linkage data indicated that R7AexoU LMW EPS contained elevated levels of t-Glc and reduced levels of 4-Glc compared to R7A and a complete absence of the 4,6-Glc branching residue linkage observed in the LMW extracts of other strains investigated including R7AexoB. In *S. meliloti* ExoU is responsible for the addition of the sixth sugar residue (1,6-Glc) to the EPS backbone (Reuber & Walker, 1993). *S. meliloti* exoU mutants do not exhibit a fluorescent halo around colony growth on agar containing Calcofluor however they reportedly produce a small amount of Calcofluor-binding material but not enough to be recovered from culture supernatants for NMR analysis (Glucksmann et al., 1993b). The linkage data obtained in this study suggest that ExoU was involved in the addition of a 1,6-Glc residue to the branching chain during R7A EPS biosynthesis. The fluorescent halo observed around R7AexoU colonies on G/ RDM containing Calcofluor (Figure 3.3F) suggests that truncated EPS produced by R7A is exported, perhaps indicating differences in transport of the truncated EPS molecule between R7A and *S. meliloti*.

ExoB is involved in the initial stages of EPS biosynthesis and carries out the conversion of UDP-Glc to UDP-Gal (Buendia et al., 1991). Linkage analysis of R7AexoB extract revealed a reduction in 4-Glc and an increase in t-Glc compared to R7A. Overall, the chemical analysis described in this chapter demonstrated that R7AexoB and R7AexoU differ not only from R7A but also from each other in EPS production. Defining whether the LMW fractions of R7AexoU and R7AexoB represent truncated wild-type EPS or alternative polysaccharides requires further chemical analysis.

Based on the composition and linkage results described in this chapter along with subsequent NMR analysis performed by Dr Artur Muszynski, a proposed structure for R7A EPS was determined. R7A produces an O-acetylated acidic EPS that consists of Glc, Gal, GlcA and RibA (Figure 4.4A). NMR analysis indicated a high degree of acetylation; however the position of the O-acetyl groups on the EPS backbone are yet to be determined. The proposed R7A EPS structure is similar to that reported for *S. meliloti* strain IFO13336 and *M. huakuii* bv. My6 (Amemura et al., 1981; Hisamatsu et
The EPS structures differ with R7A EPS containing t-ß-RibA compared to the t-α-RibA of S. meliloti strain IFO13336 and M. huakuii bv. My6 EPS.

The biosynthetic pathway of the EPS I (succinoglycan) produced by S. meliloti has been extensively characterised (Section 1.6.1.1). An R7A EPS biosynthesis pathway is proposed based on comparison of the EPS structures and the exo gene clusters identified in R7A, S. meliloti and Rhizobium sp. NGR234 (Figure 4.4). The three strains share the EPS biosynthesis genes required up to and including the step catalysed by ExoU, the formation of the hexasaccharide [Gal-(Glc)_5] after which species-specific modifications occur. The proposed R7A EPS structure correlates well with the EPS biosynthesis genes identified in the strain (Kelly, 2007).

Chemical analysis confirmed that R7A EPS does not contain succinyl and pyruvyl substituents as expected based on the absence of pyruvyltransferase (exoV) and succinyltransferase (exoH) homologues in MAFF303099 or R7A. The presence of a UDP-glucose dehydrogenase (mlr5265) and several acetyltransferase (exoZ) homologues indicated that R7A EPS may contain GlcA residues and harbour O-acetyl substituent groups, both of which were confirmed through chemical analysis. The glycosyltransferase involved in the addition of UDP-GlcA to R7A EPS is unknown. In R. leguminosarum PssC, PssD and PssE are the glycosyltransferases required for the addition of two GlcA residues (Williams et al., 2008). PssC, PssD and PssE homologues were not identified in MAFF303099 or Rhizobium sp. NGR234, which also contains GlcA in its EPS, indicating that other glycosyltransferases are capable of performing the action. Further analysis of the R7A/MAFF303099 exo gene cluster identified a glycosyltransferase of unknown function encoded by mll5268, the gene immediately downstream of mll5266-mll5267. Homologues of Mll5268 are present in other mesorhizobia and Rhizobium sp. NGR234 but not in other rhizobia, suggesting that it may be involved in the addition of GlcA to the EPS molecule.

Gene(s) responsible for the presence of RibA in R7A EPS remain unidentified with no clear candidates for the synthesis or incorporation of RibA in the EPS molecule clustered with the known EPS biosynthesis genes. Strain R7Amlr5266 produces slightly mucoid colonies clearly distinguishable from those of R7A, indicating that Mr5266 is involved in R7A EPS biosynthesis although its role has not been determined. Bioinformatic analysis suggests Mr5266 is an
oxidoreductase/dehydrogenase and based on the phenotype of R7Amlr5266 and the location of mlr5266 in the exo cluster it may be involved in the incorporation of RibA in R7A EPS; however, this has not been experimentally demonstrated.

A) *M. loti* R7A EPS

B) *S. meliloti* EPS I (succinoglycan)

C) *Rhizobium* sp. NGR234 EPS

Figure 4.4: Proposed R7A EPS biosynthesis and comparison to *S. meliloti* EPS I and *Rhizobium* sp. NGR234 EPS.

A) R7A produces an O-acetylated acidic EPS that consists of Glc, Gal, GlcA and RibA. O-acetyl group positions on the EPS molecule are yet to be determined. Proposed involvement of the identified R7A gene products in the generation of nucleotide sugar precursors and the biosynthesis of R7A EPS are indicated. The activity of the proteins in R7A EPS biosynthesis were assigned based on comparisons to the known EPS structures of B) *S. meliloti* and C) *Rhizobium* sp. NGR234 and their homologous genes for which the functions have been determined.
Preliminary analysis of EPS extracts from *M. loti* strains MAFF303099 and NZP2037 was also performed. The results of glycosyl composition and neutral sugar linkage determination indicated that their HMW EPS fractions were similar to that of R7A. However, further linkage analysis is required to confirm that uronic acid linkages are also the same as those of R7A. The EPS produced by MAFF303099 has not previously been investigated. However, the EPS produced by NZP2037 was partially characterised by (Hotter & Scott, 1991) and their results correlate well with the results obtained in this study. Hotter and Scott’s characterisation of NZP2307 EPS included paper chromatography of acid hydrolysates of EPS from NZP2037. This identified the presence of spots that co-migrating with Glc, Gal, GlcA and a spot that co-migrated with Ribose, suggesting that the EPS contained these glycosyl residues. Similar results were obtained in this study through glycosyl composition analysis of NZP2037 EPS performed by GC-MS of TMS-derivatives. Furthermore, NMR analysis performed by Hotter and Scott indicated that NZP2037 contained O-acetyl groups but lacked succinyl and pyruvyl groups. NMR analysis of R7A EPS has similarly indicated the presence of O-acetyl groups only (Artur Muszynski, personal communication).
5 Investigation R7AexoB and R7AexoU EPS mutants with contrasting symbiotic proficiencies
5.1 Introduction
The symbiotic ability of the R7A EPS mutant strains varied, ranging from proficient through to severely impaired (Section 3.2.1.1). A correlation may exist between symbiotic proficiency and the stage of EPS biosynthesis disrupted. R7A EPS mutant strains that produce no EPS (e.g. R7AexoB and R7AΔexoA) due to mutations at the beginning of EPS biosynthesis are able to form nodules. This could be due to failure of the host to recognise the strain due to the lack of EPS, or to signalling by another polysaccharide uncovered by the lack of EPS functionally complementing in the absence of EPS. Strains that are disrupted mid-way through EPS biosynthesis (e.g. R7AexoU, R7AexoO and R7Amlr5265) are symbiotically impaired. These strains may produce truncated forms of EPS that are recognised as incompatible by the host and induce plant responses that inhibit the symbiotic process. Strain R7AexoU3’ appears to produce truncated EPS similar to R7AexoU as evidenced by the strain’s colony properties and the fluorescent halo observed around colonies grown on G/ RDM containing Calcofluor. However, the symbiotic proficiency of R7AexoU3’ is likely due to the small amount of full-length EPS that chemical analysis confirmed was produced by the strain (Chapter 4).

This chapter further investigates the R7AexoB and R7AexoU strains with the aim of determining why the exoU mutant is symbiotically impaired whereas the exoB mutant is symbiotically proficient.

5.2 Results
5.2.1 Nodulation competition assays
R7AexoB is symbiotically proficient as a sole inoculum. To investigate if the mutant would be able to compete with R7A as a co-inoculant, nodulation competition assays were performed.

*L. corniculatus* seedlings were inoculated with a 1:1 mixed inoculum containing cells of R7A and R7AexoB (~1000 cells of each). The 1:1 ratio of the inoculum was verified by plating dilutions onto G/ RDM and counting the number of mucoid (R7A) and non-mucoid (R7AexoB) colonies. Twenty nodules were harvested from the co-inoculated plants 28 dpi and nodule crushes were performed (Section 2.10.6), with dilutions of the nodule exudate plated onto G/ RDM and G/ RDM containing Nm. Exudate from 13/20 nodules gave mucoid colonies on G/ RDM plates and no growth
on G/ RDM containing Nm, indicating the nodules contained R7A only. Only non-mucoid colonies were observed on both G/ RDM and G/ RDM containing Nm plates for 5/ 20 nodule exudates, indicating the nodules contained R7AexoB only. The two remaining nodules gave rise to both mucoid and non-mucoid colonies, indicating the nodules harboured a mix of both R7A and R7AexoB at an average ratio of ~100:1 R7A to R7AexoB. These results suggest that the R7AexoB mutant was only slightly impaired in competitive ability despite its complete lack of EPS.

5.2.2 Isolation and characterisation of an R7A exoB exoU double mutant

Although R7AexoB forms non-mucoid colonies on G/ RDM plates, the possibility existed that the strain was able to produce small quantities of wild-type EPS that accounted for its nodulation proficiency, as the MAFF303099 genome contains five genes annotated as encoding UDP-glucose 4-epimerase (mlr1234, mlr5697, mlr6767, mll7878 and mlr8551). R7AexoB was isolated from a transposon mutagenesis screen and is disrupted in the mll5697 homologue.

To investigate if the symbiotic proficiency of R7AexoB was in fact the result of the production of wild-type EPS, an exoB exoU double mutant strain was constructed. If the double mutant exhibited R7AexoB phenotypes, it would rule out the possibility that wild-type EPS production was responsible for the strain’s symbiotic phenotype.

5.2.2.1 Assembly of an exoU IDM construct

Primers UpFUS2L and UpFUS2R were used to amplify 394 bp of internal exoU coding sequence from R7A chromosomal DNA, with BamHI and HindIII restriction enzyme sites incorporated into either primer respectively. Purified PCR products were cloned into the suicide vector pFUS2 to form pSKUPF. A plasmid isolated from *E. coli* S17-1/λpir cells transformed with pSKUPF was confirmed by restriction analysis and sequencing.

Spot-matings were performed between S17-1/λpir/ pSKUPF and R7AexoB or R7A. Four rounds of single-colony purifications were carried out to purify putative mutant clones. Because R7AexoB is non-mucoid no change in morphology was evident in putative R7AexoB exoU::pFUS2 mutants; however, the appearance of non-mucoid colonies in the R7A background indicated that the introduction of pSKUPF had been successful.
5.2.2.2 Confirmation of R7AexoBexoU and R7AexoUPF

Clones representing five putative R7AexoB exoU::pFUS2 mutants and five putative R7A exoU::pFUS2 mutants were characterised by Southern hybridisation (Section 2.6.6) of ClaI-digested genomic DNA using the exoU 394-bp PCR product as a probe (Figure 5.1). A wild-type band of 2397 bp was expected in R7A and R7AexoB parent strains. Because pFUS2 contains one ClaI site, two bands of 2.2 kb and 7.7 kb were expected in mutant clones in which IDM had been successful. Representative clones that exhibited the expected banding in the R7AexoB and R7A backgrounds were designated as R7AexoBexoU and R7AexoUPF respectively.

![Image of Southern hybridisation](image.png)

**Figure 5.1: Confirmation of R7AexoUPF and R7AexoBexoU.**

Southern hybridisation of ClaI-digested genomic DNA probed with the 394-bp exoU PCR product. DNA size marker (M) is λ DNA digested with HindIII, with the size of the fragments indicated in kb. Lanes 1,7: R7A; lanes 2-6: R7A exoU::pFUS2 clones; lanes 8-12: R7AexoB exoU::pFUS2 clones. * Denotes isolates designated as R7AexoUPF (lane 2) and R7AexoBexoU (lane 10).
5.2.2.3 Characterisation of R7AexoBexoU and R7AexoUPF

R7AexoBexoU exhibited growth characteristics indistinguishable to those of R7AexoB. It did not form cell aggregates in G/ RDM broth and displayed R7AexoB-like properties on Congo red and Calcofluor media (Figure 5.2). It also formed nodules on L. corniculatus at rates comparable to control plants inoculated with R7A or R7AexoB (Figure 5.3). In contrast, R7AexoUPF showed the same growth and symbiotic properties as R7AexoU. These results rule out the possibility that the symbiotic proficiency of R7AexoB was due to the production of wild-type EPS.

![Image](image.png)

**Figure 5.2: Colony characteristics of R7AexoBexoU.**

TY cultures grown for 48 h were spotted (10 µL) onto media. A) Colony appearance after 48 h growth on YMB containing 0.005% Congo red and B) Fluorescence on TY containing 0.02% Calcofluor, viewed under UV light.

![Image](image.png)

**Figure 5.3: Nodulation of L. corniculatus by R7AexoBexoU and R7AexoUPF.**

Total number of nitrogen-fixing nodules formed on 10 L. corniculatus plants inoculated with the indicated strains. Results represent three independent nodulation assays with the standard error of the mean indicated.
5.2.3 Analysis of R7AexoB LPS

ExoB is a UDP-glucose 4-epimerase required for the generation of UDP-Gal, R7AexoB was therefore expected to be altered in all Gal-containing polysaccharides. Previous analysis of R7AexoB LPS did not reveal any differences from R7A (Kelly, 2007) (Figure 5.4A). However, recent detailed analysis of LPS by Dr Artur Muszynski at the CCRC indicated that R7AexoB does produce an altered LPS. Silver-stained deoxycholate polyacrylamide gel electrophoresis (DOC-PAGE) analysis of LPS extracts indicated a slight change in the lipid A/core region of R7AexoB LPS compared to R7A and R7AexoU. The R7AexoB LPS contained a slightly faster-migrating band in the lipid A/core region of the LPS (Figure 5.4B). Composition analysis of the LPS extracts by GC-MS of TMS-derivatised samples revealed that R7AexoB LPS lacked Gal compared to R7A (Figure 5.5) and R7AexoU LPS (not shown).

Figure 5.4: Silver-stained LPS isolated from R7A, R7AexoB and R7AexoU.

A) Previous DOC-PAGE of silver-stained LPS did not reveal any differences in LPS production by R7AexoB (Kelly, 2007) lane 1 R7A; lane 2 R7AexoB; lane 3 R7AexoU.

B) DOC-PAGE of silver-stained LPS recovered from the water phase by Dr Artur Muszynski revealed a faster-migrating band in R7AexoB LPS is indicated by the arrow. S, Salmonella minnesota; lane 1 R7A; lane 2 R7AexoB; lane 3 R7AexoU.
Figure 5.5: Composition of R7A and R7AexoB LPS

TMS-derivatised LPS samples were analysed by GC-MS to determine the composition. R7AexoB lacked a Gal peak, as indicated by the arrow. Rha – rhamnose; Sug - unidentified sugar; GalA - galacturonic acid; Man – mannose; Gal – galactose; Glc – glucose; Hep – heptose; Kdo - 3-Deoxy-D-manno-oct-2-ulosonic acid; Ino – inositol; C20:0 and C22:1 - fatty acids.
5.2.4 Symbiotic complementation of R7AexoU by a Nod factor mutant strain

It had been established that R7AexoU was impaired in nodulation due to an inability to form functional ITs (Kelly, 2007). To investigate if this symbiotic deficiency could be functionally complemented by an R7A strain that produced wild-type EPS yet itself was symbiotically impaired due to an inability to produce Nod factor (R7AΔnodA), co-inoculation assays with fluorescently-labelled strains were performed.

5.2.4.1 Assembly of GPF and DsRed reporter plasmids

Reporter plasmids constitutively expressing the fluorescent proteins GFP and DsRed that would be compatible with R7A and various mutant strains were constructed. GFP and DsRed were chosen as reporter genes because their differing excitation wavelengths allow for visualisation of each independently.

GFP expressed from the nptII promoter (PnptII) was PCR-amplified as a fragment of ~1.1 kb from pPR3 (Rodpothong, 2008) using primers nptII1 and PnptIgfpR1 that incorporated HindIII and EcoRV restriction sites respectively. The PCR fragment was cloned into pFAJ1700 that had been digested with HindIII and HpaI to form pSKGFP. *E. coli* S17-1/λpir was transformed with pSKGFP and the plasmid from one clone was confirmed by restriction analysis and sequencing. GFP expression was confirmed by visualisation of bright green cells by epifluorescent microscopy.

Primers 1708DsRedL and 1708DsRedR were used to amplify the dsred gene from pDSRED with BamHI and EcoRI restriction sites incorporated respectively. Primer 1708DsRedL incorporated the ribosome binding site of a strongly expressed R7A gene (msi158) to ensure strong expression of the gene in R7A strains. The plasmid pFAJ1708 encodes PnptII upstream of the MCS so the 714-bp DsRed PCR product was cloned into the MCS of pFAJ1708 as a BamHI/EcoRI fragment to form pSKDSRED and the plasmid from one *E. coli* S17-1/λpir clone confirmed by restriction analysis and sequencing. Strong DsRed expression by the S17-1/λpir clone was confirmed through the visualisation of bright red cells by epifluorescent microscopy.
5.2.4.2 Co-inoculation with R7AexoU and R7AΔnodA reporter-tagged strains

The GFP and DsRed expressing constructs were introduced into R7A, R7AexoU and R7AΔnodA to form reporter strains that could be differentiated in co-inoculation experiments. *L. japonicus* Gifu seedlings inoculated with either R7AexoU or R7AΔnodA reporter strains or a 1:1 mix (~1000 cells each) of the two were examined 7-21 dpi by epifluorescent and confocal microscopy. Control plants inoculated with R7A transformed with the reporter plasmids were included in all assays. As a sole inoculum, R7AΔnodA failed to induce any plant responses. Seedlings inoculated with R7AexoU alone displayed root hair deformation and the establishment of nodule primordia. Infection focal points of R7AexoU cells were readily observed at deformed root hair tips although no ITs developed (Figure 5.6). As expected, at 21 dpi no nodules had developed on any seedling inoculated with either the Nod factor- or EPS-deficient strain alone. In contrast, seedlings inoculated with a 1:1 mix of the two strains formed effective nodules with a slight delay compared to R7A-inoculated control plants (Figure 5.7).
Figure 5.6: R7AexoU infection focal points.

As a sole inoculum of *L. japonicus* Gifu, R7AexoU formed infection focal points in curled root hair tips however; ITs did not develop.

![Image](image.jpg)

Figure 5.7: Nodulation of *L. japonicus* Gifu co-inoculated with R7AexoU and R7AΔnodA.

Total number of nitrogen-fixing nodules formed on 10 *L. japonicus* Gifu plants inoculated with individual strains or a 1:1 mix of R7AexoU and R7AΔnodA.
The dynamics of IT development and nodule occupancy on co-inoculated *L. japonicus* Gifu were examined. Plants were inoculated with 1:1 mixed inocula of R7AΔnodA and R7AexoU harbouring the different combinations of reporter plasmids to rule out any reporter gene bias. Over 100 ITs and 10 nodules were examined on plants inoculated with each mixture to determine the occupying strain(s) (Table 5.1). On control plants inoculated with a 1:1 mix of R7A containing the different reporter constructs, ~50% of the IT were formed by each reporter strain with only one mixed IT observed. Of the nodules that formed on control plants, 90% harboured only one of the reporter strains. On R7AΔnodA/ R7AexoU inoculated plants, ~7 extended ITs were readily observed per plant root. Of these, 80% appeared to contain R7AexoU alone, 10% R7AΔnodA alone and 10% were mixed (Figure 5.8). Dual nodule occupancy was observed in 80% of the nodules that formed on R7AΔnodA/ R7AexoU inoculated plants, with individual plant cells containing either one or both strains (Figure 5.9).

**Table 5.1: IT and nodule occupancy of co-inoculated *L. japonicus* Gifu.**

<table>
<thead>
<tr>
<th>Mixed inocula (1:1)</th>
<th>Cells within IT*</th>
<th>Cells within nodule*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Green</td>
<td>Red</td>
</tr>
<tr>
<td>R7A/pSKGFP and R7A/pSKDSRED</td>
<td>94</td>
<td>100</td>
</tr>
<tr>
<td>R7AΔnodA/pSKGFP and R7AexoU/pSKDSRED</td>
<td>15</td>
<td>75</td>
</tr>
<tr>
<td>R7AexoU/pSKGFP and R7AΔnodA/pSKDSRED</td>
<td>92</td>
<td>13</td>
</tr>
</tbody>
</table>

* Each IT and nodule formed on co-inoculated plants was determined by epifluorescent microscopy to harbour either only GFP-fluorescent bacteria, or only DsRed-fluorescent bacteria or a mix of both GFP- and DsRed-fluorescent bacteria.
Figure 5.8: ITs formed on R7AexoU/R7AΔnodA co-inoculated plants.

Extended ITs were observed that appeared to contain either A) R7AΔnodA or B) R7AexoU alone. C) and D) Examples of mixed IT formed on R7AexoU/ R7AΔnodA co-inoculated plants. The epifluorescent microscopy images show an identical field of view with DsRed or GFP filters followed by a combined overlay image.

Figure 5.9: Dual nodule occupancy of R7AexoU/R7AΔnodA co-inoculated plants.

A), B) and C) Confocal microscopy of mixed nodules formed on R7AexoU/ R7AΔnodA co-inoculated plants.
To examine the ratio of R7AΔnodA to R7AexoU present within the nodules formed on co-inoculated plants, 20 nodules were harvested and dilutions of the exudates were spread onto G/ RDM containing Tc and G/ RDM containing Nm and Tc plates. Only R7AexoU cells were able to grow on the G/ RDM containing Nm and Tc plates but both strains could grow on G/ RDM containing Tc. R7AΔnodA colonies were easily distinguished from those of R7AexoU based on their larger mucoid morphology. The results confirmed what was observed by epifluorescent microscopy with both strains usually present within the nodules. The number of R7AΔnodA cells recovered from mixed nodules outnumbered R7AexoU cells by an average of 17:1. One nodule was found to harbour only R7AexoU and is likely to have been an ‘escape’ nodule as occasionally found on R7AexoU-inoculated plants.

5.2.5 Complementation of R7AexoU symbiotic impairment with R7A EPS

Co-inoculation assays indicated that R7AexoU was capable of forming functional ITs in the presence of wild-type EPS provided in trans by R7AΔnodA. This led to nodulation assays to investigate if the symbiotic impairment of R7AexoU could be overcome by the addition of wild-type R7A EPS.

*L. japonicus* Gifu seedlings were inoculated with R7AexoU and varying concentrations of wild-type EPS extracted from R7A cultures (Section 2.11.1). The R7A EPS had been partially purified and separated into HMW and LMW fractions by Sephacryl S-400 gel permeation chromatography (Section 2.11.2). Each plant was inoculated with 20 µL of OD₆₀₀ = 0.1 R7AexoU suspensions and 20 µL of EPS preparations that ranged in concentrations from 0.0625 µg/ mL to 100 µg/ mL. Some plants were inoculated with only the HMW or LMW EPS fractions and others with a mixture of both fractions. Plants were observed periodically over six weeks for nodule formation. Regardless of the EPS fraction or concentration, none of the R7AexoU/ EPS-inoculated plants formed any nitrogen-fixing nodules and all exhibited small uninfected bumps identical to those formed on control R7AexoU-inoculated plants. Plants that had been inoculated solely with EPS preparations showed no signs of symbiotic responses and appeared indistinguishable from uninoculated plants.
5.2.5.1 R7AexoU IT formation in the presence of R7A EPS

Although attempts at complementing nitrogen-fixing nodule formation by R7AexoU in the presence of R7A EPS had been unsuccessful, further plant assays were set up to investigate if the addition of R7A EPS had an effect on IT formation by R7AexoU.

*L. japonicus* Gifu seedlings were inoculated with R7AexoU/pSKGFP and purified R7A LMW EPS (5 µg/mL). Roots from 20 plants were observed by epifluorescent microscopy to follow progression of R7AexoU during the infection process in the presence of R7A EPS. Infection focal points within curled root-hairs were frequently observed (Figure 5.10A) and one IT was identified that appeared to extend the length of the root hair (Figure 5.10B).

![Figure 5.10: R7AexoU IT formation in the presence of R7A LMW EPS.](image)

*L. japonicus* Gifu plants co-inoculated with R7AexoU/pSKGFP and R7A LMW EPS (5 µg/mL) were examined by epifluorescent microscopy 14 dpi. A) Infection focal points within curled root-hairs were readily observed. B) One extended IT was observed.
5.2.6 Effects of R7AexoU pre-inoculation on symbiotic proficiency

Due to its lack of full-length EPS, R7AexoU may be recognised as foe by host plants and as a result induce plant defence responses that prevent IT development. To investigate this possibility, co-inoculation assays were performed to determine if pre-inoculation with R7AexoU would adversely affect nodulation by symbiotically-proficient strains.

*L. japonicus* Gifu seedlings were pre-inoculated with R7AexoU before inoculation with symbiotically-proficient strains R7A or R7AexoB after various time intervals (15 min, 1 h, 24 h and 72 h later) and plants were periodically observed for nodule formation. Pre-inoculation with R7AexoU did not affect nodulation by either R7A or R7AexoB when inoculated up to 24 h later. However, plants inoculated with R7A 72 h after inoculation with R7AexoU exhibited a delay in the onset of nodulation and formed fewer nitrogen-fixing nodules compared to control plants inoculated only with R7A. The inhibitory effect was more pronounced on plants inoculated with R7AexoB 72 h after inoculation with R7AexoU. The formation of nitrogen-fixing nodules was severely delayed with only a few nodules developing throughout the course of the assay (Figure 5.11).

To investigate if R7AexoU LMW EPS alone could cause the inhibitory effects, seedlings were inoculated with R7AexoU EPS (5 µg/mL) prior to inoculation with R7A or R7AexoB 72 h later. Symbiotic inhibition was not observed, with nodules forming at comparable rates to control plants that had not been pre-inoculated with the R7AexoU EPS.

To determine if the inhibitory effects of pre-inoculation were specific to R7AexoU or could be caused by other symbiotically impaired R7A strains, similar pre-inoculation assays were performed using R7AΔndvB and R7AΔnodA mutants. R7AΔndvB and R7AΔnodA are severely impaired in nodulation due to deficiencies in CBG and Nod factor production respectively. Pre-inoculation with either of these strains followed by inoculation with R7A or R7AexoB 72 h later had no effect on the onset of nodulation or the number of nitrogen-fixing nodules formed (Figure 5.11).
A) Total number of nitrogen-fixing nodules formed on 10 *L. japonicus* Gifu plants inoculated with the indicated strain(s). For co-inoculated plants, the second strain was inoculated 72 h after the initial inoculum. B) Plant appearance 6 weeks after inoculation with R7AexoB alone. C) Plant appearance 6 weeks after initial inoculation with R7AexoU followed by R7AexoB 72 h later.

**Figure 5.11: Effect of pre-inoculation with R7AexoU on symbiotically-proficient strains.**
5.2.6.1 Split-root experiments to investigate R7AexoU pre-inoculation

The severe inhibitory effect that pre-inoculation of *L. japonicus* Gifu with R7AexoU had on the symbiotic ability of R7AexoB was further investigated on split-root plants. The split-root system was used to investigate if R7AexoU was inducing systemic plant defence responses that prevented nodulation by the usually proficient R7AexoB strain.

Split-root seedlings were prepared as described in Section 2.10.2. On each split-root plant one root was inoculated with R7AexoU then 72 h later the other was inoculated with R7AexoB. Plants formed only small white uninfected bumps on the root inoculated with R7AexoU but developed nitrogen-fixing nodules on the root inoculated with R7AexoB (Figure 5.12).

![Figure 5.12: Effect of R7AexoU pre-inoculation on R7AexoB nodulation on split-root plant.](image)

*L. japonicus* Gifu split-root plant initially inoculated with R7AexoU on the left root followed by inoculation with R7AexoB on the right root 72 h later.
5.2.7  Specificity of R7AexoU symbiotic proficiency

R7AexoU was found to be severely impaired in the nodulation of *L. japonicus* Gifu and *L. corniculatus*. To investigate possible host- and environment-specificity, the symbiotic proficiency of R7AexoU on additional hosts and in varied root-lighting conditions was examined.

Nodulation of *L. japonicus* Gifu, *L. japonicus* MG-20 and *L. burttii* was investigated under differing root light-exposure conditions during plant growth (Section 2.10.4). R7A formed nodules on all hosts at comparable rates regardless of whether the roots were exposed to light or kept in the dark. *L. japonicus* Gifu and *L. burttii* inoculated with R7AexoU failed to form nodules on plants with roots grown in the dark or light. Interestingly, R7AexoU formed effective nodules on *L. japonicus* MG-20 plant roots that were kept in the dark whilst only forming ineffective nodule primordia and very few late-arising nodules on *L. japonicus* MG-20 plant grown with their roots exposed to light (Figure 5.13).

![Graph](image)

**Figure 5.13:** R7AexoU nodulation of *L. japonicus* MG-20 under differing root lighting growth conditions.

Total number of nitrogen-fixing nodules formed on 10 plants of *L. japonicus* MG-20 inoculated with R7A or R7AexoU and grown with roots exposed to the light or kept in the dark.
To further examine differences in symbiosis between R7AexoU and *L. japonicus* Gifu compared to *L. japonicus* MG-20, seedlings grown with roots in the dark or exposed to light were inoculated with reporter strains R7A/ pSKGFP and R7AexoU/ pSKGFP. Epifluorescent microscopy 21 dpi revealed that R7A formed fully developed ITs on both hosts at similar rates regardless of the root lighting conditions. As expected, only infection focal points were observed on R7AexoU-inoculated *L. japonicus* Gifu roots exposed to either lighting condition. Interestingly, R7AexoU formed a similar number of extended ITs (~3/ root) on *L. japonicus* MG-20 plants with roots grown in either lighting condition. However, only those on roots grown in the dark appeared to release R7AexoU into the nodule primordia and form infected nitrogen-fixing nodules. The extended ITs observed on roots grown in the light appeared impaired at releasing R7AexoU from the base of the IT and as a result nodule primordia exhibited only slight infection (Figure 5.14).
Figure 5.14: R7AexoU IT formation on *L. japonicus* MG-20.

Epifluorescent microscopy of R7AexoU/ pSKGFP-inoculated *L. japonicus* MG-20 roots grown exposed to light or kept in the dark. A), C) Examples of IT formed by R7AexoU on roots exposed to the light. B), D) Examples of IT formed by R7AexoU on roots kept in the dark. E) A nodule formed on a plant grown with roots exposed to the light exhibiting slight infection with R7AexoU. F) Highly infected nodules characteristic of those formed by R7AexoU on plants grown with roots kept in the dark.
5.3 Discussion

This chapter described the investigation of two ‘classes’ of EPS-deficient R7A strains through examination of the symbiotically proficient R7AexoB and severely impaired R7AexoU. R7AexoB and R7AexoU exhibit contrasting colony morphologies (Section 3.2.1.2) and chemical analyses revealed differences in their LMW EPS extract as described in Chapter 4.

Given that exoB is involved at the precursor stages of EPS biosynthesis (the generation of UDP-galactose) R7AexoB would be expected to produce no form of EPS, yet its symbiotic proficiency is comparable to that of R7A. Competition assays performed between R7A and R7AexoB indicated that R7AexoB was slightly less competitive for nodulation than R7A but it nevertheless formed 25% of the nodules when co-inoculated with R7A, with a further 10% of nodules containing both strains. Competition assays performed between an exoB mutant of *B. japonicum* and the wild-type strain revealed that the exoB mutant exhibited greatly reduced competitiveness with almost no nodules occupied by the exoB mutant when soybean was co-inoculated with equal titres of the mutant and wild-type (Parniske et al., 1993). An important difference between the reported *B. japonicum* exoB competitiveness compared to R7AexoB competitiveness observed in this study is that the *B. japonicum* exoB mutant is significantly delayed in nodulation (Parniske et al., 1993) whilst R7AexoB nodulates at rates close to wild-type. The result suggests that R7AexoB is relatively competitive with R7A and raises the question as to what factors are responsible for its symbiotic proficiency.

Although the non-mucoid colony morphology of R7AexoB suggested a lack of wild-type EPS production, the possibility remained that a small amount may be produced by the mutant that accounted for its symbiotic ability. The requirement for only small quantities of EPS to allow nodulation to proceed is supported by the symbiotic ability of R7AexoU3’. Only following prolonged incubation is slight mucoid growth apparent with R7AexoU3’ yet the symbiotic ability of the strain is comparable to R7A (Section 3.2.1). Further to this, it has previously been reported that only small quantities of EPS are required for symbiosis, an observation that is one of the key factors implicating a signalling rather than protective role for EPS (Pellock *et al.*, 2000; Urzainqui & Walker, 1992). The R7AexoBexoU double mutant was constructed to
address the possibility that the symbiotic ability of R7AexoB was due to the production of small amounts of wild-type EPS. Given the symbiotic impairment caused by mutation of *exoU*, introduction of the mutation into an R7AexoB background would be expected to prevent the potential production of any wild-type EPS. R7AexoBexoU exhibited identical colony morphologies and symbiotic proficiency to R7AexoB, ruling out the possibility of R7AexoB symbiotic proficiency being a result of wild-type EPS production.

*S. meliloti* and *R. leguminosarum* *exoB* mutants are impaired in nodulation and are defective not only in EPS production but also in other Gal-containing polysaccharides such as LPS (Canter Cremers *et al.*, 1990; Long *et al.*, 1988; Sanchez-Andujar *et al.*, 1997). Previous analysis of R7AexoB LPS did not reveal any differences from R7A (Kelly, 2007). However, detailed analysis of LPS by Dr Artur Muszynski indicated that R7AexoB does produce an altered LPS that lacks Gal. R7AexoB LPS was altered in the lipid A/ core region of the LPS rather than the O-antigen region. This suggests that Gal is present in the lipid A/ core region of R7A LPS, which is consistent with the structurally defined *M. loti* NZP2213 LPS (Ruswa *et al.*, 1995). In contrast, *R. leguminosarum* *exoB* mutants have been reported to exhibit deficiencies in LPS species containing the O-antigen (Canter Cremers *et al.*, 1990; Sanchez-Andujar *et al.*, 1997). Sachez-Andujar (1997) note that in addition to the absence of O-antigen, LPS produced by an *R. leguminosarum* bv. *trifolii* *exoB* mutant also contains a faster-migrating lipid A/ core region compared to wild-type, similar to that observed with R7AexoB compared to R7A. Based on these results, R7A LPS likely contains Gal in the lipid A/ core region that is not involved in O-antigen attachment.

There are several possible explanations for the symbiotic proficiency of R7AexoB. In *S. meliloti* symbiotic interactions, it has been shown that a lack of EPS I (succinoglycan) production can be functionally complemented by the presence of other rhizobial polysaccharides including a second EPS (EPS II or galactoglucan) and KPS (Glazebrook & Walker, 1989; Gonzalez *et al.*, 1996a; Reuhs *et al.*, 1995). Although homologues of the *S. meliloti* EPS II and KPS genes were not identified in *M. loti* (Kelly, 2007), chemical analysis of R7AexoB extracts indicate the potential presence of a LMW polysaccharide consisting of variously-linked glucose residues as discussed...
in Section 4.2.4.1. One possible explanation for the symbiotic proficiency of R7AexoB is therefore complementary signalling by an alternative polysaccharide.

An alternative explanation for the symbiotic ability of R7AexoB may be that the plant is ‘blind’ to the strain and therefore does not activate a defence response. EPS has been proposed to function in the down-regulation of host plant defence responses (Niehaus et al., 1993; Parniske et al., 1994; Wielbo et al., 2004). Furthermore, *M. truncatula* transcriptome analysis in response to inoculation with *S. meliloti* compared to an EPS mutant suggested that the EPS-deficient strain may be impaired in nodulation due to an inability to suppress plant defence responses through the signalling action of EPS (Jones et al., 2008). R7AexoB would therefore be expected to be impaired in nodulation due to an inability to suppress plant defence responses. However, as the *exoB* mutation caused pleiotropic effects on other Gal-containing polysaccharides including LPS, these altered polysaccharides may not elicit plant defence responses to begin with. In this scenario, it would be LPS that normally induces the defence response and full-length EPS would either shield the LPS or act as a positive signal to prevent induction of the response. Support for this idea is provided by studies that reveal *M. loti* LPS induces nitric oxide (NO) production in *Lotus* roots (Murakami et al., 2011; Nagata et al., 2008). NO is involved in an important signalling molecule in plant innate immunity (Grun et al., 2006). Interestingly, Murakami et al (2011) identified that the lipid A component of *M. loti* LPS was the major component involved in NO induction, which suggests that as R7AexoB LPS is affected in this region it may not be as effective at eliciting a defence response.

An alternative explanation for the symbiotic proficiency of R7AexoB could simply be that the strain avoids eliciting a defence response due to its complete lack of EPS production. In this scenario, EPS intermediates secreted by the other EPS mutants would induce the defence response and impair the symbiotic proficiency of the mutants. This explanation is consistent with the finding that *exoA* mutants were only slightly impaired on *L. corniculatus* and moderately impaired on *L. japonicus*, compared to the more severe phenotypes of other EPS mutants.

R7AexoU was found to be impaired in the symbiotic process at the stage of IT formation. The mutant appeared unaffected in its ability to bind to root hairs and
was readily observed forming infection focal points in curled root-hair tips. However, the initiation of IT development was impaired with only the very occasional irregular IT observed on *L. corniculatus* roots. Analysis of nodules that occasionally formed after a lengthy delay revealed no abnormalities in bacteroid formation, demonstrating that R7AexoU was impaired in gaining nodule entry but could successfully differentiate into bacteroids and fix nitrogen once entry into the nodule was achieved (Kelly, 2007).

Co-inoculation assays performed in this study demonstrated that R7AexoU and R7AΔnodA were able to functionally complement each other’s respective EPS- and Nod factor-deficiencies to facilitate the formation of nitrogen-fixing nodules. This result is comparable to studies performed with EPS- and Nod factor-deficient complementation demonstrated in the *S. meliloti*-alfalfa symbiosis (Kapp et al., 1990; Klein et al., 1988). Examination of the complementation though microscopy of fluorescently-tagged strains during the symbiotic process provided some interesting results. The vast majority of nodules formed on co-inoculated plants harboured both R7AexoU and R7AΔnodA, yet the majority of extended ITs observed contained solely R7AexoU. This implies that the relatively few mixed ITs that formed developed fully and allowed for release of both strains into the nodule. This observation suggests that EPS provided *in trans* by R7AΔnodA was sufficient to allow R7AexoU to initiate IT development and extension, but as few nodules were found to contain solely R7AexoU it would appear that release from the IT into the nodule cells was impaired. This result suggests that R7A EPS is required for both IT initiation and release and provides further evidence that the role of EPS is as a signalling molecule rather than just forming a protective sheath for the bacteria in the IT. The predominance of *exoU* over *nodA* mutants as sole occupants of ITs formed in the co-inoculation experiments also supports the suggestion that Nod factor production is beneficial and possibly necessary throughout IT growth (see reviews by (Downie, 2010; Murray, 2011)). For example, *R. leguminosarum* and *S. meliloti* mutants that produce Nod factors that lack certain decorations can initiate ITs but the ITs generally abort (Ardourel et al., 1994; Walker & Downie, 2000).

Perhaps the most significant evidence for EPS functioning as a signalling molecule is provided from experiments in which an EPS mutant strain’s symbiotic deficiencies
were complemented by the addition of small quantities of LMW EPS purified from the parent strain (Battisti et al., 1992; Djordjevic et al., 1987; Urzainqui & Walker, 1992). In contrast to the complementation results reported by Djordjevic et al., Staehelin et al. were unable to symbiotically complement a *Rhizobium* sp. NGR234 EPS mutant by exogenous addition of EPS under the conditions they used (Staehelin et al., 2006). Analogous experiments performed in this study to complement R7AexoU via the exogenous addition of R7A EPS did not result in the formation of nitrogen-fixing nodules. The potential for enhanced IT initiation and extension by R7AexoU in the presence of R7A EPS was examined, with only one extended IT observed in the presence of R7A EPS. Numerous explanations exist as to why the complementation proved unsuccessful. The inability to complement R7AexoU with exogenous EPS in this study may relate to differences in determinate and indeterminate nodule development, as previous reports of successful complementation were all performed in an indeterminate-nodule-forming symbiosis. Additionally, co-inoculation experiments indicated that EPS may be required for IT release in *M. loti* - *Lotus* symbiosis which would presumably require the presence of EPS at the base of the IT which is unlikely to occur when EPS is added exogenously.

Given the strong symbiotic impairment of R7AexoU, we speculated that truncated EPS produced by the strain may be recognised as incompatible by the host, causing induction of a defence response or failure to effectively dampen defence responses, thus preventing successful nodulation. Pre-inoculation of *L. japonicus* Gifu with R7AexoU inhibited nodulation by symbiotically proficient strains. Interestingly, pre-inoculation with R7AexoU almost completely prevented subsequent nodulation by R7AexoB whereas R7A, after a delay, was able to nodulate. This may be a result of R7A but not R7AexoB being able to signal compatibility through EPS production and down-regulate the defence response induced by pre-inoculation with R7AexoU. The suppression of host defence responses by wild-type EPS is supported by *M. truncatula* transcriptome analysis which indicate an initial defence response elicited 1 hpi with *S. meliloti* is subsequently dampened within 48 hpi by the wild-type strain yet expression of defence-related genes remains high in response to an *S. meliloti* EPS mutant at 72 hpi (Jones et al., 2008; Lohar et al., 2006).
The inhibitory effect of R7AexoU pre-inoculation was found to be specific for R7AexoU in that two other mutants impaired in symbiosis due to Nod factor (R7AΔnodA) or CBG (R7AΔndvB) deficiencies did not affect subsequent nodulation by R7AexoB. This finding supports the proposal that truncated EPS produced by R7AexoU is inducing a plant defence response. R7AexoU had no effect on the nodulation ability of symbiotically proficient strains as long as the symbiotically proficient strain was inoculated earlier than 72 h after R7AexoU inoculation. This suggested that R7AexoU induced systemic acquired resistance in the plant by 72 h and that it was responsible for the impaired nodulation of the symbiotically proficient strains. In an attempt to identify if the response was localised or systemic, split-root plant assays were performed. The formation of nodules by R7AexoB on lateral roots that were not pre-inoculated with R7AexoU suggests that the response was not systemic or perhaps the response invoked systemically is weaker, allowing an alternative R7AexoB signal to overcome it.

The symbiotic proficiency of R7AexoU was found to be both host- and environment-specific. Although severely impaired in nodulation of L. japonicus Gifu and L. burttii, R7AexoU was much less impaired in the nodulation of L. japonicus MG-20, depending on the root lighting conditions. In conditions where the plant was subjected to the environmental stress of roots exposed to light, R7AexoU was less successful in forming nodules whilst R7A nodulation in these conditions was unaffected. One explanation for the observed environmental effect is that R7AexoU was unable to overcome plant responses to these stresses as well as R7A. Investigation of IT formation by R7AexoU on L. japonicus MG-20 under the differing root lighting conditions revealed that the strain was able to initiate and form extended ITs on the host under either condition in comparable numbers. However, only on the plants in which roots were grown in the dark was R7AexoU released from the IT and able to infect the developing nodule. Exposure of legume roots to light has previously been shown to induce the expression of the plant hormone ethylene (Lee & Larue, 1992). Ethylene is a potent plant hormone that affects many plant growth and developmental processes. Basal ethylene levels are low however the hormone is rapidly synthesised in response to external stresses and induces the expression of many stress-associated genes that lead to impairment of nodulation by rhizobia (Shaharoona et al., 2011).
This result provides further support for a requirement for wild-type EPS at the stage of bacterial release from ITs but not for IT elongation. It also suggests that *L. japonicus* MG-20 does not detect R7AexoU as incompatible at the root-hair tip surface.

Examining the differences between *L. japonicus* MG-20 and *L. japonicus* Gifu in their response to R7AexoU should yield further insight into the role of EPS and plant genes in the symbiotic process.

### 5.3.1 R7A EPS acts as a signal to modulate host defence responses.

The following model is proposed for the functioning of R7A EPS in symbiosis as a modulator of host defence responses based on the results obtained in this chapter.

In the wild-type situation, R7A is perceived by a compatible host and an initial defence response is dampened through the signalling action of wild-type EPS, allowing for IT formation and the release of R7A into cells within the nodule primordia, resulting in the establishment of infected nodules. The EPS signal is perceived by the plant both at the stage of IT initiation and the stage of bacterial release from the IT. This second check would allow the plant to detect other bacteria that may have entered through the IT.

R7AexoB is able to effectively nodulate due to either (i) complementary signalling by an alternative polysaccharide in the absence of any form of EPS (due to the stage of ExoB action at the beginning of EPS biosynthesis); or (ii) the absence of any surface molecule that the plant normally perceives to activate a defence response. Competition assays between R7A and R7AexoB and the stronger inhibitory effect of pre-inoculation of R7AexoU on subsequent nodulation by R7AexoB compared to R7A favours the second explanation, although the possibility that a complementary signal produced by R7AexoB is not as effective as wild-type EPS cannot be discounted. For example, in *S. meliloti* EPS II and KPS are capable of complementing EPS I-deficient mutants but with reduced efficiency (Pellock *et al.*, 2000). There may also be host differences as shown by the different responses of *L. japonicus* Gifu and *L. corniculatus* to R7AΔexoA. It is likely that R7AΔexoA has normal LPS whereas R7AexoB LPS lacks a Gal residue and it is possible that this difference is the cause of the different host-specific responses.
R7AexoU is impaired due to the production of a truncated EPS molecule produced as a result of the lack of activity of ExoU midway through EPS biosynthesis. The truncated EPS either does not signal compatibility to the host or is actively perceived by the plant resulting in the activation of the defence response; in either case, the defence responses initially activated are unable to be dampened. The production of truncated EPS may prevent complementary signalling by an alternative polysaccharide that is active in the R7AexoB background. The host defence response prevents IT development by R7AexoU. Co-inoculation and pre-inoculation results suggest that the response elicited by R7AexoU can be dampened by the wild-type EPS molecule and allow nodulation to proceed. This is strongly supported by the symbiotic proficiency of R7AexoU3' that produces low amounts of wild-type EPS while still producing the truncated EPS, as visualised as a halo surrounding the colony on G/RDM containing Calcofluor plates. The cause of the symbiotic impairment of R7A exoU is investigated further in the next chapter.
6 Isolation of R7AexoU nodulation phenotype suppressor mutants
6.1 Introduction

R7AexoU exhibits severe nodulation deficiencies on various Lotus species. Microscopy revealed that R7AexoU was unable to establish ITs on L. corniculatus or L. japonicus Gifu that are required for entry into the nodule primordia. Chemical analysis of extracts from R7AexoU revealed the strain produces only a LMW EPS fraction which may consist of a truncated form of EPS as discussed in Chapter 4. This truncated EPS molecule may signal incompatibility to the host and induce a plant defence response. Alternatively, the symbiotic impairment of R7AexoU may be attributed to the absence of full-length HMW EPS, resulting in the exposure of other cell surface components normally ‘hidden’ from the plant in EPS-producing strains. In R7AexoU these cell surface components may be available to be recognised by the plant and therefore could induce plant responses inhibiting nodulation.

To gain further insight into the basis for R7AexoU symbiotic deficiencies, an mTn5 mutant library of R7AexoU was constructed and screened for suppressor mutants that were symbiotically proficient on L. corniculatus. Isolation of symbiotically-proficient R7AexoU/ mTn5 clones was expected, as previous work had shown that an exoB exoU double mutant formed effective nodules on L. corniculatus (Section 5.2.2.3). Similarly it was expected that exoU exoA double mutants would be isolated, as R7AΔexoA nodulated L. corniculatus effectively (Section 3.2.2.1). However, if double mutants that suppressed the R7AexoU symbiotic phenotype and harboured mTn5 insertions in genes not involved in EPS biosynthesis could be isolated, the screen might reveal other bacterial components involved in the R7AexoU symbiotic phenotype.

6.2 Results

6.2.1 Construction of an in-frame markerless deletion R7A exoU mutant

The original R7AexoU mutant was isolated from a transposon mutagenesis screen (Hubber, 2005) and therefore was unsuitable for use in further transposon mutagenesis. In order to construct an R7AexoU/ mTn5 double mutant library, it was necessary to first isolate a markerless deletion R7A exoU mutant that could then be subjected to mTn5 mutagenesis.
6.2.1.1 R7AΔexoU mutagenesis construct

An overlap extension PCR approach (Section 2.7.2.1) was employed to generate a ~2 kb in-frame exoU mutant product using primer sets exoULL/ exoULR and exoURL/ exoURR that incorporated SpeI sites at the outer ends. Digested PCR product was cloned into the suicide vector pJQ200SK to form pSKMU which was confirmed by restriction analysis and sequencing.

6.2.1.2 Isolation of R7AΔexoU

Spot matings between R7A and S17-1/ λpir/ pSKMU were performed and the markerless deletion process was carried out as described in Section 2.7.2.2, and clones with a non-mucoid phenotype selected. Primer set exoUcheckL/ exoUcheckR that amplifies across the joining site of the left and right arms of the ~2 kb fragment was used to confirm the mutation. Genomic DNA extracted from DXO clones was used as template DNA. If the second-crossover event occurred as desired resulting in the markerless deletion, only a 562-bp product should be amplified. However, if the second crossover resulted in the clone reverting to wild-type, a 1405-bp product should be amplified. Agarose gel electrophoresis of the PCR products indicated that the desired second-crossover occurred for all of the clones examined (Figure 6.1), and one of the clones was designated R7AΔexoU.
Figure 6.1: Confirmation of R7AΔexoU.

PCR was performed on genomic DNA with exoUcheckL/exoUcheckR primers to confirm the markerless deletion mutation. DNA size marker (M) is λ DNA digested with HindIII and ΦX174 DNA digested with HaeIII, with the size of the fragments indicated in kb. Lane 1 is genomic DNA extracted from R7A and lanes 2 to 6 represent five putative exoU mutant clones. * Denotes the isolate designated as R7AΔexoU.

6.2.2 Construction and screening of an R7AΔexoU/mTn5 mutant library

Transposon mutagenesis of R7AΔexoU was performed to introduce random secondary insertion mutations. *E. coli* S17-1/λpir harbouring the suicide plasmid pCRS487 was mated into R7AΔexoU to deliver the transposon mTn5-GNm (Reeve et al., 1999). Filter-mating growth was resuspended in sterile water and dilutions of the suspension were plated onto G/RDM containing Nm and Rif to select for transconjugants. Following ~5 days at 28°C, colony growth was resuspended from plates that contained between ~30 to ~300 transconjugants. Cells were pelleted by centrifugation and washed twice in sterile water to form the plant inocula. Eighty plant plates, each containing 10 *L. corniculatus* seedlings (Section 2.10.1), were each
inoculated with a different transconjugant resuspension from the mating dilution plates. Forty plant plates were inoculated with suspensions from dilution plates containing ~30 transconjugants each, 20 plant plates with suspensions representing ~100 transconjugants and 20 plant plates with inocula of ~300 transconjugants. In total the symbiotic screen consisted of 800 *L. corniculatus* seedlings inoculated with ~9200 R7AΔexoU/ mTn5 double mutants.

Plants were routinely observed for nodule development and as early as 14 dpi it was evident that nodules indistinguishable to those formed on R7A control plants were forming on several of the plants. At 42 dpi, 12 of the 80 plant plates inoculated with R7AΔexoU/ mTn5 clones contained plants exhibiting healthy growth due to the formation of nitrogen-fixing nodules (Figure 6.2).

![Figure 6.2: Nodulation screen for R7AΔexoU/mTn5 suppressors.](image-url)

*L. corniculatus* plants 42 dpi with A) R7A, B) R7AΔexoU and C), D) Pools of R7AΔexoU/ mTn5 clones.
Random nodules were harvested from each of the identified plant plates and rhizobia were recovered from them via nodules crushes (Section 2.10.6). All recovered isolates exhibited non-mucoid colony morphology on G/ RDM and were resistant to Nm. To ensure that the recovered isolates maintained symbiotic proficiency (and thus the nodules were not simply late-arising ‘escape’ nodules as occasionally formed by R7AexoU), each nodule isolate was inoculated onto *L. corniculatus* seedlings (Figure 6.3). The results indicated that 27 of the 37 isolates maintained symbiotic proficiency on *L. corniculatus*, with nodulation kinetics similar to that of R7A and only slight variations in the nodulation efficiency observed. The remaining 10 isolates formed only small white bumps indistinguishable to those formed on control R7AexoU-inoculated plants, indicating that these isolates were likely recovered from one of the occasional late-arising nodules that R7AexoU can form on *L. corniculatus*. 
Figure 6.3: Nodulation of *L. corniculatus* by R7AΔexoU/mTn5 isolates.

Total number of nitrogen-fixing nodules formed on 10 *L. corniculatus* plants on a single plate inoculated with the R7AΔexoU/mTn5 isolates applied as an individual inoculum. Strain names represent the suppressor screen plant plate and particular nodule from which they were recovered (e.g. 14-1 is an isolate from suppressor screen plant plate 14 and from nodule 1 recovered from the plate).
6.2.2.1 Identification of mTn5 insertion sites in symbiotically-proficient R7AΔexoU/mTn5 isolates

To determine if the R7AΔexoU/ mTn5 isolates recovered from L. corniculatus nodules contained mTn5 insertion sites in varying regions of the genome, Southern hybridisations were performed (Section 2.6.6) on genomic DNA from 38 isolates, including at least three from each of the 12 plant plates identified in the original screen. The DNA was digested with SalI, for which there are no recognition sites in mTn5.

The varying sizes of bands detected using an nptII PCR product to probe the membranes indicated that several different sites of mTn5 insertion existed amongst the isolates (Figure 6.4). In most instances nodules harvested from a particular plant plate all contained R7AΔexoU/ mTn5 isolates with the same mTn5 insertion site, based on the size of the bands. Furthermore, three isolates recovered from the same nodule appeared to contain the same insertion site. A few of the isolates displayed more than one band, possibly indicating the presence of multiple transposon insertions or tandem insertion of the vector plasmid pCRS487 rather than a ‘clean’ transposition event.

Together, Southern hybridisation banding and nodulation assays with individual isolates identified six symbiotically-proficient R7AΔexoU/ mTn5 strains that appeared to contain different mTn5 insertion sites.
Figure 6.4: Southern hybridisation of R7AΔexoU/mTn5 isolates.

Southern hybridisation of SalI-digested genomic DNA isolated from 38 R7AΔexoU/ mTn5 isolates recovered from nodules, probed with nptII to identify the genomic fragment harbouring the mTn5 insertion. DNA size marker (M) is λ DNA digested with HindIII, with the size of the fragments indicated in kb. Strain names refer to the suppressor screen plant plate and particular nodule from which they were recovered. Isolates 43-2 and 43-3 were not checked by Southern hybridisation as there were not enough lanes.
To determine the exact site of the mTn5 insertions, genomic DNA extracted from isolates representing the six potentially different insertions was digested with EcoRI and cloned into pUC19-Cm. Transformed E. coli EPI300 competent cells were plated onto LB containing Km and Cm to select for clones harbouring the mTn5-containing genomic fragment. No EcoRI recognition sites are present in mTn5 so the Km\(^{R}\)/Cm\(^{R}\) clones contained the entire transposon and flanking genomic regions. Plasmid DNA extracted from the clones was sequenced with the primer TAC-105F, which reads out from one end of mTn5 into the flanking genomic DNA (Reeve et al., 1999). The resultant sequence was compared to M. loti strain MAFF303099 using the BLASTN function of the rhizobase website (http://blast.kazusa.or.jp/blast_search/rhizobase/genomes).

MAFF303099 genome co-ordinates of the insertion sites and annotated functions of the gene products as given in Rhizobase are shown in Table 6.1. As expected, based on known exo mutant symbiotic phenotypes, R7A\(^{Δ}\)exoU/ mTn5 strains exhibiting symbiotic proficiency with insertions in the exoLAMON\((P)\) operon were isolated. However the identification of four genes not currently implicated in EPS biosynthesis in isolates 33-1 (mll2385), 37-1 (mlr2244), 43-2 (mll5197) and 43-3 (mlr4457) was of great interest.

**Table 6.1: Site of mTn5 insertion in R7A\(^{Δ}\)exoU/mTn5 isolates.**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Insertion site(^{a})</th>
<th>Gene disrupted(^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-1A</td>
<td>4201796</td>
<td><em>mll5271</em>, Glycosyltransferase (<em>exoL</em>)</td>
</tr>
<tr>
<td>16-1</td>
<td>4203109</td>
<td><em>mll5272</em>, Glycosyltransferase (<em>exoA</em>)</td>
</tr>
<tr>
<td>33-1</td>
<td>1938211</td>
<td><em>mll2385</em>, Sensory histidine kinase/ response regulator hybrid protein</td>
</tr>
<tr>
<td>37-1</td>
<td>1806149</td>
<td><em>mlr2244</em>, Sugar binding protein of sugar ABC-transporter</td>
</tr>
<tr>
<td>43-2</td>
<td>4132002</td>
<td><em>mll5197</em>, Malate dehydrogenase</td>
</tr>
<tr>
<td>43-3</td>
<td>3544869</td>
<td><em>mlr4457</em>, Two-component response regulator</td>
</tr>
</tbody>
</table>

\(^{a}\) Insertion site of mTn5 based on sequence obtained with the readout primer TAC-105F and comparison to the M. loti MAFF303099 genome.

\(^{b}\) MAFF303099 gene encoded at the insertion site and annotated gene product function from Rhizobase.
6.2.2.2 Bioinformatic analysis of the identified novel genes

The genes identified as harbouring mTn5 insertions in the symbiotically-proficient R7AΔexoU/ mTn5 isolates that had not previously been linked to EPS biosynthesis were further examined. Their context within the MAFF303099 genome is displayed in Figure 6.5.

*mlr2244* is predicted to encode a sugar binding protein of a sugar ABC-transport system, with two ABC-transporter permease encoding genes (*mlr2245* and *mlr2246*) located immediately downstream. BLASTP analysis indicates that homologous operons are present in *S. meliloti*, *R. etli*, *R. leguminosarum* and *Rhizobium* sp. NGR234 with ~60% amino-acid identity. The function of the *S. meliloti* Mlr2244 homologue, SM_b20634, was not identified in a comprehensive transportome study of sugar binding protein-dependent ABC-transporters in *S. meliloti* (Mauchline et al., 2006).

*mlr2385* encodes a complex two-component regulatory system sensory histidine kinase/response regulator hybrid protein with a second hybrid sensory histidine kinase/response regulator (*mlr2384*) located immediately downstream. The N-terminal region of Mlr2385 includes 8 HAMP domains and a GAF domain. BLASTP analysis reveals homologues in other rhizobia with *R. etli*, *R. leguminosarum* and *Bradyrhizobium* sp. sharing ~62% amino acid identity whilst *S. meliloti* and *Rhizobium* sp. NGR234 encode proteins with weaker homology of 37.2% and 25% amino-acid identity respectively. Adjacent to the regulatory genes are divergently transcribed genes encoding a membrane protein of unknown function (*mlr2383*) and a putative transmembrane efflux protein (*mlr2380*).

*mlr4457* encodes a two-component system response regulator with a sensor histidine kinase encoding gene (*mlr4459*) located immediately downstream. Homologues of the two-component regulatory system in *R. etli* and *R. leguminosarum* share 77% and 52% amino-acid identity to Mlr4457 and Mlr4459 respectively. In MAFF303099 genes encoding components of an ABC-transporter are located adjacent to the two-component regulatory however the genes are not conserved in other rhizobia.

*mlr5197* is located at the end of an operon encoding ABC-transporter components that BLASTP indicates are involved in amino acid transport. BLASTP analysis suggests Mll5197 is a malate/lactate dehydrogenase family member with
homologous proteins encoded in other rhizobia sharing ~50% amino acid identity. It shares 32% identity to the DpkA protein of *Pseudomonas putida* which is a NADPH-dependent delta(1)-piperideine-2-carboxylate/ delta(1)-pyrroline-2-carboxylate reductase that is involved in the catabolism of D-lysine and D-proline (Muramatsu *et al.*, 2005), and is the closest homologue with a characterised function.

**Figure 6.5: Genome context of the novel genes disrupted by mTn5.**

Schematic of the genomic regions encoding the novel genes identified as containing transposon insertions in the symbiotically-proficient R7AΔexoU/ mTn5 isolates. MAFF303099 genome co-ordinates, gene names and annotated gene product functions are indicated. The approximate position of the mTn5 insertion is indicated by the black triangle.
6.2.2.3 Colony characteristics of the R7AΔexoU/mTn5 isolates

Although the R7AΔexoU/ mTn5 isolates harboured insertions at different sites spread throughout the genome, they exhibited similar colony characteristics. Furthermore, these characteristics were in contrast to those displayed by R7AΔexoU and similar to those of the symbiotically-proficient R7AexoB and R7AΔexoA strains. It was immediately apparent that none of the R7AΔexoU/ mTn5 isolates displayed the cell aggregation phenotype of R7AΔexoU when grown in G/ RDM broths. The colony growth of the isolates was also easily dispersed on G/ RDM plates compared to the 'hard' colony morphology of R7AΔexoU.

To investigate the colony characteristics further, the isolates were plated onto Congo red- and Calcofluor-containing media. R7AΔexoU absorbs the dye from Congo red media and as result forms deep-red coloured colonies. The R7AΔexoU/ mTn5 isolates exhibited slight variations in Congo red absorption but all appeared less red than R7AΔexoU and more similar in colour to colonies formed by R7AexoB and the R7AΔexoU/ mTn5 isolate harbouring the transposon insertion in exoL (R7AΔexoU/ 5271) (Figure 6.6A).

On TY containing Calcofluor medium, phenotypic differences between R7AΔexoU and the symbiotically-proficient R7AΔexoU/ mTn5 isolates were clear. Whilst R7AΔexoU cultured on the medium fluoresces brightly when viewed under UV light, all of the R7AΔexoU/ mTn5 isolates remained non-fluorescent, comparable to R7AexoB and R7AΔexoU/ 5271 (Figure 6.6B). Furthermore, none of the double mutants showed the fluorescent halo characteristic of R7AΔexoU on G/ RDM containing Calcofluor (Figure 6.6C).
**Figure 6.6: Colony phenotypes of R7AΔexoU/mTn5 isolates.**

TY broth cultures of the strains were grown for 48 h then spotted (10 µL) onto Congo red or Calcofluor containing media. Colony growth after 48 h on A) YMB containing 0.005% Congo red. Colony fluorescence under UV light on B) TY containing 0.02% Calcofluor and C) G/ RDM containing 0.02% Calcofluor.
6.2.3 Re-construction of the novel R7AΔexoU/mTn5 mutations

To confirm that the observed phenotype of the R7AΔexoU/ mTn5 isolates was caused by the mTn5 insertions identified, an IDM approach (Section 2.7.1) was used to disrupt mlr2244, mll2385, mlr4457 and mll5197 in both R7A and R7AΔexoU backgrounds. PCR primer sets were designed to amplify a ~350-bp internal region of each of the target genes from R7A genomic DNA with BamH1 and HindIII sites incorporated. Digested PCR products were cloned into the suicide plasmid pFUS2 to form pSKPF2244, pSKPF2385, pSKPF4457 and pSKPF5197 which were confirmed by restriction analysis and sequencing. Biparental spot matings were performed between R7A or R7AΔexoU and confirmed E. coli S17-1/λpir clones. Selection of R7A or R7AΔexoU cells that had integrated the mutant construct was achieved through plating spot matings onto G/ RDM containing Gm plates and passaging of GmR single colonies were carried out to purify putative mutant clones.

6.2.3.1 Attempted confirmation and characterisation of the IDM strains in both R7A and R7AΔexoU backgrounds

GmR clones were plated onto Congo red and Calcofluor media to determine if the putative mutant clones exhibited the expected colony properties. In the R7A background, all clones formed mucoid colonies and were indistinguishable from R7A. In the R7AΔexoU background all putative mutant clones exhibited phenotypic properties identical to R7AΔexoU, with none exhibiting the altered properties of the symbiotically-proficient R7AexoU/ mTn5 isolates.

Southern hybridization (Section 2.6.6) was performed on GmR R7A/ pSKPF4457 and R7AΔexoU/ pSKPF4457 clones to determine whether the IDM process had been successful, using XhoI-digested genomic DNA. The membrane was initially probed with the ~350 bp mlr4457 PCR product with a band of 8.2 kb expected in R7A and R7AΔexoU parent controls and a band of ~16 kb expected in mutant clones (Figure 6.7A). The membrane was then stripped (Section 2.6.6.5) and re-probed with pFUS2 plasmid DNA with a band of ~16 kb expected in mutant clones (Figure 6.7B). The results of the Southern hybridisation indicated that the IDM was successful for three of the R7A/ pSKPF4457 clones. However none of the R7AΔexoU/ pSKPF4457 clones displayed the expected banding with either probe, indicating that the suicide plasmid was absent from their genomic DNA.
Figure 6.7: Southern hybridisation of R7A/pSKPF4457 and R7AΔexoU/pSKPF4457 clones.

A) Southern hybridisation with the ~350 bp mlr4457 PCR product. B) Southern hybridisation with the pFUS2 DNA probe. DNA size marker (M) is λ DNA digested with HindIII, with the size of the fragments indicated in kb. Lanes 1 and 7 represent R7A and R7AΔexoU respectively. Lanes 2 to 6 represent five R7A/ pSKPF4457 clones. Lanes 8 to 12 represent five R7AΔexoU/ pSKPF4457 clones.
6.2.4 Complementation of R7AΔexoU/2385 with a cosmid containing mll2385

Another approach to confirm that the genes identified as harbouring mTn5 were responsible for the observed symbiotic phenotypes was to complement the mutants and restore the R7AΔexoU phenotypes. Due to limited time available, it was decided to attempt to complement only one isolate as a proof of concept. R7AΔexoU/ 2385 was chosen as the strain to test the complementation approach and an mll2385-containing cosmid was identified from an existing R7A cosmid library.

Pools of DNA from a R7A cosmid library stored in microtitre plates were used as template DNA in PCR with primers 2385pFUS2L/ 2385pFUS2R to identify mll2385-encoding cosmids. A product of the expected ~350-bp size was amplified from microtitre plate 7B. Further PCR screening of individual columns and rows identified well 7C as containing a clone containing with an mll2385-encoding cosmid that was designated pSK2385.

E. coli EPI300 cells transformed with pSK2385 were selected on LB containing Tc. Cosmid DNA extracted from transformed clones was sequenced using primers 2385pFUS2L and 2385pFUS2R to confirm the presence of the gene. The cosmid was also end sequenced with primers pLAFRF and pLAFRR to identify the genomic region spanned in the cosmid through comparison with the MAFF303099 genome. The sequencing results confirmed the presence of mll2385 on the cosmid (Figure 6.8).

Cosmid pSK2385 was introduced into R7AΔexoU/ 2385 by electroporation and the complemented clone plated on Congo red- and Calcofluor-containing media along with appropriate controls. Surprisingly, the R7AΔexoU/ 2385/ pSK2385 clone exhibited properties identical to R7AΔexoU/ 2385, that is they remained Calcofluor-dark and did not absorb Congo red (not shown).
Figure 6.8: Genome spanning region of *mll2385*-encoding R7A library cosmid pSK2385.

Genome region spanned by cosmid pSK2385 based on cosmid end-sequencing results. MAFF303099 genome co-ordinates, open reading frames and annotated gene functions from Rhizobase are indicated.

### 6.2.5 Complementation of R7AΔexoU/2385 with an R7A cosmid library

This observation that complementation of R7AΔexoU/2385 with a cosmid harbouring *mll2385* did not result in the restoration of R7AΔexoU phenotypic properties indicated that perhaps other mutations may have contributed to the observed properties of R7AΔexoU/2385.

In another complementation approach, filter matings (Section 2.6.14.2) were performed between R7AΔexoU/2385 and pools of *E. coli* clones harbouring an R7A cosmid library. Mating growth was resuspended in sterile water and dilutions were plated onto G/ RDM containing Nm, Tc, Rf and Calcofluor. Colonies were examined under UV light and several were identified that exhibited bright Calcofluor fluorescence (Figure 6.9A). Eight Calcofluor-bright colonies were single colony purified and the strains plated onto Congo red and Calcofluor media to compare colony characteristics with those of R7AΔexoU and R7AΔexoU/2385 (Figure 6.9B and C). Of the eight isolates, six (39-1, 39-2, 39-3, 84-1, 84-3 and 84-5) displayed increased Congo red absorption and increased fluorescence on Calcofluor media compared to R7AΔexoU/2385 though to a lesser degree than R7AΔexoU. However, the isolates did not exhibit the cell-aggregation phenotype characteristic of R7AΔexoU.
Figure 6.9: Isolation of R7AΔexoU/2385 complementing cosmids and colony morphologies of complemented R7AΔexoU/2385.

A) R7A library cosmids that complemented R7AΔexoU/2385 were isolated by identifying Calcofluor-bright transconjugants (indicated by the arrow). Colony growth of R7AΔexoU/2385 with the identified cosmids after 48 h on B) YMB containing 0.005% Congo red and C) TY containing 0.02% Calcofluor.
6.2.5.1 Investigation of R7AΔexoU/2385 complementing cosmids

Cosmids were recovered from the six isolates that displayed increased Congo red absorption and Calcofluor fluorescence through triparental spot matings (Section 2.6.14.1), and were designated pSK39-1, pSK39-2, pSK39-3, pSK84-1, pSK84-3 and pSK84-5 after the isolate from which the cosmid was recovered. Cosmid DNA was digested with EcoRI and analysed by agarose gel electrophoresis (Figure 6.10). The restriction patterns indicated that three different cosmids were present in the complemented isolates.

![Restriction digests of R7AΔexoU/2385 complementing cosmids.](image)

**Figure 6.10: Restriction digests of R7AΔexoU/2385 complementing cosmids.**

Cosmid DNA recovered from two clones representing each of the complemented isolates was digested with EcoRI and checked by agarose gel electrophoresis. DNA size marker (M) is λ DNA digested with HindIII and ΦX174 DNA digested with HaeIII, with the size of the fragments indicated in kb.
Cosmids pSK39-1, pSK84-1 and pSK84-3 were end-sequenced using the primers pLAFRF and pLAFRR and the sequence was compared to that of MAFF303099 to identify the genomic region encoded on the cosmids. Cosmid pSK39-1 spanned the MAFF303099 genome co-ordinates 6507553-6525395, pSK84-1 6518208-6551021 and pSK84-3 6518695-6539972. The three cosmids contained a 6.7 kb overlapping region corresponding to MAFF303099 genome co-ordinates 6518695-6525395 (Figure 6.11). The majority of the genes on the 6.7 kb region encode products of unknown function; however, an exoB homologue (mll7878) and a dTDP-glucose 4,6-dehydratase (mll7879) are found in the region.

Figure 6.11: R7AΔexoU/2385 complementing cosmids overlapping region.
Genes encoded in the 6.7-kb overlapping region of cosmids pSK39-1, pSK84-1 and pSK84-3. MAFF303099 genome co-ordinates, open reading frames and annotated gene functions from Rhizobase are indicated.

6.2.6 Symbiotic proficiency of complemented R7AΔexoU/2385 isolates.
The symbiotic proficiency of R7AΔexoU/ 2385 strains containing pSK39-1, pSK84-1 and pSK84-3 on L. corniculatus was examined. The three complemented R7AΔexoU/ 2385 strains all formed nodules at rates comparable to control R7AΔexoU/ 2385- and R7A-inoculated plants (data not shown).

6.2.7 LPS production by R7AΔexoU/mTn5 isolates
Given that the R7AΔexoU/ mTn5 isolates displayed phenotypic properties similar to R7AexoB, which was shown to produce an altered form of LPS (Section 5.2.3), the LPS of strains R7AΔexoU/ 2385 and R7AΔexoU/ 4457 were examined at the CCRC by Dr Artur Muszynski. Silver-stained DOC-PAGE analysis of LPS recovered from the water phase indicated that R7AΔexoU/ 2385 LPS was altered in the lipid A/ core
region similar to R7AexoB, whereas R7AΔexoU/ 4457 LPS was comparable to that of R7A and R7AΔexoU (Figure 6.12). Composition analysis of the LPS samples confirmed that similar to R7AexoB, R7AΔexoU/ 2385 LPS lacked Gal whilst R7AΔexoU/ 4457 LPS composition was comparable to R7A and R7AexoU (data not shown).

Figure 6.12: Silver-stained LPS isolated from R7A and EPS mutant strains.

DOC-PAGE of Silver-stained LPS recovered from the water phase. S-Salmonella minnesota, lane 1 - R7A, lane 2 - R7AexoB, lane 3 - R7AΔexoU, lane 4 - R7AΔexoU/ 2385 and lane 5 - R7AΔexoU/ 4457.

6.2.8 Potential involvement of cellulose in EPS mutant phenotypes

Observations that the cell aggregation property of R7AexoU when grown in G/ RDM broth was lost in the symbiotically-proficient R7AΔexoU/ mTn5 double mutants as a result of the mutations in genes not previously been linked to EPS production, led to the hypothesis that alternative bacterial factors may be involved.

The production of cellulose is a factor commonly associated with a cell aggregation phenotype in various bacteria (Deinema & Zevenhuizen, 1971; Kawano et al., 2011; Matthysse et al., 1981; Sadasivan & Neyra, 1985; Ude et al., 2006). Furthermore, colony phenotypes including fluorescence on Calcofluor media and the binding of Congo
red dye have previously been recognised as a property of cellulose production by bacteria (Ausmees et al., 1999; Del Gallo et al., 1989; Matthisse et al., 1981; Matthisse et al., 1995b; Recouvreux et al., 2008). To investigate whether the cell aggregation displayed by R7AΔexoU was possibly related to cellulose production, dispersal of the cell aggregates through treatment with cellulase was examined. A cellulase treatment (Section 2.8) was performed on G/ RDM cultures of R7AΔexoU with R7A and R7AexoB included as control non-aggregating strains (Table 6.2). OD$_{600}$ values post-cellulase treatment for R7A and R7AexoB remained similar to the pre-treatment values. Absorbance values of the control R7AΔexoU suspension that was not treated with cellulase also remained similar. In contrast, the cellulase-treated R7AexoU suspension OD$_{600}$ value greatly increased compared to pre-treatment and visual inspection revealed that the cell aggregates had dispersed (Figure 6.13).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pre-cellulase OD$_{600}$</th>
<th>Post-cellulase OD$_{600}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>R7A</td>
<td>1.47</td>
<td>1.34</td>
</tr>
<tr>
<td>R7AexoB</td>
<td>2.81</td>
<td>2.62</td>
</tr>
<tr>
<td>R7AΔexoU</td>
<td>0.37</td>
<td>1.38</td>
</tr>
<tr>
<td>R7AΔexoU-control*</td>
<td>0.34</td>
<td>0.39</td>
</tr>
</tbody>
</table>

* Control R7AΔexoU sample did not receive cellulase treatment but was incubated for 1 h at 28°C with shaking.
6.2.9 **Bioinformatic analysis of cellulose production by *M. loti***

The biosynthesis of cellulose has been shown in a range of bacteria including species associated with plant hosts as discussed in Section 1.6.5. The production of cellulose by *M. loti* however has not been reported.

An operon encoding cellulose biosynthesis has been characterised in *R. leguminosarum* bv. *trifolii* (Ausmees *et al.*, 1999). The operon encodes a cellulose synthase (*celA*), cyclic diguanylic acid binding protein (*celB*) and an endoglucanase (*celC*) in similar arrangement to the homologous cellulose biosynthesis operon of *A. tumefaciens* (Romling, 2002). BLASTP analysis of the amino acid sequence of the products of these genes against the Rhizobase database was performed to identify potential cellulose biosynthesis operons in other rhizobia. Analogous operons were identified in *R. leguminosarum* bv. *viciae* 3841, *R. etli* CFN42 and *Rhizobium* sp. NGR234 (Figure 6.14) but were absent from *M. loti* and *S. meliloti*.
Figure 6.14: Cellulose biosynthesis operons identified in rhizobia.

BLASTP analysis of the known cellulose biosynthesis operon of *R. leguminosarum* bv. *trifolii* revealed similar operons in *R. leguminosarum* bv. *viciae*, *R. etli* and *Rhizobium* sp. NGR234. ORFs are not drawn to scale. Homologous genes are indicated by sharing the same colour.

Whilst no operons of similar arrangement were present in *M. loti*, a *celA*-like gene (*mlr7873*) was identified in MAFF303099. Mlr7873 shared 27% identity to the *R. leguminosarum* bv. *viciae* 3841 *celA* (*RL1646*) gene product and further analysis of the genes surrounding *mlr7873* identified an endoglucanase gene, *mll7872*, the product of which shared 31% identity to the *R. leguminosarum* bv. *viciae* 3841 *celC* (*RL1648*) gene product.

Investigation of the cluster of 8 genes in MAFF303099 (*mll7871* to *mll7879*) revealed that similar gene arrangements were present in many rhizobial species including *R. leguminosarum* bv. *viciae* 3841 (Figure 6.15) and *S. meliloti* (not shown). The genes identified in MAFF303099 encoded products that shared ~60% amino acid identity to homologues in *R. leguminosarum* bv. *viciae* 3841. Interestingly, the cluster included the
genes *mll7878* and *mll7879* that were present in the common 6.7 kb region of cosmids pSK39-1, pSK84-1 and pSK84-3 described above.

**Figure 6.15:** *M. loti* MAFF303099 putative cellulose biosynthesis genes.

BLASTP analysis identified putative cellulose synthase (*mll7873*) and cellulase (*mll7872*) genes in MAFF303099. A similar gene cluster present in *R. leguminosarum* bv. *viciae* is shown with homologous genes indicated by sharing the same colour. Rhizobase genome co-ordinates, open reading frames and annotated gene product functions are indicated.

### 6.2.10 Construction of an R7A celA mutant

If R7A does indeed produce cellulose, then *celA* (*mll7873*) represents the best candidate gene involved in its biosynthesis. To investigate if mutation of the gene had an effect on colony and symbiotic properties, an in-frame markerless *celA* deletion strain was constructed.

#### 6.2.10.1 R7AΔcelA mutagenesis construct

An overlap extension PCR approach (Section 2.7.2.1) was employed to generate a ~2 kb in-frame *celA* mutant product using primer sets celALL/ celALR and celAL/ celARR that incorporated SpeI sites at the outer ends. Digested PCR product
was cloned into the suicide vector pJQ200SK to form pSKCELA and the insert confirmed by restriction analysis and sequencing.

6.2.10.2 Isolation of R7AΔcelA
Spot matings between R7A and S17-1/λpir/pSKMU were performed and the markerless deletion process was carried out as described in Section 2.7.2.2. Primer set celAcheckL/celAcheckR that amplifies across the joining site of the left and right arms of the ~2 kb fragment were used to confirm the mutation using genomic DNA extracted from DXO clones as template. If the second-crossover event occurred as desired resulting in the markerless deletion, only a 565-bp band should be amplified. However, if the second-crossover resulted in the clone reverting to wild-type, only a 2302-bp band should be amplified. The results of the PCR indicated that the desired second-crossover occurred for five of the nine clones examined (Figure 6.16). A clone that exhibited the expected mutant band was designated as R7AΔcelA.

![Figure 6.16: Confirmation of R7AΔcelA.](image)

PCR was performed on genomic DNA extracted from DXO clones with celAcheckL/celAcheckR primers to confirm the markerless deletion mutation. DNA size marker (M) is λ DNA digested with HindIII and ΦX174 DNA digested with HaeIII, with the size of the fragments indicated in kb. Lane 1 is genomic DNA extracted from R7A and lanes 2 to 10 represent nine putative celA mutant clones. * Denotes the isolate designated as R7AΔcelA.
6.2.11 Construction of an R7A celA exoU double mutant

To investigate what effects the mutation of celA would have in an exoU mutant background, a double mutant strain was constructed. Biparental spot matings between R7AΔcelA and S17-1/λpir/pSKUPF were carried out with transconjugants selected on G/ RDM containing Gm. Following single-colony purification genomic DNA was extracted from non-mucoid GmR clones, digested with ClaI and the digested-DNA transferred from agarose gels to a nylon membrane for use in Southern hybridisation (Section 2.6.6). The membrane was probed with the exoU 394-bp PCR product to allow visualisation of a shift in band size in the putative mutant clones compared to R7A (Figure 6.17). A band of 2.4 kb was expected in R7A representing the exoU-containing ClaI genomic fragment. Because pFUS2 contains one ClaI site, two bands of 2.4 kb and 7.7 kb were expected in mutant clones in which the IDM had been successful. A representative clone that exhibited the expected banding was designated as R7AΔcelAexoU.

Figure 6.17: Confirmation of R7AΔcelAexoU.

Southern hybridisation of ClaI-digested genomic DNA probed with the 394 bp exoU PCR product. DNA size marker (M) is λ DNA digested with HindIII, with the size of the fragments indicated in kb. Lane 1 is R7A; lanes 2-12 represent genomic DNA extracted from 11 putative mutant clones. * Denotes isolate designated as R7AΔcelAexoU.
6.2.12 Colony characteristics and symbiotic proficiency of R7A ΔcelA and R7A ΔcelA ΔexoU

R7A ΔcelA exhibited colony characteristics indistinguishable to those of R7A on G/ RDM, Congo red- and Calcofluor-containing media (Figure 6.18). R7A ΔcelA ΔexoU displayed colony phenotypes identical to R7A ΔexoU on including the production of a halo on G/ RDM containing Calcofluor (Figure 6.18). R7A ΔcelA ΔexoU also formed ‘hard’ colony growth on G/ RDM agar and exhibited R7A ΔexoU-like cell aggregation in broth culture. L. corniculatus inoculated with R7A ΔcelA formed nodules at rates comparable to R7A whilst R7A ΔcelA ΔexoU-inoculated plants formed only small uninfected bumps indistinguishable to those formed by R7A ΔexoU (data not shown).

Figure 6.18: Colony phenotypes of R7A ΔcelA and R7A ΔcelA ΔexoU.

TY broth cultures of the strains were grown for 48 h then spotted (10 µL) onto Congo red or Calcofluor containing media. Colony growth after 48 h on A) YMB containing 0.005% Congo red. Colony fluorescence under UV light on B) TY containing 0.02% Calcofluor and C) G/ RDM containing 0.02% Calcofluor.
6.3 Discussion

This chapter described the isolation of R7AΔexoU/ mTn5 strains that suppressed the R7AΔexoU symbiotic impairment. As expected, strains harbouring transposon insertions in exo genes involved in the early stages of EPS biosynthesis were isolated. However, the isolation of R7AΔexoU/ mTn5 strains disrupted in genes seemingly unrelated to EPS biosynthesis suggested other bacterial factors may be involved in the symbiotic impairment of R7AΔexoU.

Six different sites of transposon insertion were identified amongst the symbiotically-proficient R7AΔexoU/ mTn5 double mutants and the position of the insertion was determined for representative isolates. Insertions within exoL and exoA were identified with the transposon insertions likely polar and therefore disrupting the entire exoLAMON(P) operon. The suppression of the symbiotic impairment by these isolates is likely due to preventing the generation of truncated EPS by R7AΔexoU through the disruption of EPS biosynthesis at an early stage. Interestingly, no isolates were recovered with mTn5 insertions in the exoB gene, suggesting that the recovery of further symbiotically-proficient R7AΔexoU/ mTn5 isolates is likely possible. The remaining isolates were found to harbour transposon insertions in genes mll2385, mlr2244, mlr4457 and mll5197 which have not previously been implicated in EPS production. Functions have not been reported for these genes and they do not seem to share much in common other than perhaps their location within or adjacent to ABC-transporter operons. This however could merely be a reflection of the large number of ABC transporters encoded by M. loti, with more than 200 reported (Davidson et al., 2008).

The symbiotically-proficient R7AΔexoU/ mTn5 isolates all displayed contrasting colony properties to R7AΔexoU and were more similar to R7AexoB. The strains did not form cell aggregates, formed easily dispersed colonies on G/ RDM, did not fluoresce on Calcofluor containing media and did not absorb Congo red dye to the same extent as R7AΔexoU. Interestingly, the degree of Congo red absorption varied amongst the isolates with R7AΔexoU/ 2244 and R7AΔexoU/ 2385 forming darker red colony growth than R7AΔexoU/ 4457 and R7AΔexoU/ 5197. This observation would suggest that although the isolates share common characteristics there are perhaps subtle differences between them, as a result of the different mutations.
IDM constructs designed to disrupt *mll2385, mlr2244, mlr4457* and *mll5197* were successfully introduced into an R7A background and single mutant strains were isolated. The single mutants exhibited colony phenotypes indistinguishable from R7A indicating that the genes did not affect wild-type EPS production. The IDM constructs were unable to be successfully introduced into R7AΔexoU to reconstruct the double mutant strains with investigation of putative double mutant clones by Southern hybridisation revealing that the IDM construct was absent from the clones. It is possible that *exoU* mutants exhibit a higher frequency of mutation to Gm® and therefore true IDM mutants were unable to be isolated.

Due to the difficulties encountered in reconstruction of the suppressor mutants, complementation approaches were undertaken using R7AΔexoU/2385 as a test strain. Surprisingly, introduction of an *mll2385*-encoding cosmid did not restore any of the R7AΔexoU phenotypes, with complemented clones indistinguishable to R7AΔexoU/2385 in all properties examined. This led to attempted complementation of R7AΔexoU/2385 via the introduction of R7A library cosmids *en masse*. Several transconjugants exhibiting R7AΔexoU-like Calcofluor fluorescent properties were identified and the three different cosmids they contained were examined. Sequencing revealed that the cosmids shared a small overlapping genomic region of 6.7 kb that encoded seven ORFs, most of unknown function. Two genes in the region, *mll7878* and *mll7879*, annotated in Rhizobase as encoding UDP-glucose 4-epimerase and dTDP-glucose 4-6-dehydratase respectively were of particular interest. BLASTP analysis of Mll7878 and Mll7879 revealed homologous proteins in various rhizobia including the *S. meliloti* proteins SM_b20459 and SM_b20458 which shared 61.8% and 68.6% amino-acid identity, respectively. The *S. meliloti* proteins have recently been reported to function in the biosynthesis of UDP-xylose (Xyl) and UDP-arabinose (Ara) (Gu et al., 2011). SM_b20458 functions as a UDP-xylose synthase and catalyses the decarboxylation of UDP-GlcA to form UDP-Xyl (Bar-Peled et al., 2001) and SM_b20459 functions as a UDP-xylose 4-epimerase to catalyse the reversible reaction that forms UDP-Ara from UDP-Xyl (Burget & Reiter, 1999). Xyl and Ara have previously been identified as components of rhizobial polysaccharides. *B. japonicum* LPS contains both sugars (Puvanesarajah et al., 1987) and when grown on certain carbon sources *B. japonicum* produces EPS containing Xyl (Karr et al., 2000). Chemical
analysis of R7A EPS extracts in this study did not reveal the presence of Xyl or Ara (Chapter 4).

Coincidentally, an mll7878 mutant strain (R7AΔmll7878) had previously been constructed to examine if the gene was involved in R7A EPS biosynthesis due to its homology (67.9% amino-acid identity) to S. meliloti ExoB (Kelly, 2007). R7AΔmll7878 exhibited mucoid colony on G/ RDM agar and was indistinguishable from R7A in all colony and symbiotic phenotypes examined. Furthermore, an R7AΔmll7878exoB double mutant was constructed and found to exhibit characteristics identical to R7AexoB, indicating that mll7878 was not involved in R7A EPS production (Kelly, 2007).

R7AΔexoU/2385 harbouring the mll7878 and mll7879-encoding cosmids exhibited partial restoration of R7AΔexoU phenotypes. Calcofluor fluorescence was not fully restored to R7AΔexoU levels and the cosmids did not cause reversion to the R7AΔexoU cell aggregation phenotype, indicating that the symbiotic properties of R7AΔexoU/2385 are not related to disruption of mll7878 and mll7879. The partial complementation observed may be due to the presence of mll7878 and mll7879 in trans affecting their expression and/or activity in Xyl and Ara production, which may result in changes to LPS or other cell surface components.

To further examine R7AΔexoU/2385, LPS produced by the strain was investigated at the CCRC by Dr Artur Muszynski. LPS profiles indicated a slight change in the core region of the LPS compared to R7A and R7AΔexoU controls, with the altered LPS profile comparable to that of R7AexoB. In contrast, the LPS profile of another suppressor strain, R7AΔexoU/4457, was comparable to R7A and R7AΔexoU. As discussed in Section 5.2.3, R7AexoB produced an altered LPS that is lacking Gal. Composition analysis confirmed that R7AΔexoU/2385 LPS also produced LPS lacking Gal. The results indicate that R7AΔexoU/2385 is deficient in Gal production, similar to R7AexoB. This deficiency in Gal production may either be due to the mutation in mll2385 affecting regulation of UDP-Gal production or R7AΔexoU/2385 may contain an additional mutation in exoB.

The isolated R7AΔexoU/mTn5 strains faced strong selection pressure due to the screening process. Only strains able to successfully nodulate were recovered,
meaning that any strains that contained further mutations allowing nodulation to proceed would be selected for. Despite this strong selection pressure, plant nodulation assays performed with R7A \textit{exoU} mutants do not support the idea that further spontaneous mutations occurred in the R7A\textDelta exoU/mTn5 isolates, accounting for their symbiotic proficiency. R7A \textit{exoU} mutants are subjected to this same selection pressure on plants, yet all isolates recovered from any late-arising nodules in this study, and in previous nodulation assays (Hubber, 2005; Kelly, 2007), have been found to maintain R7A \textit{exoU} mutant colony and symbiotic phenotypes.

Mll2385 is a complex two-component regulatory system sensory histidine kinase/ response regulator hybrid protein that contains multiple HAMP domains (so called on the basis that the domain is present in histidine kinases, adenylyl cyclases, methyl-accepting proteins and phosphatases). The role of multiple HAMP domains in regulatory proteins is not well understood, although it has been proposed that the domains may interact in an intramolecular manner to form a structure necessary for interaction with target molecules (Aravind & Ponting, 1999). To gain insight into the possible regulatory function of Mll2385, homologous proteins in other bacteria that had been investigated were identified. Mll2385 shares 63\% amino-acid identity to the \textit{Myxococcus xanthus} sensory histidine kinase/ response regulator hybrid protein MXAN\_0712 that also contains multiple HAMP domains. \textit{M. xanthus} disrupted in MXAN\_0712 exhibited normal motility but was unable to aggregate to form fruiting bodies and was severely reduced in sporulation (Shi et al., 2008). The inability to of the mutant to form aggregates is interesting given the non-aggregating phenotype of R7A\textDelta exoU/mTn5. The \textit{B. cereus} regulatory protein RsbK shares homology with MXAN\_0712 (de Been et al., 2011) with BLASTP analysis of RsbK revealing the protein shares 37\% amino-acid identity to both MXAN\_0712 and Mll2385. In response to stress, RsbK phosphorylates a response regulator (RsbY) to induce expression of the alternative transcription factor $\sigma^b$ (de Been et al., 2010). In \textit{B. cereus} $\sigma^b$ mediates the transcription of $\sigma^b$-dependent genes that are involved in aiding the cells to respond to environmental stresses including changes in temperature, pH or osmolarity (Hecker et al., 2007). Investigations of multiple HAMP-domain containing hybrid regulatory proteins in fungi also suggest a role for the proteins in gene regulation in response to environmental stress including osmolarity (Jones et al., 2007; Meena et al., 2010). Meena et al (2010) suggest that the multiple HAMP domains
may function as sensors for different physiochemical stimuli. In general, MII2385 homologues function in perception of changing environmental stresses and the regulation of genes involved in combating such stresses. Given that cell surface polysaccharides provide protection to rhizobia from environmental stresses it is plausible that MII2385 is involved in the regulation of genes involved in their production, as evidenced by the lack of truncated EPS and altered LPS production by R7AΔexoU/2385.

Further characterisation of the R7AΔexoU/ mTn5 isolates is on-going. R7AΔexoU/5197 has recently been complemented through matings with R7A cosmid library clones. The complemented isolates exhibit fully-restored R7AΔexoU phenotypes, including bright Calcofluor fluorescence, ‘hard’ colony morphology on G/RDM agar and cell aggregation in G/RDM broth (Todd Wightman, personal communication). Characterisation of the complementing cosmid is currently being pursued. The ability to successfully complement R7AΔexoU/5197, which is disrupted in a non-regulatory gene, suggests that the unsuccessful complementation of R7AΔexoU/2385 in this study may be due to the regulatory nature of mll2385. For example, the transposon insertion may cause a dominant-negative regulatory form of Mll2385 that cannot be complemented by the presence of a wild-type copy of the gene.

The reported involvement of bacterial cellulose in cell aggregation and the dispersal of R7AΔexoU cell aggregates following treatment with cellulase provided evidence for the potential involvement of cellulose in the strains properties (Deinema & Zevenhuizen, 1971; Kawano et al., 2011; Matthysse et al., 1981; Sadasivan & Neyra, 1985; Ude et al., 2006). Furthermore, cellulose production has been shown to alter Congo red and Calcofluor-binding properties of producing strain’s (Ausmees et al., 1999; Del Gallo et al., 1989; Matthysse et al., 1981; Matthysse et al., 1995b; Recouvreux et al., 2008).

Cellulose production by M. loti has not previously been reported, however bioinformatic analysis performed in this study identified a possible cellulose synthase (celA) gene, mll7873, in the MAFF303099 genome. The possible involvement of the gene in cellulose biosynthesis was enhanced by the presence of a divergently transcribed endoglucanase (celC) homologue. Strains R7AΔcelA and R7AΔcelAexoU
were constructed and their colony and symbiotic phenotypes examined. R7AΔcelA exhibited properties identical to R7A and R7AΔcelAexoU was indistinguishable from R7AΔexoU in all phenotypes examined. These results indicate that cellulose production by R7A is not a determinant of the R7AΔexoU phenotype or its production is encoded by genes with little homology to known cellulose biosynthesis genes. Given that cellulose production by R7A appears unlikely, a model is proposed to account for the observed R7AΔexoU and R7AΔexoU/ mTn5 phenotypes in which truncated EPS produced by R7AΔexoU in essence mimics cellulose production. Dispersal of R7AΔexoU cell aggregates through treatment with cellulase could be due to the action of the enzyme on the 1,4-β-Glc linkages of the truncated EPS. Although cellulase is generally considered to specifically cleave cellulose due to its composition of solely 1,4-β-Glc, the enzyme may potentially exhibit activity on any 1,4-β-Glc containing polysaccharides including EPS, which is supported by reports demonstrating that *Pseudomonas* strains EPS is susceptible to cleavage by cellulase (Cescutti *et al.*, 1998; Loiselle & Anderson, 2003; Nielsen *et al.*, 2011).

The isolated R7AΔexoU/ mTn5 strains may therefore be deficient in production or secretion of the truncated EPS, resulting in their colony and symbiotic phenotypes being comparable to EPS mutant strains disrupted at the early stages of EPS biosynthesis such as R7AexoB. The secretion pathway for R7AΔexoU LMW EPS would therefore differ from that of wild-type HMW EPS, an hypothesis that could be tested by determining the effect of mutation of genes involved in HMW EPS transport (e.g. *exoT*) on the fluorescence and halo production of R7AΔexoU on G/ RDM containing Calcofluor.

Although the genetic determinants of the observed R7AΔexoU/ mTn5 phenotypes were not conclusively defined in this study, phenotypic analysis demonstrated that they all share common properties that indicate they no longer secrete the LMW truncated EPS secreted by R7AΔexoU. It is possible that the site of mTn5 insertion is not responsible for the observed phenotypes and that further unselected mutations occurred in early stage EPS biosynthesis genes (Subsequent to the submission of this thesis, this was found to be the case).
7 Characterisation of *M. loti* NZP2037 and its *exo* mutants
7.1 Introduction

*Mesorhizobium loti* strain NZP2037 exhibits symbiotic characteristics that differentiate it from other *M. loti* strains, including its ability to enter into a successful symbiotic partnership with both indeterminate and determinate nodule-forming hosts. This characteristic was the basis of an isogenic study performed by Hotter and Scott into the requirement for EPS in symbiosis, which concluded that EPS was required for nodulation of indeterminate hosts only (Hotter & Scott, 1991). Another unique characteristic of NZP2037 is the strain’s ability to form nitrogen-fixing nodules on the determinate host *Lotus pedunculatus*, a host that most *M. loti* strains (including R7A) only induce uninfected nodule primordia on (Pankhurst et al., 1979).

The NZP2037 EPS mutants investigated by Hotter and Scott (1991) were determined to harbour transposon insertions in either the *exoA* or *exoB* EPS biosynthesis genes (Kelly, 2007). Based on the findings that disruptions in these genes involved at the early stages of EPS biosynthesis may not cause symbiotic deficiencies in an R7A background, an *exoU* NZP2037 mutant was constructed using an IDM approach. Plant nodulation assays with the NZP2037 *exoA*, *exoB* and *exoU* mutants confirmed that none of the mutations had an effect on nodulation of the determinate hosts *L. corniculatus*, *L. japonicus* Gifu or *L. pedunculatus* (Kelly, 2007).

We hypothesised that NZP2037 may produce a secondary polysaccharide, perhaps under symbiotic conditions, that allowed nodulation to proceed in the *exoU* mutant background. The production of an alternative polysaccharide by NZP2037 could also account for the strain’s unique ability to form infected nodules on *L. pedunculatus* as previously postulated by Jones et al. (1987). These authors identified a NZP2037 polysaccharide associated with the cell surface that was capable of binding a prodelphinidin-rich flavolan that is present within *L. pedunculatus* nodules (Jones et al., 1987). Alternatively, NZP2037 may differ from other *M. loti* in additional bacterial components that allow for the overcoming of any EPS deficiencies such as Nod factor or effector molecules secreted by Type III or Type IV secretion systems.

This chapter details experiments performed to investigate the bacterial components responsible for the observed differences in symbiotic abilities of both wild-type and EPS-deficient strains of R7A and NZP2037.
7.2 Results

7.2.1 Construction of in-frame markerless deletion NZP2037 \( \text{exoU} \) and \( \text{exoB} \) mutants

To confirm the nodulation ability of NZP2037 EPS mutants and allow for the assembly of double mutants and the ability to host various plasmids, in-frame markerless deletion \( \text{exoB} \) and \( \text{exoU} \) mutants of NZP2037 were constructed.

7.2.1.1 NZP2037\( \Delta \text{exoU} \) and NZP2037\( \Delta \text{exoB} \) mutagenesis constructs

An overlap extension PCR approach (Section 2.7.2.1) was employed to generate \( \sim2 \) kb in-frame \( \text{exoU} \) and \( \text{exoB} \) mutant products using the primer sets \( \text{exoULL/ exoULR} \) and \( \text{exoURL/ exoURR} \) for \( \text{exoU} \) and \( \text{exoBLL/ exoBLR} \) and \( \text{exoBRL/ exoBRR} \) for \( \text{exoB} \). Both \( \text{exoU} \) and \( \text{exoB} \) primer sets incorporated SpeI recognition sites at the outer ends. Digested PCR products were cloned into pJQ200SK to form pSKM2U and pSKM2B. Restriction analysis and sequencing confirmed that the plasmids harboured the correct inserts.

7.2.1.2 Isolation of NZP2037\( \Delta \text{exoB} \) and NZP2037\( \Delta \text{exoU} \)

NZP2037 was spot-mated with the appropriate \( E. \ coli \) S17-1/\( \lambda \)pir clones and the markerless deletion process carried out as described in Section 2.7.2.2, with non-mucoid isolates selected.

Primer sets \( \text{exoUcheckL/ exoUcheckR} \) and \( \text{exoBcheckL/ exoBcheckR} \) that amplify across the joining site of the left and right arms of the \( \sim2 \) kb \( \text{exoU} \) and \( \text{exoB} \) fragments respectively, were used to confirm the mutations. Genomic DNA extracted from DXO clones was used as template DNA. If the second-crossover event occurred as desired resulting in the markerless deletion, only products of 562 bp \( (\text{exoU}) \) and 557 bp \( (\text{exoB}) \) should be amplified. However, if the DXO clones reverted to wild-type, products of 1405 bp \( (\text{exoU}) \) and 1457 bp \( (\text{exoB}) \) should be amplified. Agarose gel electrophoresis of the PCR products indicated that the desired second-crossover occurred for all of the clones examined (Figure 7.1). Representative clones were designated NZP2037\( \Delta \text{exoU} \) and NZP2037\( \Delta \text{exoB} \).
Figure 7.1: Confirmation of NZP2037ΔexoB and NZP2037ΔexoU.

PCR was performed on genomic DNA with exoUcheckL/exoUcheckR (lanes 1-7) and exoBcheckL/exoBcheckR (lanes 8-11) primers to confirm the markerless deletion mutations. DNA size marker (M) is λ DNA digested with HindIII and ΦX174 DNA digested with HaeIII, with the size of the fragments indicated in kb. Lanes 1 and 7 contain products from NZP2037 genomic DNA; lanes 2 to 6 represent five exoU DXO clones; lanes 8-11 represent 4 exoB DXO clones. * Denotes the isolates designated as NZP2037ΔexoU (lane 2) and NZP2037ΔexoB (lane 8).

7.2.1.3 Characterisation of NZP2037ΔexoB and NZP2037ΔexoU

Both NZP2037ΔexoB and NZP2037ΔexoU exhibited non-mucoid colony morphologies on G/ RDM plates. Analogous to the respective R7A mutants, NZP2037ΔexoU formed cell aggregates in G/ RDM broth culture whilst NZP2037ΔexoB did not. Also similar to the R7A mutants, NZP2037ΔexoB did not absorb Congo red dye and did not fluoresce on Calcofluor-containing media whereas NZP2037ΔexoU formed deep red colonies on Congo red and exhibited Calcofluor fluorescence (Figure 7.2). On G/ RDM containing Calcofluor, a fluorescent halo was observed around NZP2037ΔexoU colonies comparable to that observed with R7AexoU (Figure 7.2C).
**Figure 7.2: Colony morphologies of NZP2037ΔexoB and NZP2037ΔexoU.**

TY broth cultures of the strains were grown for 48 h then spotted (10 µL) onto Congo red or Calcofluor containing media. Colony growth after 48 h on A) YMB containing 0.005% Congo red. Colony fluorescence under UV light on B) TY containing 0.02% Calcofluor and C) G/ RDM containing 0.02% Calcofluor.

Both NZP2037ΔexoB and NZP2037ΔexoU formed nodules on *L. corniculatus* and *L. pedunculatus* at rates comparable to NZP2037 (Figure 7.3). The NZP2037 strains were delayed and formed fewer nodules overall on *L. corniculatus* compared to R7A. As expected, R7A did not form any nitrogen-fixing nodules on *L. pedunculatus*. 
A)

B)

Figure 7.3: Symbiotic proficiency of NZP2037ΔexoB and NZP2037ΔexoU.
Total number of nitrogen-fixing nodules formed on A) 10 L. corniculatus plants and B) 10 L. pedunculatus plants inoculated with the indicated strains.
7.2.2 Construction of an NZP2037 cosmid library

A cosmid library of genomic DNA from NZP2037 was constructed to be used in complementation experiments to identify the factors responsible for the strain’s ability to establish a successful symbiosis with determinate hosts regardless of any EPS deficiencies, as well as to investigate the factors allowing nodulation of *L. pedunculatus*.

Chromosomal DNA extracted from NZP2037 was partially digested with Sau3A to obtain chromosomal fragments of ~22 kb that were then cloned into pIJPAR, a modified pIJ3200 plasmid that encodes the stability locus *parDE* (Miller, 2005). The ligated mix was packaged into lambda phage and used to transfect *E. coli* HB101 as described in Section 2.6.13.4. In total ~3000 transduced *E. coli* were isolated and stored to form the NZP2037 cosmid library. Clones were stored in 17 pools, each consisting of ~175 clones, with 1632 clones also stored individually in 17 microtitre plates (Section 2.6.13.5).

7.2.2.1 Testing whether the NZP2037 cosmid library was representative of the genome

Cosmid DNA was extracted from 12 random clones and digested with EcoRI to determine if there was a random distribution of NZP2037 genomic DNA amongst the clones (Figure 7.4). For each clone a band of ~22 kb was observed representing pIJPAR along with multiple smaller bands representing NZP2037 genomic DNA insert. The restriction pattern differed among the 12 clones, indicating that the cosmid library contained clones with varying NZP2037 genomic inserts.
Figure 7.4: Restriction digest of cosmid DNA from random NZP2037 library clones.

Agarose gel electrophoresis of EcoRI-digested cosmid DNA extracted from 12 random NZP2037 library clones (lanes 1-12). DNA size marker (M) is λ DNA digested with HindIII and ΦX174 DNA digested with HaeIII, with the size of the fragments indicated in kb.

Non-symbiotic *M. loti* CJ6 lacks a functional C4-dicarboxylate transport (*dct*) operon that is required for the utilisation of succinate as a sole-carbon source. When plated on RDM containing succinate as the sole carbon source (Succ/RDM), the strain only forms very small colonies due to its inability to transport succinate into the cell. In comparison, strains harbouring the *dct* operon are able to grow on Succ/RDM at a rate similar to that observed on G/RDM (Weaver, 2003).

To test the representativeness of the NZP2037 cosmid library, pooled clones were mated into CJ6 by triparental filter matings (Section 2.6.14.2) and dilutions of the matings were plated onto Succ/RDM. The fraction of transconjugants carrying a NZP2037 cosmid harbouring the *dct* operon was 1 in 380, calculated by dividing the average number of succinate-utilising colonies by the average total number of colonies per plate. Theoretically, based on an average cosmid insert of 22 kb and an estimated NZP2037 genome size of 7 Mb, the fraction of clones expected to
complement the succinate utilisation phenotype would be 1 in 318. The closeness of the observed complementation fraction compared to the theoretically expected fraction suggests that the constructed NZP2037 cosmid library is representative of the genome.

7.2.3 Complementation of R7AexoB and R7AexoU non-mucoid colony morphology with the NZP2037 cosmid library

Triparental matings were performed to complement R7AexoB and R7AexoU with the NZP2037 cosmid library. Growth from filter matings was resuspended and dilutions spread onto G/ RDM containing Nm and Tc. Following 5 days growth at 28°C, larger mucoid colonies developed against the background of small non-mucoid colonies. NZP2037 cosmids that conferred the mucoid phenotype were recovered from five mucoid colonies in each of the R7AexoB and R7AexoU backgrounds.

Based on their EcoRI restriction profiles, two different exoB-complementing NZP2037 cosmids were recovered, whilst all complemented R7AexoU harboured the same NZP2037 cosmid (Figure 7.5A). End-sequencing of the two different exoB cosmids (pSKB1 and pSKB4) and one of the exoU cosmids (pSKU1) confirmed that the cosmids spanned the regions encoding exoB and exoU respectively (Table 7.1). It was expected that more than one cosmid able to complement R7AexoU would be present in the NZP2037 cosmid library. To identify if alternative cosmids were indeed present in the library, a PCR-based screening approach was employed to identify them among the individually-stored NZP2037 cosmid clones. Pools of NZP2037 cosmid clone DNA, each representing half of a microtitre plate, was prepared. The half-plate pooled DNA was used as the template in PCR reactions with the primer pair exoUpFUSL/ exoUpFUSR that amplify ~350 bp of exoU. This identified half-plates 5B, 10A and 13B as containing cosmids that encoded exoU. Further PCR-screening of the columns and then the rows of these half-plates identified microtitre plate wells 5-7-C, 10-5-B and 13-9-F as containing clones that harboured NZP2037 exoU cosmids. The cosmids were designated pSK57C, pSK105B and pSK139F respectively. Their EcoRI profiles indicated that pSK139F was similar to pSKU1 recovered previously, whilst pSK57C and pSK105B represented different exoU-encoding NZP2037 cosmids (Figure 7.5B). End-sequencing of the three cosmids confirmed all were different and all spanned the exoU-coding region (Table 7.1).
Figure 7.5: Restriction profiles of exoB- and exoU-encoding NZP2037 library cosmids.

Agarose gel electrophoresis of EcoRI-digested cosmid DNA isolated from exoB and exoU-complementing NZP2037 library clones. DNA size marker (M) is λ DNA digested with HindIII and φX174 DNA digested with HaeIII, with the size of the fragments indicated in kb. A) Lanes 1-5 represent cosmids extracted from complemented R7AexoB including pSKB1 (lane 1) and pSKB4 (lane 4). Lanes 6-10 are cosmids isolated from complemented R7AexoU; The cosmid in lane 6 was designated pSKU1. B) EcoRI-digests of exoU-encoding NZP2037 cosmids identified by PCR screening. Lane 1 is pSK57C; lane 2 is pSK105B and lane 3 is pSK139F.

Table 7.1: Co-ordinates of exoB and exoU-encoding NZP2037 library cosmids.

<table>
<thead>
<tr>
<th>NZP2037 library cosmid</th>
<th>MAFF303099 genome co-ordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSKB1</td>
<td>4578147-4597888</td>
</tr>
<tr>
<td>pSKB4</td>
<td>4582426-4605556</td>
</tr>
<tr>
<td>pSKU1</td>
<td>4191648-4220522</td>
</tr>
<tr>
<td>pSK57C</td>
<td>4184761-4208836</td>
</tr>
<tr>
<td>pSK105B</td>
<td>4184340-4203771</td>
</tr>
<tr>
<td>pSK139F</td>
<td>4191653-4220736</td>
</tr>
</tbody>
</table>

*MAFF303099 genome co-ordinates for exoB are 4583943-4584944 and for exoU 4191750-4192722.
To confirm that the four *exoU*-encoding NZP2037 cosmids were able to complement R7AexoU, they were mated into both R7A and R7AexoU backgrounds. As expected, all cosmids restored mucoid colony morphology to R7AexoU; however, the colonies exhibited an abnormal morphology which was also apparent in the R7A background. The colonies initially appeared to grow normally but after several days they began to exhibit a ‘watery’ appearance. This phenotype was more pronounced in strains harbouring the pSKU1 or pSK139F cosmids (Figure 7.6).

![Figure 7.6: Abnormal colony morphology of R7A and R7AexoU harbouring NZP2037 *exoU*-encoding cosmids.](image)

Both R7A and R7AexoU exhibited ‘watery’ colonies following several days growth on G/ RDM when NZP2037 *exoU*-encoding cosmids were present. A) R7A/ pSKU1; B) R7A/ pSK57C; C) R7A/ pSK105B; and D) R7A/ pSK139F.
7.2.4 Complementation of R7A and R7A EPS mutant strains symbiotic phenotypes with the NZP2037 cosmid library.

One of the main reasons for constructing the NZP2037 cosmid library was to introduce it into R7A, R7AexoB and R7AexoU in an attempt to identify NZP2037 cosmids that influenced the symbiotic proficiency of the strains on *L. japonicus* Gifu and *L. pedunculatus*.

7.2.4.1 Complementation of R7AexoU on *L. japonicus* Gifu with the NZP2037 cosmid library

R7AexoU only induces the formation of small uninfected bumps on *L. japonicus* Gifu whereas NZP2037ΔexoU is symbiotically proficient on this host. NZP2037 cosmids were introduced into R7AexoU by triparental filter matings (Section 2.6.14.2) and dilutions were plated on G/RDM containing Nm and Tc. Transconjugants were prepared for plant inoculation as described in Section 2.10.3.

Forty plant plates, each containing five *L. japonicus* Gifu seedlings, were inoculated with suspensions representing 50-100 transconjugant R7AexoU/ NZP2037 cosmid clones or appropriate control strains. Nitrogen-fixing nodules began developing on some plant plates as early as 10 dpi and these plants exhibited growth indistinguishable to that of control R7A-inoculated plants. Twenty random nitrogen-fixing nodules were harvested from plants six weeks post-inoculation and the inhabiting rhizobia were recovered from nodule crushes (Section 2.10.6). All recovered isolates exhibited the ‘watery’ mucoid colony morphology on G/RDM described above. The complementing NZP2037 cosmids were recovered from six isolates by mating into *E. coli* HB101. Cosmid DNA isolated from these clones all exhibited the same restriction pattern as pSKU1 and pSK139F, indicating that the complementation of the R7AexoU symbiotic phenotype was due to the presence of an *exoU*-encoding NZP2037 cosmid.

7.2.4.2 Attempts to complement the *L. pedunculatus* symbiotic phenotype of R7A, R7AexoB and R7AexoU with the NZP2037 cosmid library

In attempts to identify the factors responsible for NZP2037’s unique ability to form effective nodules on *L. pedunculatus*, the NZP2037 library was mated into R7A, R7AexoB and R7AexoU backgrounds with transconjugants screened for their symbiotic ability on *L. pedunculatus*. The reasoning for including the EPS-deficient
R7AexoB and R7AexoU strains was that production of any alternative EPS or similar factors encoded on NZP2037 cosmids should not be masked by wild-type EPS in these strains.

Three screening experiments were performed for each of the three R7A backgrounds. In each experiment, 20 plant plates containing five *L. pedunculatus* seedlings each were inoculated with suspensions representing 50-100 transconjugant colonies for each of the three different R7A backgrounds. If averaged out to 75 transconjugants per plate, that equated to 1500 different transconjugants in each background examined per screening experiment. NZP2037-inoculated control plants began to form nodules by 10 dpi, whilst only small white bumps and no nitrogen-fixing nodules developed on any of the plants inoculated with the transconjugant clones in any of the R7A backgrounds.

One possible reason for the inability of the complemented R7A strains to successfully nodulate *L. pedunculatus* was that the inoculation with ~75 different transconjugants may mask any potential symbiotically proficient clone due to competition from the vast majority of impaired clones in the inoculum. To test this, matings were carried out to introduce the NZP2037 cosmid library into R7A and R7AexoU as described previously and individual colonies were used to inoculate individual *L. pedunculatus* seedlings. A sterile toothpick was used to streak the colony alongside and below the tip of the root. In total 400 plants (40 plant plates) were inoculated each with either R7A or R7AexoU transconjugants. Again, only small ineffective bumps were observed on plants with no nitrogen-fixing nodules evident.

### 7.2.5 Multiple factors may be involved in the NZP2037 symbiotic phenotypes

There are several possible explanations as to why the complementation of R7A strains with the NZP2037 cosmid library to allow nodulation of *L. pedunculatus* was unsuccessful. The NZP2037 cosmids library encode a maximum of 30 kb of genomic DNA each and it is possible that a gene cluster responsible for the symbiotic ability spans a larger region or the genes may not be clustered. Another possibility is that multiple NZP2037 factors may be required to complement R7A and complementation may therefore not be possible with just one NZP2037 library cosmid.
Investigating NZP2037 genomic regions potentially involved in the strain’s symbiotic phenotypes is difficult due to the fact that the genome sequence of the strain is not yet available. However, a symbiosis island integrated into the genome of NZP2037 has been sequenced and annotated (T. Kaneko, personal communication to C. Ronson). One particular gene of interest present on this island that is absent from the genomes of R7A and MAFF303099 is *nodU*, encoding a carbamoyltransferase. The presence of *nodU* in NZP2037 is likely responsible for the strain producing a Nod factor that differs from other *M. loti* through the incorporation of an additional carbamoyl group on the non-reducing terminal residue (Lopez-Lara et al., 1995). Given the crucial role Nod factor plays in establishment of a successful symbiosis, we speculated that *nodU* may be an important factor in determining the symbiotic phenotype of NZP2037. However, other factors in addition to the modified Nod factor may additionally be required, as suggested by the lack of complementation of R7A strains with the NZP2037 cosmid library.

### 7.2.5.1 Isolation of a NZP2037 genomic region encoding *nodU*

Primers nodUL and nodUR were designed to amplify 409 bp of *nodU* based on *Rhizobium spp.* NGR234 sequence to screen the NZP2037 cosmid library for a *nodU*-containing clone. A product of the expected size was amplified from NGR324 template DNA but no product was amplified from NZP2037 template DNA. This indicated that the sequence of the *nodU* genes differed between NGR234 and NZP2037 in the primer regions.

As a result of the inability to obtain a PCR product using NZP2037 template DNA, a Southern hybridisation based approach was employed to isolate the NZP2037 genomic region encoding *nodU*. NZP2037 genomic DNA was digested with nine frequent-cutter restriction enzymes, separated on an agarose gel and then Southern hybridization performed (Section 2.6.6) with the NGR234 *nodU* PCR product as probe. The results showed that *nodU* was present on a ~4 kb EcoRV fragment. A large-scale EcoRV restriction digest of NZP2037 genomic DNA was then performed and run on an Eckhart agarose gel. Four thin slices of the gel (2-3 mm each) were cut out with the first slice in line with the 4.4 kb marker. The remaining slices represented progressively smaller EcoRV genomic fragments. DNA was extracted from the gel slices (Section 2.6.10.1), run on an agarose gel and again hybridized to
the NGR234 \textit{nodU} probe. The results indicated that gel slice 2 contained the \textit{nodU}-encoding NZP2037 genomic fragment. The genomic fragments from gel slice 2 were then cloned into EcoRV-digested pBLUESCRIPT and 80 of the transformed clones stored in a microtitre plate in individual wells (Section 2.6.13.5).

DNA extracted from the stored clones using the colony-crack protocol (Section 2.6.3) was probed with the NGR234 \textit{nodU} PCR product by Southern hybridization. This led to the identification of clones in microtitre plate wells B6 and F5 as potentially harbouring NZP2037 \textit{nodU} DNA and these were named pSKB6 and pSKF5. Sequencing of pSKB6 and pSKF5 plasmid DNA was performed using M13 forward and M13 reverse primers as well as primers that read out from \textit{nodU} (\textit{nodUROL}/ \textit{nodUROR}). The results indicated that the plasmids were identical and encoded the entire \textit{nodU} gene and 483 bp of the upstream region.

\textbf{7.2.5.2 Introduction of NZP2037 \textit{nodU} into R7A and R7AexoB strains}

In order to be able to introduce the NZP2037 \textit{nodU} gene into R7A strains, it was necessary to first clone the genomic fragment into suitable plasmids that would be compatible with the NZP2037 cosmid library. Due to their stability, antibiotic-resistance genes and compatibility with pIJPAR, plasmids pPROBE-KT (Nm\textsuperscript{R}) and pPROBE-GT (Gm\textsuperscript{R}) were chosen. The \textit{nodU} fragment was gel-extracted as an EcoRV fragment from pSKB6 and pSKF5 and cloned into pPROBE plasmids that had been digested with EcoRI/ Asp718 (pPROBE-KT) or PstI/ SalI (pPROBE-GT) and end-filled using Klenow (Section 2.6.10.2) to form pSKNU-KT and pSKNU-GT. Clones were confirmed by BamHI digestion. \textit{E. coli} S17-1/\lambda pir cells transformed with pSKNU-KT and pSKNU-GT were then used as donors in biparental spot matings to transfer the plasmids to R7A and R7AexoB.

\textbf{7.2.5.3 Effect of NZP2037 \textit{nodU} on the symbiotic phenotypes of R7A and R7AexoB}

Plant nodulation assays were carried out to investigate the symbiotic effects of NZP2037 \textit{nodU} in R7A and R7AexoB backgrounds in either the presence or absence of clones from the NZP2037 cosmid library. Matings to introduce the NZP2037 cosmid library were performed as described above. Dilutions of matings were spread on G/ RDM media containing antibiotics to select for both the NZP2037 \textit{nodU} constructs (pSKNU-KT or pSKNU-GT) and the NZP2037 library cosmid. Inocula
consisting of between 10-300 transconjugants were prepared as described in (Section 2.10.3) and used to inoculate plant plates, each containing five *L. pedunculatus* seedlings. For the R7A background, 20 plant plates were inoculated with suspensions representing ~300 transconjugants and 20 plates representing ~30 transconjugants per plate. For the R7AexoB background, 20 plates were inoculated with suspensions representing ~150 transconjugants and 20 plates with ~15 transconjugants.

NZP2037-inoculated control plants formed nitrogen-fixing nodules whilst control R7A and R7AexoB plants formed only small bumps. Plants inoculated with either R7A or R7AexoB harbouring the NZP2037 *nodU* plasmids formed mainly small bumps but also larger, abnormally-shaped nodule-like structures. Plants inoculated with R7A or R7AexoB containing both the NZP2037 *nodU* constructs and the NZP2037 cosmid library exhibited nodule formation indistinguishable to plants inoculated with R7A or R7AexoB harbouring only the NZP2037 *nodU* construct. The abnormal nodule-like structures induced by R7A and R7AexoB expressing NZP2037 *nodU* appeared elongated and lacked the characteristic spherical shape of determinate nodules. The nodules appeared rough on the surface with callous growth evident (Figure 7.7).

![Figure 7.7: Abnormal nodules formed by R7A and R7AexoB with NZP2037 nodU.](image)

Examples of the nodules formed on *L. pedunculatus* 42 dpi with A) and B) NZP2037; C) and D) R7A; E) R7A/ pSKNU-KT; F) and G) R7A/ pSKNU-KT/ NZP2037 library and H) R7AexoB/ pSKNU-GT/ NZP2037 library.
Only the NZP2037-inoculated control plants appeared healthy at the end of the nodulation assay with all plants inoculated with the R7A strains exhibiting the effects of nitrogen starvation, including severely limited growth and yellowing leaves (Figure 7.8). Nodule crushes (Section 2.10.6) were performed on several of the larger abnormally shaped nodule-like structures formed on plants inoculated with R7A or R7AexoB harbouring NZP2037 \textit{nodU} but in all cases no rhizobia were able to be recovered, indicating that these nodules were uninfected.

\textbf{Figure 7.8: Nodulation of \textit{L. pedunculatus} by R7A and R7AexoB harbouring NZP2037 \textit{nodU}.}

\textit{L. pedunculatus} plant appearance and nodule development 42 dpi with the indicated strains. Plants were inoculated with R7A/ pSKNU-KT and R7AexoB/ pSKNU-GT in both the presence and absence of the NZP2037 cosmid library.
7.2.5.4 R7A and R7AexoB strains harbouring the NZP2037 nodU plasmids are symbiotically proficient on L. corniculatus

R7A and R7AexoB strains expressing NZP2037 nodU were used to inoculate L. corniculatus to see if the nodU gene affected their nodulation ability. R7A and R7AexoB nodulate L. corniculatus at comparable rates with nitrogen-fixing nodules appearing within 10 dpi. NZP2037 is also able to nodulate L. corniculatus but is less efficient with the first nitrogen-fixing nodules not appearing until ~21 dpi. L. corniculatus seedlings were observed over a 6-week nodulation assay which revealed that the expression of NZP2037 nodU had no effect on L. corniculatus nodulation by R7A or R7AexoB.

7.3 Discussion

This chapter investigated the requirement for EPS in the establishment of symbiosis between NZP2037 and determinate-nodule-forming hosts. Previous studies had revealed that EPS mutant strains of NZP2037 including exoU mutants remained symbiotically proficient on determinate hosts (Hotter & Scott, 1991; Kelly, 2007).

In-frame markerless deletion mutant strains NZP2037ΔexoB and NZP2037ΔexoU were constructed and analysed. NZP2037ΔexoB exhibited Congo red and Calcofluor properties analogous to the R7AexoB mutant. Interestingly, the cell aggregation and Congo red absorption phenotypes of NZP2037ΔexoU were analogous to those of R7AexoU; however, the Calcofluor fluorescence of NZP2037ΔexoU was not as intense as that of NZP2037 or the R7AexoU mutant. This observation may indicate differences in truncated EPS production or cell surface components of NZP2037ΔexoU compared to R7AexoU. Preliminary analysis of NZP2037 EPS indicated that it is very similar and perhaps identical to that produced by R7A and MAFF303099 (Section 4.2.5). This suggests that any truncated form of EPS produced by NZP2037ΔexoU should be comparable to that produced by R7AexoU, as was evidenced by the Calcofluor-binding halo around NZP2037ΔexoU colonies grown on G/ RDM containing Calcofluor (Figure 7.2C). Evidence was presented in Chapters 5 and 6 that R7A exoU mutants induce uninfected nodules on L. corniculatus because the truncated EPS secreted by the mutant induces a plant defence response in the absence of full-length EPS. If this hypothesis is correct, then NZP2037ΔexoU either must secrete reduced amounts of the truncated EPS or another polysaccharide
produced by NZP2037 must be able to signal to suppress the plant defence response that would otherwise be induced. Interestingly, differences in the nodulation of *L. corniculatus* by R7A and NZP2037 were observed, with nodule formation delayed and overall nodule numbers reduced on NZP2037-inoculated plants compared to R7A-inoculated plants. This suggested that symbiotic factors produced by NZP2037 are not as efficient as those of R7A on *L. corniculatus*.

If NZP2037 produces an alternative polysaccharide that allows nodulation to proceed in the *exoU* mutant strain, this factor may also be responsible for the strain’s unique ability to form infected nodules on *L. pedunculatus*. Inoculation of *L. pedunculatus* with R7A results in the formation of small uninfected nodule primordia comparable to those induced by symbiotically-impaired R7A EPS mutants on *L. corniculatus* and *L. japonicus*. The ability of NZP2037 to form effective nodules on *L. pedunculatus* has been correlated with the strain’s tolerance of a prodelphinidin-rich flavolan (condensed tannin) present within *L. pedunculatus* nodules that is not found in other *Lotus* host nodules, including *L. corniculatus* and *L. japonicus* (Pankhurst & Jones, 1979). The tolerance of NZP2037 to the prodelphinidin-rich flavolan was attributed to binding of the compound by a polysaccharide that was absent from the symbiotically-impaired strain NZP2213 (Jones *et al.*, 1987). The flavolan-binding polysaccharide (FBP) was covalently linked to the peptidoglycan layer of NZP2037 and was only able to be extracted from exponential growth phase cultures, although it remained attached to the cell surface of stationary phase cultures. Addition of the FBP to NZP2213 cell suspensions afforded concentration-dependent protection of the NZP2213 cells from the bactericidal effects of the *L. pedunculatus* prodelphinidin-rich flavolan, although application of the FBP to *L. pedunculatus* inoculated with NZP2213 did not allow NZP2213 to form effective nodules. However, co-inoculation of *L. pedunculatus* with NZP2037 and NZP2213 resulted in the development of effective nodules that contained both strains, suggesting that NZP2037 FBP provided protection from the flavolan to NZP2213 cells. The polysaccharide responsible for the observed tolerance has not been identified although the authors note that the growth-phase dependent ability to extract FBP may relate to observations that both LPS and KPS exhibit changes as cells progress through the growth cycle.
The production of LPS or KPS by NZP2037 that differs or is absent from R7A could account for the contrasting phenotypes of the \textit{exoU} mutants, if the unique polysaccharide is capable of supressing a defence response elicited by the truncated EPS produced as a result of the \textit{exoU} mutation. \textit{S. meliloti} LPS has been demonstrated to supress \textit{M. truncatula} defence responses (Tellstrom \textit{et al.}, 2007) providing support for the idea that altered LPS production by NZP2037 may be responsible for the nodulation ability of NZP2037\textdelta exoU. KPS production by NZP2037 may also be potentially involved as functional complementation of EPS-deficient \textit{S. meliloti} by KPS has been demonstrated (Pellock \textit{et al.}, 2000).

The genome of NZP2037 is presently being sequenced and the sequence when available should facilitate the identification of potential genes involved in the production of a polysaccharide or alternative factor involved in the strain’s symbiotic proficiency. In the absence of a genome sequence, an NZP2037 cosmid library was constructed and used in complementation-based approaches to attempt to identify NZP2037 genomic regions responsible for the strain’s symbiotic ability. The ability of the library to complement \textit{Mesorhizobium} strain CJ6 to Dct+ and to complement R7A \textit{exoU} and \textit{exoB} mutants indicates that the library was representative of the genome.

Interestingly, the presence of the various NZP2037 \textit{exoU}-encoding library cosmids resulted in abnormal mucoid colony growth in both R7A and R7AexoU backgrounds. Potentially, the NZP2037 \textit{exoU} library cosmids contain other EPS biosynthesis gene(s), either structural or regulatory, that affect normal EPS production by R7A. Certain \textit{R. leguminosarum} quorum-sensing mutants exhibit increased expression of the \textit{plyB} gene that encodes an extracellular glycanase, resulting in changes to the appearance of mucoid colonies due to cleavage of the EPS (Edwards \textit{et al.}, 2009; Frederix \textit{et al.}, 2011). The \textit{exoK} gene that encodes an extracellular glycanase is located downstream of \textit{exoU} in both MAFF303099 and R7A, suggesting that the NZP2037 \textit{exoU} cosmids that cause the abnormal colony morphology likely also contain an \textit{exoK} homologue. Therefore, the observed colony morphology may be the result of increased extracellular glycanase production resulting in the degradation of the mucoid HMW EPS, although ExoK is thought to cleave only nascent EPS chains, not mature EPS (York & Walker, 1998b).
The successful complementation of R7AexoU in planta leading to recovery of cosmids containing exoU demonstrated that symbiotic complementation of impaired R7A strains with the NZP2037 cosmid library was feasible. The main aim was then to complement R7A and R7AexoB with the NZP2037 cosmid library and isolate strains symbiotically proficient on L. pedunculatus to identify NZP2037 genes responsible for the strain’s unique symbiotic phenotype. R7AexoB was included in the complementation assays on the basis that any potential polysaccharide produced by gene products encoded by NZP2037 would not be masked by the production of R7A EPS. Despite multiple complementation assays, no developed nodules formed on any L. pedunculatus plants inoculated with either R7A or R7AexoB containing the NZP2037 library cosmids. This suggested that multiple factors may be involved in the symbiotic proficiency of NZP2037.

Nod factors produced by R7A and NZP2037 share a similar structure, except that NZP2037 Nod factor differs through the incorporation of a carbamoyl group on the C6 of the non-reducing terminal residue (Lopez-Lara et al., 1995). It was speculated that this extra carbamoyl group may be one of the factors R7A was missing that was influencing the symbiotic response on L. pedunculatus. The symbiosis island of NZP2037 contains a nodU gene (T. Kaneko, personal communication to C. Ronson). Previous studies have shown that nodU gene product is responsible for the addition of a carbamoyl group to Nod factor (Gottfert et al., 1990; Jabbouri et al., 1995), suggesting that the gene was responsible for the difference in Nod factor structure observed between NZP2037 and R7A. Plasmids containing a ~4.2 kb NZP2037 genomic fragment that contained the nodU gene were constructed and introduced into R7A and R7AΔexoB.

The results obtained from L. pedunculatus nodulation assays suggested that the presence of NZP2037 nodU in R7A and R7AΔexoB resulted in the production of a modified Nod factor as larger elongated structures with callous-like growth were formed by the strains. These nodule-like structures were distinct from the small nodule primordia usually formed by R7A and from the spherical nodules formed by NZP2037. The stunted growth of plants harbouring the abnormal nodules and the inability to recover any bacteria from within them indicated that they remain uninfected. This result suggests that the modified Nod factor induced nodule
development altered from that of R7A Nod factor but that the R7A strains remained unable to infect the nodules. Introduction of the NZP2037 cosmid library into R7A and R7AexoB strains carrying the nodU-expressing constructs did not yield isolates showing improved nodule development compared to R7A strains harbouring only the nodU plasmids.

The results of experiments performed in this chapter suggest that if a unique polysaccharide produced by NZP2037 is responsible for the strain’s exoU mutant and *L. pedunculatus* symbiotic phenotypes, it is encoded by a large cluster of genes or genes located apart on the genome, given that the NZP2037 library cosmids contain ~22 kb genomic fragments. The biosynthesis of both LPS and KPS by rhizobia involves multiple gene loci spread around the genome (Kereszt *et al*., 1998; Noel & Duelli, 2000). Alternatively, multiple factors may be involved in the symbiotic phenotypes and therefore R7A strains would need to harbour multiple NZP2037 library cosmids to establish a successful symbiosis with *L. pedunculatus*. Obtaining the entire genomic sequence of NZP2037 and comparing it to that of the MAFF303099 and recently available R7A sequences would greatly assist in determining the potential for alternative polysaccharide production by NZP2037. The possibility also exists that rather than NZP2037 producing a unique factor that accounts for its nodulation ability, R7A and other *M. loti* may produce a factor, absent from NZP2037, that signals incompatibility to *L. pedunculatus*. 
8 Concluding remarks
This study investigated whether *M. loti* EPS functions as a secondary signalling molecule in the establishment of a determinate-nodule-forming symbiosis with *Lotus* hosts. Although a symbiotic association, the invasion of plant nodules by rhizobia bears parallels to plant pathogen interactions with the success of the outcome in both instances requiring either avoiding recognition by the host plant’s innate immune response or suppressing its activation.

A model for the functioning of R7A EPS as a suppressor of plant innate immunity in response to perception of microbe-associated molecular patterns (MAMPs, also known as PAMPs for pathogen-associated molecular patterns) is proposed (Figure 8.1). In the wild-type situation, R7A MAMPs are perceived by the *Lotus* plant through pattern recognition receptors (PRR) and PAMP-triggered immunity (PTI) is induced. This initial defence response is suppressed through the signalling action of R7A EPS, allowing IT formation and nodule organogenesis to proceed. Transcriptome analysis of host plants following inoculation with wild-type rhizobia suggests that suppression of an initial defence response occurs. Investigation of *M. truncatula* gene expression 1 h post-inoculation with *S. meliloti* indicated an increase in the expression of plant defence-related genes; however, expression of these genes was suppressed by 6 h and remained low for several days (Lohar et al., 2006). Down-regulation of defence genes was also observed in *L. japonicus* 24 h post-inoculation with *M. loti*, indicating the strain was recognised as compatible (Hogslund et al., 2009). Evidence for the involvement of EPS in suppression of the initial defence response is provided by analysis of *M. truncatula* gene expression following inoculation with wild-type or EPS-deficient strains. At three days post-inoculation, defence-related gene expression remained up-regulated in plants inoculated with the EPS-deficient strain compared to plants inoculated with the wild-type strain (Jones et al., 2008).

Aslam et al. (2008) suggest that bacterial EPS suppresses PTI via chelation of Ca\(^{2+}\) ions, which function as messengers following MAMP perception and are required for the induction of PTI. However results obtained in this study suggest that this mechanism is unlikely to account for the suppression of PTI in the *M. loti - Lotus* symbiosis. R7AexoU3’ was symbiotically proficient on all hosts examined and produced EPS similar in composition to that of R7A. The strain’s colony morphology
indicated that only small quantities of full-length EPS were produced while the halo around colonies of the strain grown on G/ RDM agar containing Calcofluor indicated that the strain still produced the LMW EPS characteristic of R7AexoU. Furthermore, co-inoculation experiments indicated that the production of wild-type EPS in trans by R7AΔnodA was sufficient to allow R7AexoU to form extended ITs. Taken together, these results support a signalling role for full-length EPS in suppressing the defence response. In addition, the symbiotic effectiveness of EPS-deficient strains R7AexoB and R7AΔexoA (R7AΔexoA less effective on L. japonicus) that lack any wild-type EPS also suggests that chelation of Ca\(^{2+}\) by EPS is not responsible for PTI suppression. Evidence contrary to the functioning of EPS as a Ca\(^{2+}\) sequestering molecule in rhizobia-legume interactions is also provided by numerous studies that indicate it is LMW EPS that is the symbiotically-active form (Battisti et al., 1992; Djordjevic et al., 1987; Urzainqui & Walker, 1992; Wang et al., 1999). Furthermore, an exoK mutant of Rhizobium sp. NGR234 that produced only HMW EPS was symbiotically impaired despite the fact that its Ca\(^{2+}\)-sequestering potential is presumably unaffected (Staehelin et al., 2006).

**Figure 8.1: Model for R7A EPS acting as a signal to suppress the induction of PTI.**

Perception of MAMPs by the host plant induces PTI. Wild-type R7A EPS acts as a signal and is perceived by a host receptor that leads to suppression of PTI. IT formation and nodulation proceeds through the perception of compatible Nod factor.
The symbiotic effectiveness of R7AexoB was determined to not be due to wild-type EPS production by examining the phenotypes of R7AexoBexoU. Therefore, the symbiotic ability of R7AexoB is due either to complementary signalling by an alternative polysaccharide in the absence of any form of wild-type EPS or to the absence of surface molecules that normally act as MAMPs to activate a plant defence response (Figure 8.2).

Chemical analysis performed in this study revealed that the LMW fraction of R7AexoB contained 2-Glc representing CBG as well as other variously-linked Glc residues that may represent an alternative polysaccharide involved in complementary signalling. Functional complementation of EPS-deficient rhizobia by alternative polysaccharides has previously been reported (Glazebrook & Walker, 1989; Gonzalez et al., 1996a; Pellock et al., 2000; Reuhs et al., 1995). Alternative polysaccharides have also been shown to act as suppressors of plant defence responses in pathogenic interactions. A Xanthomonas campestris ndvB mutant deficient in CBG production was found to be compromised in the infection of Nicotiana benthamiana, due to induction of defence responses. The addition of purified CBG to either the same leaf or a different leaf to that inoculated with the ndvB mutant restored virulence, indicating that CBG induced systemic suppression of defence responses (Rigano et al., 2007).

Previous investigations of LPS produced by the R7A EPS mutants revealed no differences compared to R7A (Kelly, 2007). However, more refined analysis (performed by Dr Artur Muszynski) has indicated that R7AexoB produces LPS that lacks Gal in the lipid A/core region. A pleiotropic effect of the exoB mutation on the biosynthesis of LPS has been reported in other rhizobia (Canter Cremers et al., 1990; Long et al., 1988; Sanchez-Andujar et al., 1997). The symbiotic ability of R7AexoB may be the result of the production of an altered LPS that is no longer recognised as a MAMP and therefore a defence response is not induced. The LPS of various plant-pathogenic bacteria have been shown to induce plant defence responses including nitric oxide production and up-regulation of defence-related genes (Desaki et al., 2006; Silipo et al., 2005; Zeidler et al., 2004). The observation that non-pathogenic X. campestris produces an altered LPS that does not induce plant defence responses (Silipo et al., 2008) provides support to the idea that modified LPS production by
R7AexoB may be involved in the strain’s symbiotic proficiency. R7AexoB was not able to overcome the symbiotic inhibitory effect of pre-inoculation with R7AexoU unlike R7A, suggesting that R7AexoB lacks the ability to suppress defence responses.

**Figure 8.2: Proposed models accounting for the symbiotic proficiency of R7AexoB.**

A) An alternative polysaccharide functionally complements for the lack of EPS, leading to suppression of PTI in the absence of any form of wild-type EPS production by R7AexoB. B) R7AexoB is affected in other surface components that normally act as MAMPs and as a result the plant is ‘blind’ to R7AexoB and a defence response is not initiated, allowing nodulation to proceed.

R7AexoU is severely impaired in nodulation at the stage of IT formation, possibly due to the production of a truncated EPS molecule that either does not signal compatibility to the host or is actively perceived by the plant resulting in the activation of a defence response (Figure 8.3). In addition, the production of truncated EPS may prevent complementary signalling by an alternative polysaccharide that is active in the R7AexoB background.
Co-inoculation and pre-inoculation results suggest that the response elicited by R7AexoU can be dampened by the wild-type EPS molecule and allow nodulation to proceed. EPS provided \textit{in trans} by R7AΔnodA was sufficient to allow R7AexoU to initiate IT development and extension, but as few nodules were found to contain solely R7AexoU, it would appear that release from the IT into the nodule primordia was inhibited. This suggests that R7A EPS is required for IT initiation and release as well as providing further evidence that EPS acts as a signalling molecule rather than forming a protective sheath. Induction of a defence response at the stage of IT release provides a further checkpoint for the plant to prevent pathogens ‘hitchhiking’ in the IT. R7AexoU formed extended ITs on \textit{L. japonicus} MG-20 plants regardless of root-lighting conditions but release from ITs was impaired on roots that were exposed to light while R7AexoU were released and infected nodules on roots that were kept in the dark, supporting the idea of a second-checkpoint.

![Figure 8.3: Proposed models accounting for the symbiotic impairment of R7AexoU.](image)

A) R7AexoU produces truncated EPS that fails to signal for suppression of PTI. The truncated EPS may be sufficient to bind to an EPS receptor but fail to effectively signal and therefore block access to the receptor by alternative polysaccharides. B) Truncated EPS produced by R7AexoU may signal incompatibility and promote activation of PTI.
The isolation of symbiotically effective R7AΔexoU suppressor mutants, several of which contained transposon insertions in genes that had not previously been implicated in EPS biosynthesis, was of great interest. Investigations into the genetic determinants of the R7AΔexoU/ mTn5 suppressor strains proved difficult due to an inability to reconstruct the strains by directed mutagenesis and the results of complementation approaches. However, the suppressor mutants all shared common colony characteristics including non-aggregating broth culture growth and easily dispersed G/ RDM agar colony growth as well as a Calcofluor-dark phenotype and the absence of a fluorescent halo around colonies grown on G/ RDM agar containing Calcofluor. These characteristics are in contrast to those displayed by R7AΔexoU and similar to those of the symbiotically proficient R7AexoB strain and suggest that the suppressor mutants no longer secrete the LMW EPS secreted by R7AΔexoU.

The finding that directed mutation of the novel genes identified in the suppressor screen in an R7A background had no effect on wild-type EPS production suggested that alternative bacterial factors may be involved in the observed phenotypes. The colony properties of R7AexoU and the dispersal of cell aggregates by cellulase suggested that cellulose may be involved. However, mutation of the only cellulose synthase homologue in M. loti had no effect on the strain’s phenotypes. An alternative explanation is that the R7AexoU truncated EPS may in effect ‘mimic’ cellulose production with chemical analysis results supporting this as R7AexoU LMW extract was found to consist solely of Glc residues. Based on obtained linkage data and the proposed R7A EPS biosynthesis pathway, truncated EPS produced by R7AexoU would be expected to consist of [(1,4-Glc),-1,6-Glc]. The absence of Gal may be due to export of the truncated molecule requiring cleavage from the lipid carrier.

The symbiotic proficiency of the R7AΔexoU/ mTn5 suppressor strains may therefore be due to a lack of production of the truncated EPS, as supported by the absence of the Calcofluor-fluorescent halo around suppressor mutant strain colonies grown on G/ RDM containing Calcofluor. The question remains as to how the identified genes disrupted by transposon insertions cause a lack of truncated EPS production with one possible explanation being that export of the truncated EPS occurs by a different mechanism than wild-type EPS, perhaps involving ABC transporters, although further work is required to demonstrate this.
M. loti NZP2037 EPS mutants were found to be symbiotically proficient regardless of the stage at which the EPS biosynthesis pathway was disrupted. NZP2037ΔexoU exhibited colony characteristics comparable to R7AexoU, including the cell aggregation property and the presence of a fluorescent halo around the strain when grown on G/ RDM containing Calcofluor, suggesting that the strain also produces a truncated EPS molecule. Attempts to identify NZP2037 factors responsible for the symbiotic proficiency of the EPS mutants and the ability of NZP2037 to nodulate L. pedunculatus proved unsuccessful. It may be that multiple factors are responsible for the symbiotic ability of NZP2037 or that the genes that encode for one factor responsible are not clustered on the genome. Genetic loci involved in LPS production by rhizobia are spread around the genome (Noel & Duelli, 2000) and given that results obtained in this study indicate LPS production may influence the symbiotic phenotype of R7A EPS mutant strains, it is plausible that the differing symbiotic properties of NZP2037 strains relate to altered LPS production compared to R7A. Results obtained by Jones et al. (1987) indicate that the FBP identified as responsible for the ability of NZP2037 to nodulate L. pedunculatus may be LPS-related.

The results presented in this thesis are part of a collaborative effort into investigating carbohydrate signalling in the establishment of symbiosis between M. loti and Lotus. In parallel to the work reported here, investigations into the requirement for R7A EPS in symbiosis with a focus on the Lotus hosts has been carried out in Professor Jens Stougaard’s laboratory at Aarhus University, Aarhus, Denmark, largely by Drs Yasuyuki Kawaharada and Niels Sandal.

Genes involved in the contrasting symbiotic phenotype of R7AexoU on L. japonicus Gifu and L. japonicus MG-20 (depending on growth lighting conditions) are being investigated through crosses of the two species and subsequent map-based cloning. A large-scale plant suppressor mutant screen was also undertaken to identify L. japonicus Gifu mutants that formed nitrogen-fixing nodules in association with R7AexoU. Several suppressor mutants have been isolated that form full-length ITs when inoculated with R7AexoU, resulting in an increase in the number of infected nodules formed. Determining the genes mutated in these suppressor mutant plants through map-based cloning and deep sequencing techniques is on-going and may
lead to the identification of plant receptors for EPS and/or components of the innate immune system.

8.1 Future directions

Characterisation of the R7AΔexoU/ mTn5 suppressor mutants and determining how they are affected in truncated EPS production will be of interest. Complementation of the strains is currently being pursued and an R7A library cosmid that fully restores R7AΔexoU phenotypes to R7AΔexoU/5197 has been isolated and is being characterised. Results obtained in this study suggest that R7AΔexoU/2385 does not produce truncated EPS due to a deficiency in the production of UDP-Gal. This could be examined by the introduction of a plasmid containing exoB expressed from an inducible promoter. If the induction of exoB expression results in truncated EPS production, it would indicate that R7AΔexoU/2385 either contains a mutation in the chromosomal copy of exoB or that the transposon insertion in mll2385 affects regulation of the gene.

In order to determine if R7AexoU truncated EPS is exported by a mechanism different to that responsible for wild-type EPS transport, the colony phenotypes of R7A and R7AexoU strains with mutations in genes associated with EPS transport (e.g. exoT and exoQ) could be examined. Determining if such mutations would prevent cell-aggregation and the production of a halo on G/ RDM containing Calcofluor in the R7AexoU background would be of particular interest.

Further experiments are being performed by Dr Artur Muszynski at the CCRC to confirm the proposed R7A EPS structure and identify the site of O-acetyl modifications. Characterisation of R7AexoU and R7AexoB LMW EPS fractions requires up-scaling of the extraction process to gather sufficient quantities of material for purification and detailed structural analysis. This may allow for confirmation of truncated EPS production by R7AexoU and identify if R7AexoB produces an alternative polysaccharide. It would also be of interest to analyse the EPS fractions of the R7AΔexoU/ mTn5 suppressor mutants.

From the host-plant perspective, continuing characterisation of the L. japonicus Gifu suppressor mutants may identify potential plant receptors for EPS that would lead to various genetic and biochemical experiments to characterise ligand interactions and
downstream signalling events. Examination of plant defence-related gene expression in response to inoculation with R7A, R7AexoU and R7AexoB, similar to the studies performed by Jones et al. (2008), would be of interest and may provide support for the proposed models presented in this study.
9 References


Bertram-Drogatz, P. A., Quester, I., Becker, A. & Puhler, A. (1998). The Sinorhizobium meliloti MucR protein, which is essential for the production of high-
molecular-weight succinoglycan exopolysaccharide, binds to short DNA regions upstream of exoH and exoY. Mol Gen Genet 257, 433-441.


Morgan, S. (2007). Surface polysaccharides as signalling molecules in the Mesorhizobium-Lotus symbiosis. BSc Hons Diss Department of Microbiology and Immunology, University of Otago, Dunedin.


Parniske, M., Kosch, K., Werner, D. & Muller, P. (1993). exoB mutants of
Bradyrhizobium japonicum with reduced competitiveness for nodulation of Glycine
max. Mol Plant Microbe Interact 6, 99-106.

of host plants with determinate nodules induced by EPS-defective exoB mutants of
Bradyrhizobium japonicum. Mol Plant Microbe Interact 7, 631-638.

formed by a surface polysaccharide defective mutant of Rhizobium sp strain TAL1145
are delayed in bacteroid development and nitrogen fixation. Mol Plant Microbe Interact 9, 364-372.

efficiency is dependent on Sinorhizobium meliloti polysaccharides. J Bacteriol 182, 4310-4318.


Petrovics, G., Putnoky, P., Reuhs, B., Kim, J., Thorp, T. A., Noel, K. D., Carlson, R.
W. & Kondorosi, A. (1993). The presence of a novel type of surface polysaccharide in
Rhizobium meliloti requires a new fatty acid synthase-like gene cluster involved in
symbiotic nodule development. Mol Microbiol 8, 1083-1094.

released from alfalfa seeds activate NodD2 protein in Rhizobium meliloti. Plant
Physiol 99, 1526-1531.

biology 5, 308-316.

lacking phosphotransferase system enzyme HPr or EIIA are altered in diverse
processes, including carbon metabolism, cobalt requirements, and succinoglycan


Studholme, R. E. (1995). Role of C4-Dicarboxylate transport genes in competitive ability of *Rhizobium leguminosarum* bv *viciae*. MSc Thesis Department of Microbiology and Immunology, University of Otago, Dunedin.


Walker, S. A. & Downie, J. A. (2000). Entry of Rhizobium leguminosarum bv. viciae into root hairs requires minimal Nod factor specificity, but subsequent infection thread growth requires nodO or nodE. Mol Plant Microbe Interact 13, 754-762.


10 Appendices
Appendix A

Growth media

All media and media-stock solutions were autoclaved unless indicated. Davis agar was added (16 g/ L) for solid media. After autoclaving, media was cooled in a 55°C water bath prior to the addition of antibiotics.

Rhizobia defined media (RDM) (per litre)

10 mL Salts solution
10 mL BTB
6 mL NH₄Cl
1 mL Trace elements
100 mg L-histidine

Dissolve and for G/ RDM or S/ RDM adjust to pH 6.5-7.0 with 2 M KOH. For Succ/ RDM add 10 g MES and adjust to pH 6.1-6.3 with solid KOH.

Autoclave

For G/ RDM, add 20 mL 20% Glucose [w/ v] and 10 mL Phosphates solution
For S/ RDM, add 200 mL 20% Sucrose [w/ v] and 10 mL Phosphates solution
For Succ/ RDM, add 20 mL 0.5 M Succinate and 4 mL Phosphates solution

RDM stock solutions

Phosphate solution (per litre)

100 g  K₂HPO₄
100 g  KH₂PO₄

Salts solution (per litre)

25 g  MgSO₄·7H₂O
2 g CaCl$_2$.2H$_2$O

1.5 g FeEDTA (or 0.66 g FeCl$_3$ and 1.5 g EDTA)

20 g NaCl

**Trace Elements** (200 mL)

3 mg ZnSO$_4$.7H$_2$O

40 mg Na$_2$MoO$_4$.2H$_2$O

50 mg H$_3$BO$_3$

40 mg MnSO$_4$.H$_2$O

4 mg CuSO$_4$.5H$_2$O

1 mL COCl$_2$.6H$_2$O (0.2 g/ L)

**Vitamins** (50 mL)

50 mg thiamine HCl

100 mg Ca panthothenate

1 mL biotin (1 mg/ mL, dissolve solution by heating before adding to the other ingredients)

18 g/ L NH$_4$Cl

Make up to 50 mL and filter-sterilise

**Bromothymol Blue (BTB)** (per litre)

2 g BTB

**LB** (per litre)

10 g Bacto-tryptone

5 g Yeast extract

5 g NaCl
**SOB (per litre)**

- 20 g Bacto-tryptone
- 5 g Yeast extract
- 0.5 g NaCl
- 10 mL 0.25 M KCl

Adjust to pH 7 with 10 M NaOH

**TY (per litre)**

- 5 g Bacto-tryptone
- 3 g Yeast extract
- 2 mL CaCl$_2$·6H$_2$O (0.65 g/mL)

For TY containing 0.02% Calcofluor, 200 mg of fluorescent brightener-28 (Sigma) that had been dissolved in 10 mL of milli-Q water and filter-sterilised was added after autoclaving.

**YMB (per litre)**

- 0.5 g K$_2$HPO$_4$
- 0.2 g MgSO$_4$
- 0.1 g NaCl
- 1.0 g Yeast extract
- 10 g Mannitol

For YMB containing 0.005% Congo red, 5 µL of a 1% [w/v] stock solution was added prior to autoclaving
**Jensen’s seedling agar** (per litre)

To 500 mL distilled water add

- 1 g CaHPO$_4$
- 0.2 g K$_2$HPO$_4$
- 0.2 g MgSO$_4$·7H$_2$O
- 0.1 g NaCl
- 0.1 g FeCl$_3$

Mix to dissolve solutes.

**Autoclave**

To 500 mL distilled water add

- 12 g agar

**Autoclave**

Add molten agar to dissolved solutes and pour approximately 40 mL into square (10 x 10 cm) petri dishes sitting on an angle to create a slant of the media. If plants are to be examined by microscopy, place an autoclaved piece of Whatmans lens cleaning tissue on top of the agar slant when it has set.
Appendix B

Buffers and Solutions

Unless otherwise stated, buffers and solutions were made up to 1 L in distilled water and autoclaved.

20x SSC (per litre)

(1x SSC is 0.15 M NaCl, 15 mM sodium citrate [pH 7])

88.2 g Trisodium citrate

175.3 g NaCl

Adjust to pH 7 with concentrated HCl and make up to 1 L with distilled water

1 M TrisHCl (per litre)

121.1 g tris

Adjust to pH 8 with ~45 mL of concentrated HCl and make up to 1 L with distilled water

5 M NaCl (per litre)

292.2 g NaCl

Dissolve on a heated stirrer and make up to 1 L with distilled water

10% SDS (per litre)

100 g sodium dodecyl sulphate

Dissolve at 37°C and make up to 1 L with distilled water, do not autoclave

10 M NaOH (50 mL)

20 g NaOH pellets

Make up to 50mL with distilled water, do not autoclave
**0.5 M EDTA** (200 mL)

37.22 g EDTA (disodium salt)

4 g NaOH

Adjust to pH 8 and make up to 200 mL with distilled water

**50x TAE buffer** (per litre)

(1x TAE is 40 mM Tris-acetate, 1 mM EDTA)

242.9 g tris base

57.1 mL glacial acetic acid

100 mL of 0.5 M EDTA

Make up to 1 L with distilled water, do not autoclave

**Tracking dye** (per mL)

300 μL bromophenol blue (10 mg/ ml)

300 μL glycerol

300 μL Milli-Q water