Characterisation of genes involved in exopolysaccharide biosynthesis and transport in the *Mesorhizobium loti* strain R7A.

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BSc

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Abstract

Rhizobia are bacteria that are important in fixing gaseous nitrogen into a plant-available form via a symbiotic interaction with leguminous plants. In order for this to occur, the legume and the rhizobia must communicate with each other using molecular signals. One molecule that rhizobia produce that has been implicated as a signal is exopolysaccharide (EPS). *Mesorhizobium loti* strain R7A EPS consists of an octasaccharide repeating subunit, comprising a backbone that contains one galactose and three glucose (Glc) residues and a side-branch containing two Glc, one glucuronic acid and a terminal riburonic acid residue. The biosynthesis of EPS is carried out by proteins encoded by genes termed *exo* genes that are arranged in a cluster. The EPS is believed to play a role in promoting infection thread formation by signalling a suppression of the host immune defences. The purified low molecular weight (LMW) form of EPS comprising a single subunit has been shown to bind strongly to the *Lotus japonicus* receptor EPR3. Mutants of *M. loti* R7A affected in the synthesis of the EPS backbone, e.g. *exoYF* and *exoB* mutants, induce effective nodules, albeit with reduced efficiency, while most mutants in genes affecting synthesis of the branch portion of the EPS, e.g. *exoU* mutants, induce uninfected nodules. It has been hypothesised that the latter mutants secrete a truncated form of EPS that is recognised by EPR3 to induce a defence response. The aims of this study were to test these hypotheses by determining whether EPS transport genes were required for the negative phenotype *exoU* mutants and by determining the phenotype of *exoK* mutants. The *exoK* gene encodes a glycanase thought to be necessary for the production of the EPS subunit monomers.

In past experiments, obtaining an in-frame markerless deletion *exoK* mutant in R7A has proven difficult, although polar mutants containing mutations with potential negative effects on downstream genes were acquired using insertion duplication mutagenesis (IDM). In this study, further attempts were made to generate R7A mutants deficient in the production of ExoK as well as attempts to generate a Δ*exoK* mutant in an EPS-deficient background in order to investigate the possibility of lethality caused by the deletion of *exoK*. The results demonstrated that deletion of *exoK* had a lethal effect on R7A when the strain was expressing *exoYF* and growth inhibition was relieved on deletion of the *exoYF* genes. A polar *exoK* mutant obtained by Kelly (PhD thesis) was partially complemented with the *exoK* gene, confirming that *exoK* was deleted and expression of downstream genes was affected in the mutant.
Investigation of a plasmid, pSKU1, was undertaken to identify the involvement of *exoK* expression in regards to a “watery” colony morphology in R7A caused by the plasmid. This involved creating an expression vector to induce over-expression of *exoK*. Over-expression of *exoK* had no perceived effect on any strain tested. Expression of *exoK* from the vector was confirmed showing that the *exoK* gene was not the cause of the phenotype induced by pSKU1. Additionally, it was found that the effect caused by pSKU1 was greatly increased when grown on medium with glycerol as carbon source.

This study also included experiments involving the deletion of the EPS transport gene *exoT* in R7A Δ*exoU* mutants. It was found that a non-polar deletion mutant of *exoT* could only be obtained in strains deficient in *exoYF* expression. This suggested that *exoT* is involved in the secretion of the truncated EPS produced by the Δ*exoU* mutant and that there is no other secretion system that performs that role.
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
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<td>bp</td>
<td>base pair</td>
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<td>bv.</td>
<td>biovar</td>
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<td>dH₂O</td>
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<tr>
<td>dNTP</td>
<td>deoxynucleoside triphosphate</td>
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>EPS</td>
<td>exopolysaccharide</td>
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<td>glucuronic acid</td>
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<tr>
<td>HMW</td>
<td>high-molecular-weight</td>
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<tr>
<td>ICE</td>
<td>integrative conjugative element</td>
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<td>IDM</td>
<td>insertion duplication mutagenesis</td>
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<tr>
<td>IT</td>
<td>infection thread</td>
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<td>kb</td>
<td>kilobase</td>
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<td>L</td>
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<tr>
<td>LMW</td>
<td>low-molecular-weight</td>
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<td>µL</td>
<td>microliter</td>
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<td>N²</td>
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<td>Nod</td>
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<td>NF</td>
<td>nodulation factor</td>
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<tr>
<td>p</td>
<td>plasmid designation</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RibA</td>
<td>riburonic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<tr>
<td>sp.</td>
<td>species</td>
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<tr>
<td>succ</td>
<td>succinate acid</td>
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<tr>
<td>Tris</td>
<td>tris (hydroxymethyl) aminomethane</td>
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<tr>
<td>TAE</td>
<td>tris-acetate</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
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<td>Volt</td>
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<td>w/v</td>
<td>weight to volume ratio</td>
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1. Introduction

1.1. Introduction to the legume-rhizobium symbiosis

The *Rhizobiaceae* comprise a family of alphaproteobacteria, commonly known as rhizobia which form a symbiotic interaction with plants of the *Leguminosae* family. The symbiosis involves the development of root nodules which house a differentiated form of rhizobia known as bacteroids. Bacteroids fix nitrogen through the actions of nitrogenase, a complex enzyme which is extremely sensitive to oxygen and requires a large amount of energy to function as 16 moles of ATP are required to fix 1 mole of $N_2$. In return for fixing nitrogen, the host plant supplies the bacteroids with a carbon source and other nutrients and a microaerophilic environment to facilitate nitrogenase function. This is a mutually beneficial relationship between the rhizobium and its host plant; however the process also bears a resemblance to a pathogen evading the innate immunity of the host. As a result, during the rhizobial attachment to the root hair, the plant host will likely induce an immune response that must be overcome (Vasse *et al*., 1993).

Initiation of the symbiosis involves a two-way signalling process. The plant host produces flavonoids that are detected by the rhizobia which in turn produce a pool of lipochitin oligosaccharide molecules known collectively as Nod factor (NF). NF is detected by the host plant leading to the initiation of nodule development (Oldroyd & Downie, 2008). It has also recently been confirmed that exopolysaccharide (EPS) plays a role as a secondary signalling molecule in nodule development in the *Mesorhizobium-Lotus* symbiotic interaction (Kelly *et al*., 2013; Kawaharada *et al*., 2015).

1.2. The nodule infection process

In order for the symbiotic relationship to establish, the rhizobia must first attach to an emerging root hair which undergoes a morphological change, forming a structure known as a shepherd’s crook. The bacterial cell is then surrounded by the root hair while the root hair tip grows to place the cell wall at the tip in apposition to the rhizobial attachment site. Originating at the hair tip the plant cell wall grows inward to form a tube through the root hair known as an infection thread, which provides a portal for rhizobial entry into the host cortex cells. The rhizobia travel down the infection thread by cell division and, upon reaching the cortical cells which have entered a state of division and have enlarged, they enter plant
cells surrounded by a plant cell-derived peribacteroid membrane to form a symbiosome. Finally, the rhizobia differentiate within the symbiosome becoming nitrogen fixing bacteroids (Gibson et al., 2008). The vascular cells of the plant extend into the nodule during development in order to allow the transport of nutrients and ammonia between the plant host and the rhizobial cells. Within the nodule the plant host produces leghaemoglobin to bind oxygen to produce the required microaerophilic conditions needed for nitrogen fixation (Torres, 2000).

1.3. Nodule structure

There are two different types of nodules that arise during the rhizobium-legume symbiosis: determinate and indeterminate (Figure 1.1). They are differentiated on the basis of the origin of the meristem. Spherical determinate nodules (formed by Lotus and Vigna) derive a temporary meristem from the outer cortical cells while elongated indeterminate nodules (formed by, for example, Trifolium, Viciae, Pisum and Medicago spp.) derive a persistent meristem from inner cortical cells (Gibson et al., 2008). Mesorhizobium loti forms determinate nodules on Lotus and Phaseolus species, though it is also capable of forming indeterminate nodules on Carmichaelia flagelliformis (Desbrosses & Stougaard, 2011; Pankhurst et al., 1987) and strain NZP2037 has been shown to form indeterminate nodules on Leucaena leucocephala (Hotter & Scott, 1991).
Figure 1.1: Determinate and indeterminate nodule structure.

A) Structure of an indeterminate nodule formed on *Medicago truncatula*. B) Structure of a determinate nodule formed on *L. japonicus*. Areas represented in this diagram include the epidermis (green), the nodule meristem (pink), rhizobial infection zone (light blue) and nitrogen fixation zone (dark blue). Figure adapted from Suzaki et al., 2015.

1.4. Rhizobium-derived signalling molecules involved in establishment of the symbiosis

NF consists of an oligosaccharide comprised of β-1,4-linked *N*-acetyl glucosamine residues. A fatty acid residue is located on the non-reducing end of the backbone. There may be variation in the number of *N*-acetyl-D-glucosamine residues forming the backbone and variation in the length and degree of saturation of the fatty acid component. The Nod factor backbone is decorated with a range of species-specific molecules which can be attached at the reducing and non-reducing ends or less commonly on the central residues of the backbone (Perret et al., 2000).

Collectively these structural differences contribute to host-range specificity. Legumes are able to sense and respond to compatible NFs by utilising NF receptor kinases that contain LysM domains in their extracellular regions. LysM receptor kinases were first discovered in *Lotus japonicus* (NFR1 and NFR5) and *Medicago truncatula* (LYK3 and NFP); subsequently
they have been characterised for other legumes such as soybean (Amor et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003).

1.5. The *Mesorhizobium-Lotus Symbiosis*

In this study, the species of rhizobia examined is *Mesorhizobium loti*. Rhizobia isolated from *Lotus* nodules were originally separated into fast- and slow-growing strains (Pankhurst, 1977), with fast-growing strains being termed *Rhizobium loti* (Jarvis et al., 1982). Eventually the *Rhizobium* genus was revised and the *Mesorhizobium* genus arose with ‘meso’ to indicate a slower growth rate than the other fast-growing rhizobia (Jarvis et al., 1997). Within the *Mesorhizobium* genus there are an ever-expanding range of species including the relatively well-characterised members of the genera *M. loti*, *M. huakuii*, *M. ciceri*, *M. tianshanense*, and *M. mediterraneum*. Within the *M. loti* species, the most well-characterised strains include NZP2213, NZP2037, NZP2235, NZP2238, MAFF303099 and R7A. The genomes of strains R7A, MAFF303099, NZP2037, R88b, CJ3Sym and *M. erdmanii* USDA3471 have been sequenced. Additionally, three more strains, SU343, NZP2042 and NZP2014, that nodulate *L. pedunculatus* effectively have been sequenced though this data is currently unpublished (J. T. Sullivan and C. W. Ronson, unpublished data). The symbiosis genes are encoded in clusters termed symbiosis islands on the chromosome in *M. loti* strains (Sullivan & Ronson, 1998). This is a distinguishing feature in *Mesorhizobium spp.* as, aside from *Bradyrhizobium*, other rhizobia, including *S. meliloti*, *Rhizobium* sp. strain NGR234 and *R. leguminosarum*, generally encode symbiosis genes on large plasmids (Freiberg et al., 1997; Glucksmann et al., 1993b). It has been demonstrated that *M. loti* strain R7A is capable of transferring its 502-kb symbiosis island, which is classified as an integrative and conjugative element (ICE) and hence named ICEMiSymR7A, to non-symbiotic strains in both the environment and in laboratory matings (Sullivan et al., 1995; Sullivan & Ronson, 1998).

The ICE contains 414 genes, including all of the genes required for NF synthesis, nitrogen fixation and transfer of the ICEMiSymR7A (Sullivan et al., 2002). This study focuses on strain R7A.
1.6. The genus *Lotus*

The *Lotus* genus of legumes contains over 200 species that generally grow in temperate regions, though some species grow in tropical or subtropical regions. The species *L. japonicus* is a perennial temperate species on which *M. loti* is able to induce the formation of determinate nodules. This legume was designated a model legume for molecular studies due to a short life cycle (2-3 months), self-fertility, a relatively small (472.1 Mb) diploid genome (2n = 12) and it is transformable by *Agrobacterium*, making it suitable for genomic research (Handberg & Stougaard, 1992). The sequence for *L. japonicus* (build 2.5) has been determined for 315 Mb representing 67% of the genome (Sato et al., 2008). There are various DNA, recombinant inbred lines, and TILLING mutant libraries available for investigations involving *L. japonicus* that are available as reviewed by (Sato & Tabata, 2006; Udvardi et al., 2005). Additionally, the endogenous retrotransposon *Lotus* retrotransposon 1 (LORE1) (Madsen et al., 2005) has been used to generate *L. japonicus* mutant libraries that are available for investigating plant phenotypes for particular gene disruptions (Fukai et al., 2012; Mun et al., 2016; Urbanski et al., 2012). There are several ecotypes utilised for molecular studies including *L. japonicus* Gifu and Miyakojima MG20. The closely related species *L. filicaulis* and *L. krylovii* and *L. burttii* can be used as crossing partners with Gifu for genetic analysis (Sandals, 1998; Kawaguchi, 2005; Tabata and Stougaard, 2014). Another *Lotus* species that *M. loti* strain R7A nodulates is *L. corniculatus* (birdsfoot trefoil). This species is used for pastures and hay, making it agriculturally significant (Aerts et al., 1999).

1.7. Rhizobial Exopolysaccharide

Rhizobia produce a range of polysaccharide molecules including exopolysaccharides (EPS), lipopolysaccharide (LPS), K-antigen (KPS), cellulose, glucomannan and cyclic-β-glucans (CBGs) (Figure 1.2). Rhizobial EPS production has been best characterised for *Sinorhizobium meliloti*, a microsymbiont of *Medicago* legume species. Strains of *S. meliloti* produce three symbiotically active yet structurally distinct exopolysaccharides, succinoglycan (EPSI), galactoglucan (EPSII) and capsular polysaccharide or K antigen (KPS). EPSI is an EPS that is produced in both HMW and LMW forms. It consists of an octasaccharide repeating unit composed of 1 galactose and 7 glucose residues decorated with acetyl, succinyl and pyruvyl groups (Figure 1.3) (York & Walker, 1998a).
Rhizobium-derived EPS forms an extracellular slime that is particularly evident when cells are cultured on solid media containing sugars, due to the formation of high molecular weight (HMW) polymers (Skorupska et al., 2006).

EPS has been proposed to have several roles in the rhizobium-legume symbiosis; however the exact contributions are not yet fully understood. The EPS may function to protect the cell from stressful conditions that the plant creates (Islam & Lam, 2013). Other roles include assisting adhesion to the host root hairs and allowing the formation, progression and release of bacteria from infection threads. One suggested role for EPS is that it functions as an immune suppressor and/or provides protection against the antimicrobial compounds that the plant produces (Skorupska et al., 2006). Studies performed on S. meliloti, R. leguminosarum and S. fredii NGR234 have provided evidence to suggest that LMW forms of EPS may function as a signalling molecule (Djordjevic et al., 1987; Urzainqui & Walker, 1992; Gonzalez et al., 1998; Staehelin et al., 2006). Mutations in S. meliloti which entirely eradicate EPS synthesis and some that modify EPSI structure affect nodule formation. Inactivation of exoY which initiates the first step in EPSI biosynthesis allows development of infection foci in curled root hairs and initiation of infection threads, but their progress through nodule primordia cells is arrested. Mutation of exoZ which acetylates EPSI causes a reduced efficiency of infection thread formation and an exoH mutant, which produces a HMW EPSI derivative lacking succinyl groups and no LMW EPS fails to form extended infection threads (Cheng and Walker, 1998). Inactivation of the glycanase exoK results in both a decrease in LMW EPS and a defect in plant symbiotic relationships (Becker et al., 1993a; York & Walker et al., 1997; York & Walker et al., 1998a; York & Walker et al., 1998b; Urzainqui & Walker, 1992). The S. meliloti EPSI trimer was used to partially complement symbiotically defective EPS mutants on alfalfa (Battisti, 1992; Gonzalez et al., 1998; Wang et al., 1999). Studies show that strains unable to synthesise either EPSI or EPSII can be rescued on alfalfa by the addition of picomolar amounts of LMW EPSII consisting of 10-15 disaccharide subunit repeats. It was also demonstrated that non-succinylated EPSI and EPS from different species were unable to promote nodule invasion, indicating that EPS structure contributes to host specificity and that succinylation is an important feature of the EPS molecule (Battisti et al., 1992; Gonzalez, 1996). Further studies have suggested that EPS signalling may suppress the host’s defence responses. S. meliloti exoY mutants form mostly small, white ineffective nodules, plus an occasional Fix’ nodule on M. sativa. The cortical cell walls of the ineffective nodules were abnormally thick and contained increased amounts of phenolic compounds.
which suggested that EPS suppresses host defences (Neihaus et al., 1993). Studies have shown that EPS mutants in *R. leguminosarum* bv. *trifolii* TA1 (Wielbo et al., 2004) and *Bradyrhizobium japonicum* (Parniske et al., 1994) are able to elicit plant defence responses in their respective hosts.

The proposed function of EPS as a modulator of plant defence responses is further supported by transcriptome studies of *Medicago trunculata* gene expression induced by either the wild-type or an EPS-deficient *exoY S. meliloti* mutant. The expression of two putative EPS receptors were upregulated when the plant host was inoculated with the wild-type. These two EPS receptors were called TC104170 and TC103114 (Jones et al., 2008). TC103114 has been identified as a receptor involved in plant defence responses (Cote et al., 2000). On the other hand, inoculating the plant with the *exoY* mutant results in an up-regulation of genes that are involved in pathogenesis/plant defence responses (Jones et al., 2008), suggesting that the EPS-deficient strain triggers a plant response that prevents nodulation of the plant host that is possibly suppressed by the wild-type EPS. More recently a receptor that has been shown to bind the LMW EPS of *M. loti* R7A directly has been identified in *Lotus japonicus* (discussed in detail in section 1.10). Some strains of *S. meliloti* (such as an *expR101* derivative of the Rm1021 strain) are able to produce a second polysaccharide, known as galactoglucon or EPS II. EPS II can substitute for EPS I, to form functional nodules on *Medicago sativa*, but not on all plants that the strain can nodulate (Glazebrook & Walker, 1989; Her et al., 1990). In low phosphate conditions, the production of EPS II is upregulated. This suggests that the EPS II molecule functions primarily when *S. meliloti* is present in a soil environment (Zhan et al., 1991; Mendrygal and Gonzalez, 2000).
Figure 1.2: Rhizobial polysaccharides.

Schematic of various rhizobial polysaccharides displayed on the cell surface that have been identified as being involved with symbiotic interactions with the host legumes. Based on figure from (Rodriguez-Navarro et al., 2007).
Figure 1.3: Rhizobial exopolysaccharide structures

Examples of repeating units of characterised exopolysaccharide structures by the indicated rhizobial species. Figure reproduced from Janczarek et al., 2014.
1.7.1. EPS biosynthesis in S. Meliloti

The EPS biosynthesis pathway in rhizobia is best characterised in *S. meliloti*. Assembly of succinoglycan starts with the addition of a UDP-galactose molecule produced by the *exoB* gene product to the membrane-bound lipid carrier by the galactosyltransferase encoded by *exoY* (Müller *et al*., 1993; Reuber & Walker, 1993). Next the *exoA* gene product adds the first glucosyl residue to the EPS followed by the sequential addition of further glucosyl residues by the *exoL*, *exoM*, *exoO* and *exoU* gene products. Strain-specific modifications involving the addition of acetyl, succinyl and pyruvy groups are carried out by the *exoZ*, *exoH* and *exoV* gene products respectively (Becker *et al*., 1993a; Becker *et al*., 1993b; Glucksmann *et al*., 1993a; Glucksmann *et al*., 1993b). The *exoP*, *exoQ*, *exoF* and *exoT* gene products mediate polymerisation and secretion of the EPS (York & Walker, 1998b). Finally, extracellular glycanase enzymes encoded by *exoK* and *exsH* cleave the HMW form of succinoglycan to produce LMW monomers, dimers and trimers of the octasaccharide repeat (York & Walker, 1997).

When the *S. meliloti exoK* gene was overexpressed by introduction of a plasmid vector containing *exoK*, *exoH* (succinyl transferase responsible for succinylation of EPSI) and *exoL* (glycosyltransferase responsible for ligation of the third sugar (glucose) to the EPS molecule), the ratio of LMW to HMW EPS was increased from 20% to 60%. It is possible that overexpression of *exoH* is required as well as *exoK* for the observed increase in LMW EPS as interrupting either gene on the plasmid with a transposon produced plasmids that failed to change the LMW:HMW ratio, but the effect observed for the *exoH* insertion may have been due to a polar effect on the *exoK* gene (Urzainqui & Walker, 1992). It was later shown that nonsuccinylated succinoglycan produced by an *exoH* mutant has a higher resistance to cleavage than wild-type succinoglycans (York & Walker, 1998a). A further extracellular glycanase *exsH*, and a type I secretion system required for its transport encoded by *prsDE* are also involved in cleavage of HMW EPSI to produce LMW EPSI (York & Walker, 1998b).
1.7.2. **EPS biosynthesis in *Mesorhizobium loti* strain R7A**

The structure of R7A EPS has recently been reported by Muszynski *et al.*, 2016. It consists of a repeating O-acetylated octasaccharide subunit of similar structure to that reported for *S. meliloti* strain IFO 13336 (Amemura *et al.*, 1981) and *M. huakuii* bv. My6 (Hisamatsu *et al.*, 1997). The R7A EPS biosynthesis pathway begins with a number of enzymes facilitating the synthesis of various nucleotide sugar precursors. These UDP-linked sugars are used to synthesise the EPS octasaccharide subunit consisting of both a backbone and a side-branch. The backbone consists of a 1,3-linked Gal and three 1,4-linked Glc residues linked together by the glycosyltransferases ExoY, ExoA, ExoL and ExoM. The side-branch consists of two 1,6-linked Glc, one 1,6-linked GlcA and one 1,6-linked RibA residues linked together by the glycosyltransferases ExoO, ExoU, Mlr5268 and Mll5269. (Kelly *et al.*, 2012; Wightman, 2014) (Figure 1.4). Acetyl decorations have been found on R7A LMW EPS on the 2 and 3 positions of the second Glc, 2 position of the third Glc, the 3 position of the fourth Glc, and the 2 and 3 positions of the terminal RibA residues (Figure 1.4) (Muszynski *et al.*, 2016). The function of the majority of these enzymes have not been directly demonstrated, but were proposed by Kelly *et al.* (2012) based on comparisons drawn between R7A exo gene clusters and EPS structures with those of MAFF303099, *S. meliloti* and *Rhizobium* sp. NGR234.

HMW EPS can be formed by linking the Gal and third Glc residue by a 1,4-linkage. The particular mechanism of subunit polymerisation is unknown, but is thought to occur as the EPS is transported from the cytosol across the periplasm and outer membrane.
Figure 1.4: *M. loti* R7A EPS biosynthesis.

A) Biosynthesis of *M. loti* R7A EPS. R7A produces an O-acetylated octasaccharide EPS repeating unit that is polymerised to form HMW EPS. The repeating unit consists of glucose
(Glc), galactose (Gal), riburonic acid (RibA) and glucuronic acid (GlcA). First the nucleotide sugar precursors are synthesised by a number of gene products (red). The sugar precursors are then used in the biosynthesis of the octasaccharide subunit via a number of glycosyltransferases (Red). The repeating units are polymerised by a currently unconfirmed transport mechanism. Adapted from (Wightman, 2014). B) Structure of the R7A EPS showing the O-acetyl group positions on the octasaccharide subunit. Acetylation occurs on the 2 and 3 positions of the second Glc, 2 position of the third Glc, the 3 position of the forth Glc, and the 2 and 3 positions of the terminal RibA residues. Adapted from (Muszynski et al., 2016). C) The position of the exo (green) and exs genes (red) involved in EPS biosynthesis within the exo clusters in S. meliloti 1021, Rhizobium sp. NGR234 and M. loti MAFF303099. Genes in white are not known to be involved in EPS biosynthesis. Strain R7A has the same genetic organisation as MAFF303099. Adapted from (Streit et al., 2004). Note that while the position of exoZ in R7A is unknown, there are at least four candidate genes based on sequence searches, only one of which has been inactivated with no change in phenotype (J. Sullivan, personal communication).

1.7.3. Other rhizobial polysaccharides

In addition to exopolysaccharides, rhizobia produce other polysaccharides as mentioned above including LPS, KPS, cellulose, glucomannan and CBGs. LPS structures have an overall hydrophobic property and consist of three main components: the core oligosaccharide, Lipid A and the O-chain polysaccharide. LPS is produced by rhizobia in two forms, rough LPS that lack the O-chain polysaccharide and a smooth form containing all three components (Carrion et al., 1990).

The lipid A component of the LPS is highly varied not only in comparison to enteric species, but also between different rhizobial species. This component consists of a carbohydrate backbone and contains fatty acyl components that vary in both length and number (Carlson et al., 1987; Kannenberg & Carlson, 2001). The lipid A component contains a very long chain fatty acid (VLCFA) which, when disrupted, results in a delayed nodule formation and nitrogen fixation, despite playing a role however, the VLCFA does not appear to be a requirement for the initiation of rhizobia-legume symbiosis (Brown et al., 2011). The VLCFA of the lipid A component has been theorised to play a role in the adhesion to the host
and suppressing host immune responses much like the EPS molecule (Carlson et al., 1987; Carlson et al., 2010; Wolpert & Albersheim, 1976).

The O-chain polysaccharide can vary, not only between rhizobial species, but even strains can contain significant differences (Carlson et al., 2010). The O-chain polysaccharide is the dominant antigen of the LPS molecule and it has been shown that the O-chain polysaccharide is required for \textit{Rhizobium leguminosarum} biovar \textit{phaseoli} to form functional nodules, possibly due to adhesion caused by the hydrophobicity of the O-chain polysaccharide component (Carlson et al., 1987; Kannenberg & Carlson, 2001).

The core oligosaccharide contains the O-chain polysaccharide attachment. If alterations are made to the core, specifically the lack of the galactosyluronic residue on the P2-2 core oligosaccharide, then O-chain polysaccharide attachment may be prevented and the resulting mutants will be defective in nodule formation. When this was first observed, two possibilities were considered, either the O-chain polysaccharide is required for symbiosis, or that the altered core itself causes the symbiotic defect (Carlson et al., 1989). It was later shown by Carlson et al. (1995) that the latter possibility was the case as a complete core devoid of O-chain polysaccharide is insufficient for an effective nodule formation.

CBGs are a unique molecule produced almost exclusively by rhizobia and their production is present in all rhizobia studied to date (Breedveld & Miller, 1994). CBGs seem to be produced exclusively in organisms that share a symbiotic relationship with a host, suggesting these molecules play a role in symbiotic interactions (Amemure & Cabrera-Cresbo, 1986; Bundle et al., 1988; Talega et al., 1994; Talega et al., 1996). The structure of CBGs is cyclic and consists of a greatly varying number of glucose residues linked via β-glycosidic bonds. This variation in residue number is species dependent and ranges from 17 in \textit{R. leguminosarum} to 40 in \textit{S. meliloti} (Benincasa et al., 1987; Koizumi et al., 1984). Rhizobia can add anionic substituent groups including phosphoglycerol and succinyl groups in order to increase CBG variation (Miller et al., 1988). The synthesis of CBGs is performed by two genes that have been identified and named \textit{ndvB} and \textit{ndvA} which encode a glycosyltransferase and a second protein that transports the CBG to the periplasm (Dylan et al., 1986; Breedveld & Miller, 1994). It has been demonstrated that in order for nitrogen-fixing nodules to form on the host plant, CBG production is a requirement and most nodules formed by \textit{S.meliloti ndvA} or \textit{ndvB} mutants on \textit{M. sativa} are unable to fix nitrogen (Dylan et al., 1986). Despite this symbiotic defect, however, a small proportion of nitrogen-fixing nodules are still formed by the \textit{ndvA}
and ndvB mutants (Dylan et al., 1990). In the _M. loti_ species, mutants defective in CBG production also display a symbiotic defect forming ineffective nodules and fewer infection threads on the host plant _L. tenuis_ (D’Antuono et al., 2005). _M. loti_ mutants containing a mutation in a putative cell envelope protein showed reduction of CBG production and similar symbiotic deficiencies (Kawaharada et al., 2007). Observations from mutagenesis studies have led to the proposal of several possible functions of CBGs. When cultures are grown in conditions of low osmolarity, the production of CBGs is upregulated, suggesting a possible role in protecting the cell from osmotic stress (Breedveld et al., 1990; Dylan et al., 1990). In addition, proposed functions of CBGs include rhizobial attachment to host root hairs and modulation of host plant defences (Dylan et al., 1990; Planque & Kijne, 1977; Mithöfer et al., 1996; Mithöfer et al., 2001).

KPS are a group of polysaccharides produced by rhizobia that are tightly associated with the outer membrane. The KPS produced by rhizobia share structural similarities to the group II KPS produced by _E. coli_, though the KPS molecule differs between rhizobial strains. The structure of rhizobial KPS molecules will generally form an acidic linear disaccharide repeat consisting of a 3-deoxy-D-manno-2-octulosonic acid (kdo) or related sugar and a neutral hexose or uronic acid (Le Quere et al., 2006; Reuhs et al., 1993). Despite this general structure found across most rhizobia, the KPS of _S. meliloti_ strain 1021 contains only β-(2-7) linked kdo molecules (Frasysse et al., 2005).

There are three gene clusters that have been identified in _S. meliloti_ Rm41 that are responsible for KPS production and are termed _rkp-1, rkp-2_ and _rkp-3_ (Kereszt et al., 1998; Kiss et al., 1997; Kiss et al., 2001; Petrovics et al., 1993). It is difficult to determine the role KPS plays in nodulation however, as the biosynthesis of KPS is also linked to LPS. Despite these difficulties, studies have been performed on the KPS of _Rhizobium_ sp. NGR234 showing that a mutation in the _rkpMNO_ genes causes a symbiotic deficiency with its host plant (Le Quere et al., 2006) and that the KPS of _S. fredii_ plays an important role in the symbiotic interactions with host plants (Parada et al., 2006). Despite studies indicating the importance of KPS in the rhizobia-legume symbiosis, the stage that KPS is involved in has not been widely studied, though it has been demonstrated that KPS plays a role in the elongation of ITs in the _S. meliloti-_alfalfa symbiosis and that _S. meliloti_ KPS can facilitate the rhizobial invasion of nodules when hosts are inoculated with strains deficient in both EPSI and EPSII, though the process is far more efficient when mediated by EPSI (Rheus et al.,
1995; Putnoky et al., 1990; Pellock et al., 2000). It has been proposed that, while KPS allows nodule formation in the absence of EPSI and EPSII, its function is not as a signalling molecule and does not substitute for the function of EPSI. It may instead alter the properties of the cell surface in order to enhance the formation of nodules when a strain is deficient in EPS production (Sharypova et al., 2006).

Cellulose is a well-characterised polysaccharide playing an important role in plant structure and is also known to be produced in algae, fungi and bacteria. (Amikam & Benziman, 1989; Ausmees et al., 1999; Ross et al., 1991). The basic structure of cellulose consists of linear polymers of 1,4-β-linked glucose in a parallel arrangement. The linear polymers are linked by intra- and inter-chain bonds in order to facilitate the formation of a wide range of crystalline structures (O’Sullivan, 1997). Therefore, the specificity in cellulose structure is not achieved by changing its chemical composition, but instead by altering the degree of polymerization. A model bacterial organism for cellulose biosynthesis studies is *Gluconacetobacter xylinus* (formerly called *Acetobacter xylinus*) with the genes responsible for cellulose biosynthesis in this species forming an operon known as *bcxABCD*. This operon encodes proteins that form a cellulose synthase complex (Lin et al., 1990; Ross et al., 1987; Wong et al., 1990). In the rhizobia-legume symbiosis, cellulose contributes to bacterial adhesion to the host plant cells. In the species *R. leguminosarum* mutants unable to produce cellulose fail to form a cap and strains that over-produce cellulose form larger caps; however cellulose is not essential for successful nodulation to occur (Ausmees et al., 1999; Laus et al., 2005; Smit et al., 1986; Williams et al., 2008).

Some bacteria, including rhizobia, produce glucomannan, a water-soluble heteropolymer located on polar cell surfaces. This polysaccharide is comprised of 95% glucose and mannose residues with the remainder made up of galactose and rhamnose residues (Laus et al., 2006). The majority of glucomannan residues are linked by 1,4-β-linkages with side-chains (Katsuraya et al., 2003). The role of glucomannan in the rhizobial-legume symbiosis is poorly understood, but in *R. leguminosarum* it has been shown to give a competitive advantage for nodule occupancy against strains deficient in glucomannan production. It is thought to be involved in root hair attachment and biofilm formation on the *Vicia hirsuta* host plant (Laus et al., 2006; Williams et al., 2008).
1.8. EPS Transport

Polysaccharides are very highly diverse molecules, with monomers in rhizobial EPS even differing from strain to strain, though retaining the backbone structure (Leigh & Coplin, 1992). Despite this, polysaccharide transport mechanisms can be divided into three types that polymerise saccharide subunits and export the polysaccharide to the cell surface, known as the Wxz/Wzy, ATP-binding cassette (ABC) transporter and synthase-dependent pathways (Figure 1.5)

Figure 1.5: Polysaccharide transport mechanisms
A cartoon schematic depicting the three main transport mechanisms by which polysaccharides are transported across the inner membrane, periplasmic space and outer membrane. Figure reproduced from (Whitney & Howell, 2013).
The Wzx/Wzy transporter dependent pathway is a wide spread transport system that simultaneously syntheses and transports molecules, including polysaccharides, across the inner membrane from the cytoplasm. This mechanism is present in both Gram-positive and Gram-negative bacterial species though polysaccharides can be transported via the ABC transporter or synthase transporters as alternative mechanisms (Islam & Lam, 2013; Alaimo et al., 2006; Whitney & Howell, 2013).

The Wzx/Wzy transporter-dependent pathway involves an undecaprenyl-phosphate lipid carrier. The precursor of the polysaccharide monomer is assembled on this carrier and the polysaccharide is synthesised by a series of membrane-bound glycosyltransferases, such as those responsible for the production of the EPS monomer mentioned previously (McGrath & Osborn, 1991; Price & Momany, 2005). A flippase protein known as Wzx then translocates the precursor monomer across the inner membrane where it is polymerised onto the reducing end of the HMW polysaccharide chain by the Wzy polymerase (Figure 1.5) located within the periplasm, via a catch and release mechanism (de Kievit et al., 1995; Islam et al., 2011; Robbins et al., 1967). The polysaccharide’s length is regulated by a polysaccharide co-polymerase (PCP) to ensure that the polysaccharide chains are the correct modal length to perform the molecule’s function. The PCP protein is also associated with an outer membrane export (OPX) protein that transports the polysaccharide chain across the outer membrane and secretes it from the cell (including KPS and EPS). Alternatively, membrane-bound polysaccharides (such as lipopolysaccharides) are anchored into the inner membrane by the WaaL protein. The Wzx/Wzy transporter pathway is responsible for the synthesis and secretion of the EPS polysaccharide in a number of species including *R. leguminosarum*, *Rhizobium* sp. strain NGR234, and *S. meliloti* (Gonzalez et al., 1998; Jofre and Becker, 2009; Staehelin et al., 2006; Marczak et al., 2013; Marczak et al., 2014).

An alternate transport mechanism is the ABC transport mechanism. This system differs from the Wzx/Wzy transporter pathway in that the entire polymer is synthesised on the lipid carrier rather than single monomeric units polymerised onto a growing chain (Cuthbertson et al., 2010; Greenfield & Whitfield, 2012; Raetz & Whitfield, 2002). A dedicated ATP-dependent ABC transporter protein transports the polysaccharide chain across the inner membrane. Like the Wzx/Wzy transporter system, the ABC transporter pathway can be associated with an OXC-PCP complex that can transport the mature polysaccharide chain across the outer membrane and secrete the molecule from the cell or anchor membrane-bound
polysaccharides to the inner membrane via the WaaL protein (Collins et al., 2007; Cuthbertson et al., 2009; Dong et al., 2006). The ABC transporter pathway is wide-spread across Gram-negative species, and is particularly well-studied in *E. coli*, with the mechanisms to synthesise and secrete group 2 KPS and LPS being particularly well-studied examples (Cuthbertson et al., 2009; Greenfield and Whitfield, 2012; Raetz and Whitfield, 2002; Whitfield, 2006). Mutations in the ABC transporter in *R. leguminosarum* bv. *viciae* 3841 causes EPS production to be downregulated, however the EPS structure remains identical to that of the wild-type, suggesting the ABC transporter plays a regulatory role, but not direct transport or synthesis of the EPS chain (Vanderlinde et al., 2010). ABC transporters are also a particularly prevalent family in plants, with *Lotus japonicus* containing 91 members. The ABC transporters have been shown to secrete other signalling molecules including flavonoids secreted from the roots of soybeans (Sanchez-Fernandez et al., 2001; Garcia et al., 2004; Sugiyama et al., 2006; Sugiyama et al., 2007; Sugiyama et al., 2008).

The third transport system is the synthase-dependent pathway and is not as well-characterised as the Wzx/Wzy and ABC transporter dependent pathways. In the synthase-dependent pathway, a lipid carrier is sometimes but not always associated with the polysaccharide which is simultaneously synthesised and translocated across the inner membrane using a membrane-bound synthase. The synthase in this system is a glycosyltransferase (Cartee et al., 2001; Ryjenkov et al., 2006). Often membrane-bound co-polymerases are involved in the regulation of this process and are associated to the synthase protein. The synthesis of the polysaccharide by synthase-dependent transport mechanisms is regulated by levels of c-di-GMP (Merighi et al., 2007). A tetratricopeptide repeat (TPR)-containing scaffold protein prevents the degradation of the polymer in the periplasm. Unlike the Wzx/Wzy and ABC transporter-dependent pathways, the synthase dependent pathway is not associated with a OPX-PCP protein and instead transports the TPR through a β-barrel porin. As such, molecules synthesised via the synthase-dependent pathway can be distinguished from those synthesised by Wzx/Wzy and ABC transporter-dependent pathways (Jain & Ohman, 1998; Keiski et al., 2010; Whitney et al., 2011). The synthase-dependent pathway family is known to synthesise LPS and EPS in a wide range of Gram-negative bacteria (Keenleyside & Whitfield, 1996; Whitney & Howell, 2013); however the synthase-dependent pathway family does not have any known members within rhizobial species.
In *M. loti*, EPS is thought to be transported out of the cell by the Wzx/Wzy pathway with all required genes, including the *exoT* flippase gene responsible for EPS export, being identified using bioinformatics approaches. Their role in EPS export has not been directly demonstrated however (Figure 1.6; Wightman, 2014) and it is currently unknown if the Wzx/Wzy pathway is responsible for the translocation of the truncated EPS molecule produced by Δ*exoU* mutants (Wightman, 2014).
1.9. EPS biosynthesis gene mutation effects on nodule formation

The structure of the EPS has been shown to be important in the formation of root nodules but its role in host range is unclear. For example, *R. leguminosarum* strains are categorised into biovars including *trifolii* and *viciae*, which are capable of nodulating different plants hosts. Despite this different range of host plants, the octasaccharide repeating structure is shared between biovars (Skorupska *et al.*, 2006).

Mutations in the biosynthesis genes of *S. meliloti* can cause a range of symbiotic defects. If *exoY* is inactivated, the rhizobia are still able to induce root hair curling and the initiation of infection threads, however the infection threads are aborted shortly after, halting their
progress into the nodule primordia. It was found that EPS may not be required to colonise root hairs, but it may increase the rate at which root hair colonisation occurs. Mutations in the \textit{exoZ} gene, a gene responsible for the acetylation of EPS in \textit{S. meliloti}, still result in Fix\textsuperscript{+} nodules, however the efficiency of nodule invasion by strains containing this mutation is reduced. The EPS produced by \textit{exoH} mutants does not contain the succinyl modification and is symbiotically dysfunctional. These mutants are still capable of inducing root hair curling, but will not form the extended root hair necessary for nodule invasion (Cheng and Walker, 1998).

EPS is able to mediate the formation of infection threads and it was previously thought that this made EPS production a requirement for rhizobial symbiosis with its host plant (Jones \textit{et al.}, 2008); however a study by Kelly (2012) has shown that \textit{M. loti} R7A strains deficient in EPS production will still result in nitrogen-fixing nodules forming. The delayed formation of nodules formed by EPS deficient mutants is likely due to a defect in the efficiency of ITs forming to reach the base of the root hairs and thus restricting rhizobial entry into the cortical cells, in addition these mutants produce colonies with a soft, non-mucoid phenotype when grown on solid medium (Wightman, 2014).

In \textit{R. leguminosarum} \textit{bv. trifolii}, \textit{pss} mutations can either cause a complete deficiency in EPS production and the strain will induce uninfected nodules and show the symptoms of a plant host immune response or \textit{pss} mutations will result in an altered regulation of EPS production compared to the wild-type and give rise to partially infected nodules. The bacteroids present in these nodules are either Fix\textsuperscript{−} or Fix\textsuperscript{+}. Mutations in the \textit{pssA}, \textit{pssD} and \textit{pssP} genes fall under the former category (Skorupska \textit{et al.}, 1995; Wielbo \textit{et al.}, 2004). The PssT protein is an integral inner membrane protein and is proposed to be the Wzy polymerase for EPS subunit and is the counterpart to ExoQ in R7A. In \textit{R. leguminosarum} \textit{bv. Trifolii} \textit{pssT}, Mazur \textit{et al.}, 2003 constructed a \textit{RtAH1pssT::pAH1} mutant through plasmid integration. The resulting mutant contained a \textit{pssT} fragment that synthesised a PssT protein lacking the C-terminal part when expressed. In this mutant EPS production was increased and a higher ratio of HMW of EPS was present than that of the wild-type, suggesting the PssT protein has a regulatory function. In addition to an increase in HMW EPS, the \textit{RtAH1pssT::pAH1} mutant formed more nitrogen fixing nodules when inoculated on \textit{Trifolium pratense} cv. Ulka (Marczak \textit{et al.}, 2013; Mazur \textit{et al.}, 2003).

\textit{Sinorhizobium fredii} HH103 mutants deficient in the production of EPS are symbiotically
functional in soybean (Parada et al., 2006). It was determined that S. fredii HH103 exoA mutants unable to produce EPS are more competitive than the wild-type when nodulating soybean cv. Williams. When used to inoculate Vigna unguiculata hosts however, the mutants are out-competed by the wild-type strain. The HH103 exoA mutant was impaired in surface motility and demonstrated poor surface translocation. Additionally, the exoA mutant was more sensitive to osmotic stress than the wild-type, suggesting that the EPS provides osmotolerance (Rodriguez-Navarro et al., 2014).

In Rhizobium sp. strain NGR234, mutations in the genes exoF, exoL, exoP, exoQ, exoY, exoK and exoZ were generated by Staehelin et al. (2006) via the insertion of Ω transposons. Mutations in exoF, exoL, exoP, exoQ and exoY result in non-mucoid phenotype and red colonies when plated on GMS medium supplemented with Congo red dye, suggesting no EPS is produced in these strains. In contrast the exoK and exoZ mutants were mucoid suggesting the strains still produced EPS. When V. unguiculata is inoculated with NGR234 or the different exo mutants, nitrogen fixing nodules are formed. This suggests that the exo genes are not required to form a symbiotic relationship with the V. unguiculata host plant. On the other hand, when the non-mucoid mutants are used to inoculate L. leucocephala, the mutants are unable to form nodules with a Fix+ phenotype, while NGR234 and NGRΩexoZ successfully form Fix+ nodules.

Inoculation of Caesalpinioideae fasciculata with the NGRΩexoK mutant results in a lower symbiotic proficiency than that of NGR234. NGRΩexoK forms smaller nodules that are fewer in number and forms an increased number of pseudonodules. In addition, nodules formed by this mutant fix less nitrogen than those formed by NGR234. The NGRΩexoK mutant was able to produce HMW EPS, but lacked the ability to produce LMW EPS. Complementation with the NGR234 exoK gene in the NGRΩexoK mutant successfully restores the symbiotic defects caused, however complementing the exoK gene with the S. meliloti exoK gene failed to restore symbiotic defects, demonstrating that the S. meliloti exoK cannot replace the function of NGR234 exoK. These observations support LMW EPS playing a role in the symbiotic interactions between NGR234 and its plant hosts (Staehelin et al., 2006).

In S. meliloti, ExoK cleaves the EPS into octasaccharide trimers which have been implicated as the symbiotically active form that functions as a signalling molecule for symbiosis (Wang
et al., 1999; York & Walker 1998a). It has been shown by Mendis et al. that ExoK is required by the rhizobia for successful symbiosis with mutants displaying severe defects in symbiotic relationships (Mendis et al., 2013). Modifications are made to the EPS by the proteins ExoZ and ExoH (York & Walker, 1998b) which affect ExoK cleavage of the EPS (York & Walker, 1998a). When the \textit{exoK} gene was overexpressed by insertion of a vector containing \textit{exoK}, \textit{exoH} and \textit{exoL}, the ratio of LMW and HMW EPS was affected. It is possible that overexpression of \textit{exoH} is required as well as \textit{exoK} for more LMW EPS to be produced than the wild type, as interrupting either gene failed to change the LMW:HMW ratio. However the effects may have been due to a polar effect on the \textit{exoK} gene (Urzainqui & Walker, 1992). It was later shown that mutating the \textit{exoH} gene decreases the LMW EPS produced, but has a highly polar effect of the transcription of \textit{exoK}, leaving the possibility that the lowered LMW EPS is due to decreased glycanase expression (York & Walker, 1998a). A deletion of \textit{exoH} decreases the activity of the ExoK glycanase while a deletion in \textit{exoZ} increases ExoK activity in \textit{S. meliloti}. The \textit{exoH} gene encodes a protein which adds a succinyl group to the EPS, while the protein encoded by \textit{exoZ} adds an acetyl to the EPS. It was found by York & Walker (1998a) that the succinyl group is required for effective EPS cleavage by ExoK while the acetyl group inhibits ExoK activity. Even though succinylation is required for effective ExoK activity, non-succinylated EPS was cleaved in low amounts (York & Walker, 1998b).

It was also found through in-vitro experiments, that purified ExoK (and ExsH) is unable to cleave cell-free EPS extract. They can however cleave EPS when added exogenously to cultures suggesting that ExoK cleaves nascent EPS (York & Walker, 1998b).

The \textit{exoK} gene has also been successfully knocked out in \textit{S. meliloti} using random Tn5 transposon mutagenesis, but had deleterious polar effects on the downstream \textit{exoLAMON} operon, shown by the inability the complement the resulting phenotype with \textit{exoK} alone (York & Walker, 1997; Mendis et al., 2013).

A number of mutants with mutations in the \textit{exo} genes have been constructed in \textit{M. loti} strain R7A (Kelly et al., 2013). These include mutants constructed via IDM in the \textit{mlr5265}, \textit{mlr5266}, \textit{exoA}, \textit{exoO} and \textit{exoK} genes. Additionally strains containing mutations in the \textit{exoB} and \textit{exoU} genes were constructed by a Tn5 insertion approach and isolated by (Hubber, 2005). The colony morphology of the strains were mostly non-mucoid in contrast with the mucoid phenotype of R7A. The one exception was R7Amlr5266 which had a slightly mucoid phenotype. The LMW EPS extract of the R7AexoB mutants contained only Glc. The lack of
Gal residues in the R7AexoB mutant is due to exoB encoding an UDP-galactose 4-epimerase involved in synthesising UDP-Gal from UDP-Glc and as a result also produced a LPS that differed from the R7A wild-type in that it lacked Gal. R7AexoB is able to form effective nodules at a slightly lower rate than the wild-type R7A strain; this differs from the R7AexoU, R7AexoO and R7Amlr5265 mutant strains which induce mostly white nodule primordia with the occasional functional nodule after a prolonged incubation period, and the R7AexoA strain that formed effective nodules in similar quantities to the R7A wild-type strain though after a delay in the incubation period. The plants inoculated with the R7AexoK and R7Amlr5266 strains displayed a phenotype that was an intermediate between that of those inoculated with R7AexoU and R7AexoB strains. Mutants that displayed nodulation impairment when inoculated on L. japonicus Gifu and L. corniculatus revealed growth defects on both plants that included stunted growth and yellowing leaves characteristic of nitrogen starvation. Two models were proposed to explain the symbiotic ability of the R7AexoB mutant despite its EPS deficiency. The first suggests a complementary signal from another polysaccharide in EPS deficient strains, while the other possible explanation is that a surface molecule usually on the EPS that is recognised and triggers a defence response is absent (Kelly et al., 2013). It has been hypothesised that R7A mutants that produce no EPS do not trigger a defence response, however mutants that produce an EPS with a truncated side-chain do and wild-type strains cause a lowered expression of plant host genes involve in the defence response. When R7AexoU was used to inoculate epr3 mutants, an increase in infection threads resulted. This suggests that the host receptor EPR3 recognises the side-chain of the EPS molecule and initiates a defence response when inoculated with R7A strains with a truncated side-chain but inhibits the defence response when inoculated with a strain that produces wild-type EPS. In strains that produce no EPS, the EPR3 receptor receives no signal and neither inhibits nor initiates a defence response, resulting in delayed nodulation (Kawaharada et al., 2015).

Difficulties have arisen in acquiring an in-frame marker-less deletion M. loti R7A ΔexoK mutant as discovered by Simon Kelly, who noted the possibility that an exoK in-frame marker-less deletion may be lethal (Kelly, 2012). Due to the role of exoK in cleaving nascent EPS, one possible explanation for this may be due to EPS being over-produced and resulting in a lethal accumulation of EPS.
1.10. EPR3 host receptor

The receptor for EPS in *Lotus japonicus* has been identified as EPR3 by Kawaharada *et al.* (2015). EPR3 is a 70 kDa transmembrane receptor kinase in the 17-member LysM receptor kinase family present in *L. japonicus*. It was shown that truncated EPS produced by R7AΔexoU causes premature termination of infection-thread development, preventing infection threads from reaching the epidermal-cortical cell boundary.

A strain of *L. japonicus* containing a mutated allele of *epr3* (*L. japonicus epr3-10*) was isolated by Kawaharada *et al.* (2015) in a screen for mutants able to form infected nodules with R7AΔexoU. Mutation of the *epr3* gene results in an increase of infection threads reaching the cortical boundary when inoculated with R7AΔexoU as well as an increase in infection by crack entry, suggesting that *epr3* functions by regulating rhizobial infection. This study also found that R7AΔexoB, a mutant that produces no EPS and forms fewer nodules than the wild-type displays no significant change in the number of infection threads formed when inoculated on a host with a mutation in *epr3* compared to infection of wild-type Gifu. In contrast, the *exoB* mutant forms significantly less infection threads on Gifu compared to R7A wild-type.

It is proposed that EPR3 can perceive both the truncated and the wild-type EPS, giving a negative and positive response respectively (Figure 1.7).

![Receptor-mediated recognition of rhizobial EPS](image)

**Figure 1.7:** Receptor-mediated recognition of rhizobial EPS.
A) Outcome of inoculation with an EPS deficient strain. B) Outcome of inoculation with a
compatible wild-type strain. C) Outcome of inoculation with a strain producing EPS lacking the branching side chain. D) An alternative mechanism for the inhibition of infection by strain producing truncated EPS involving a co-receptor. Adapted from Kawaharada et al., 2015.

The negative response to EPS by *L. japonicus* is not immediate and if a plant is co-inoculated with a rhizobial strain producing the wild-type EPS, the wild-type strain can outcompete the strain producing the truncated EPS. It was demonstrated *in vitro* that EPR3 can recognise and directly bind to the octasaccharide monomer.

EPR3 was demonstrated as not being required to detect Nod factor, nor was it able to substitute the role of Nod factor receptor 1 (NFR1) or NFR5. The *epr3* gene is induced by Nod factor and it was shown that *epr3* is not expressed when *L. japonicus* Gifu is inoculated with the strain R7AnodC which is unable to produce Nod factor (Kawaharada et al., 2015).

It has not been demonstrated that the LMW form of the R7A EPS is the symbiotically active form *in vivo*. This may be determined by purifying LMW EPS and complementing various EPS mutants or by isolating a *exoK* mutant unable to produce LMW EPS.

### 1.11. The aims of this work

The first aim of this project was to determine the role of *exoK* in the *M. loti - L. japonicus* symbiosis. In particular, if *exoK* mutants are found not to produce LMW EPS and found to induce uninfected nodules, then this would indicate that the LMW EPS is the symbiotically active form.

Previous studies have shown *exoK* to be responsible for the production of LMW EPS and implicated LMW EPS as the signalling molecule in other rhizobial species as mentioned earlier. It will be useful to construct an in-frame marker-less deletion a *M. loti* R7AΔexo*K* mutant in order to determine if the octasaccharide monomers are the recognised component of EPS. This has however proven difficult in *M. loti* due to difficulties in isolating recombinants (Kelly, 2012) and so the first objective in this study was to make additional attempts to isolate an in-frame marker-less deletion mutant of *M. Loti*, strain R7AΔexo*K*. It
was also decided to make a $\Delta$exoK mutant in a $\Delta$exoYF strain that produces no EPS, as the mutation should not be lethal in that background. Complementation of the exoYF mutation in the double mutant would confirm if the loss of exoK activity is lethal in the wild-type strain.

Once a deletion mutant is generated and confirmed as non-polar by complementation, a mutant capable of exoK induction would be generated. This would allow the phenotype of exoK expression to be directly observed and also allow large amounts of LMW EPS to be produced in vitro or in vivo by either isolation of ExoK or isolation of the LMW EPS produced by this strain as a result. Isolation of LMW EPS would allow further studies on its interaction with EPR3. If LMW EPS is required, then an exoK knockout should inhibit nodule formation. The function of exoK will also provide valuable knowledge in the EPS recognition of the L. japonicus receptors and provide great insight into the function of EPS in other plant-bacteria symbiosis relationships and if purified, may be used in the confirmation of putative plant receptors for R7A EPS in other species.

The third objective was to create an inducible exoK expression vector to determine the phenotype that occurs when exoK is over-expressed. When the NZP2037 cosmid library pSKU1 is conjugated into R7A, the consistency of the colony morphology is thinner (Kelly, 2012) and may be due to the activity of ExoK. A vector that can be induced to over-express exoK will confirm whether exoK is the gene on the pSKU1 plasmid responsible for this phenotype.

The final aim of this project was to determine if the exoT gene is required for transporting pentasaccharide EPS subunits produced by the exoU mutant across the inner membrane or if another system is involved in the transport of this truncated EPS. To achieve this, a M. loti R7AΔexoYFΔexoU pFUS2::exoT triple mutant will be constructed. The mutant would then be complemented for the exoYF mutation. Previous attempts at generating a M. loti R7AΔexoYFΔexoU pFUS2::exoT triple mutant have been unsuccessful; however an M. loti R7AΔexoYF pFUS2::exoT double mutant has been constructed (Wightman, 2014). When the exoYF gene is complemented it would produce the truncated EPS, allowing observation of whether or not the exoT gene is required.
2. **Materials and Methods**

2.1. **Bacterial Strains and Plasmids**

The bacteria used in this study are described in Table 2.1. The 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* were Tryptone yeast (TY) (Beringer, 1974) broth and agar, and Luria Bertani (LB) (Miller, 1972) broth and agar. LB agar was supplemented with 5% sucrose [w/v] or 0.5% arabinose [w/v] or 50 µg/ml aminolevulinic acid when required. *M. loti* strains were incubated at 28°C. Media used for the growth of *M. loti* were TY agar and broth or rhizobium defined media (RDM) (Ronson *et al*, 1987) and were either supplemented with 0.4% glucose (G/RDM), 5% sucrose (Suc/RDM), or 0.4% glycerol (Gly/RDM) as the sole carbon sources where appropriate. Antibiotics were used in the appropriate concentrations when required (Table 2.3). RDM media were supplemented with 1 mM IPTG when necessary.

2.3. **Storage of Bacterial Strains**

Bacterial strains were stored at -70°C in cryo-vials (Nalgene USA). Cultures were prepared for long term storage by adding 70 µL of dimethyl sulfoxide (DMSO) to 800 µL of stationary-phase culture grown in an appropriate broth medium.

2.4. **Enzymes and Chemicals**

All enzymes were purchased from Roche Diagnostics (Mannheim, Germany) and New England Biolabs (Beverly, MA, USA). Antibiotics and chemicals were purchased from Sigma (St Louis, USA). Antibiotic stock solutions were made by dissolving the antibiotic in Milli-Q water and filter-sterilised using a 0.45µm syringe filter with the exception of tetracycline which was dissolved in absolute methanol.
Table 2.1: Bacterial Strains used in this study.

<table>
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<tr>
<th>Strains</th>
<th>Description</th>
<th>Reference</th>
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<td><em>Escherichia coli</em></td>
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<td></td>
</tr>
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<td>EPI300</td>
<td>$F^{-}$ mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZAM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL nupG trfA tonA, StrR</td>
<td>Epicenter</td>
</tr>
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<td>ST18</td>
<td>E. coli S17 containing λpir ΔhemA. Auxotrophic for ALA.</td>
<td>(Thoma and Schobert, 2009)</td>
</tr>
<tr>
<td><em>Mesorhizobium loti</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZP2037</td>
<td>Wild-type strain; Nod $^+$Fix$^+$ on several Lotus species including L. pedunculatus</td>
<td>(Jarvis <em>et al.</em> 1982)</td>
</tr>
<tr>
<td>R7A</td>
<td>Field reisololate of ICMP 3153; wildtype symbiotic strain</td>
<td>(Sullivan <em>et al</em>., 1995)</td>
</tr>
<tr>
<td>R7AΔexoYF</td>
<td>Contains marker-less in-frame deletion of exoYF</td>
<td>(Wightman, 2014)</td>
</tr>
<tr>
<td>R7AΔexoU</td>
<td>Contains marker-less in-frame deletion of exoU</td>
<td>(Kelly, 2012)</td>
</tr>
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<td>R7AΔexoYFΔexoK</td>
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</tr>
<tr>
<td>Strain</td>
<td>Description</td>
<td>Source</td>
</tr>
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<td>-------------</td>
<td>--------</td>
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<td>R7AΔexoYFΔexoU</td>
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</tr>
<tr>
<td>R7AΔexoYFΔexoUexoT</td>
<td>ΔexoYFΔexoU containing pFUS2::exoT, Gm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>R7AΔexoYFexoT</td>
<td>ΔexoYF containing pFUS2::exoT, Gm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Wightman, 2014)</td>
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Table 2.2: Plasmids used in this study.

<table>
<thead>
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<th>Plasmids</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td>pBH474</td>
<td>Expresses Flp recombinase, sacB, GmR.</td>
<td>(House et al., 2004)</td>
</tr>
<tr>
<td>pSKKSG2</td>
<td>pFAJ1700 containing exoK and preceding 387-bp of DNA, TcR.</td>
<td>(Kelly, 2012)</td>
</tr>
<tr>
<td>pFAJ1700::exoY-F</td>
<td>pFAJ1700 containing exoY-exoF complementation product, TcR.</td>
<td>(Wightman, 2014)</td>
</tr>
<tr>
<td>pFUS2::exoT</td>
<td>Suicide vector, oriV_ColE1, oriTRP1, containing 350-bp region of exoT IDM mutagenesis construct, GmR.</td>
<td>(Wightman, 2014)</td>
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<td>pSKKFRT2</td>
<td>pIJ3200 IncP plasmid containing exoK-FRT-nptII construct, TcR, KmR/NmR.</td>
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<td>pSKIJSAC</td>
<td>pIJ3200 IncP plasmid containing sacB, TcR.</td>
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<td>pSKKFRT</td>
<td>pJQ200SK plasmid containing ΔexoK nptII construct, GmR, KmR, NmR.</td>
<td>(Kelly, 2012)</td>
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<td>pSKMU</td>
<td>pJQ200SK plasmid containing ΔexoU construct, GmR.</td>
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<td>pSKU1</td>
<td>NZP2037 library cosmid encoding <em>exoU</em>, *Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Kelly, 2012)</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------------------</td>
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</tr>
<tr>
<td>pSDZ</td>
<td>IPTG inducible expression vector, *Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Ramsay <em>et al.</em> 2015)</td>
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<td>pBF1</td>
<td>pSDZ plasmid containing <em>exoK</em> preceded by natural ribosomal binding site.</td>
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<td>pBF2</td>
<td>pSDZ plasmid containing <em>exoK</em> preceded by optimised ribosomal binding site derived from pFAJ1700.</td>
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Table 2.3. Antibiotics used in this study

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<td>Used for</td>
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</table>
2.5. DNA Isolation and Manipulation

2.5.1. Plasmid DNA Extraction

Plasmid DNA was extracted from *E. coli* LB broth cultures grown to stationary phase and supplemented with the appropriate antibiotics using either the Zyppy Plasmid Miniprep Kit (Zymoresearch USA) or the QIAGEN Midiprep Kit (Qiagen, Germany), following the protocols provided.

2.5.2. Genomic DNA Isolation

2.5.2.1. Ultra-quick genomic DNA preparations

Genomic DNA of *M. loti* strains were isolated using the ultra-quick genomic DNA preparation method described by (Gonzales-y-Merchand *et al.*, 1996). Bacterial strains were grown in 5 mL TY cultures at 28°C for 48 hours with shaking. Aliquots (3 mL) of the culture were harvested by centrifugation at 13,000 rpm for 3 mins in 1.5 mL microfuge tube. The supernatants were discarded and the bacterial pellets were resuspended in 400 μL of lysis buffer. The lysis buffer contained 4 M guanidium thiocyanate, 10 mM EDTA, 0.1% [w/v] Tween-80. The cell suspensions were then snap-frozen at -70 °C for 15 min before being incubated at 65°C in a water bath for 5 min. The snap-freezing/heating step was repeated once. Aliquots (100 μL) of dH₂O and (500 μL) phenol/chloroform (1:1) were added to the cell lysates. The lysates were shaken vigourously by hand for 1 min and centrifuged at 13,000 RPM for 5 min. The aqueous phases were then extracted and 500 μL aliquots of chloroform were added to each lysate. The lysates were shaken vigorously by hand for 1 min and centrifuged at 13,000 RPM for 3 min. The aqueous phases were extracted and 1 mL aliquots of absolute ethanol and 50 μL aliquots of 3M Na acetate were added to the lysates. The lysates were inverted 20 times and centrifuged at 13,000 RPM for 10 min. The supernatants were discarded and 1 mL aliquots of 70% ethanol were added. The lysates were recentrifuged and the supernatants were discarded. The genomic DNA pellets were then dried at 37°C and resuspended in 100 μL dH₂O.

2.5.2.2. PrepMan™ Ultra genomic DNA preparations

Aliquots (200μL) of an *M. loti* TY culture broth were centrifuged at 13,000xg for 1 min and the supernatants were discarded. The remaining cells were resuspended in 100μL of PrepMan Ultra Sample Preparation Reagent by vortex for 10-30 s. Samples were then heated at 100°C
for 10 mins in a boiling water bath and centrifuged at 13,000xg for 2 mins. Aliquots (75µL) of the supernatants were transferred to sterile 1.5mL microcentrifuge tubes.

2.5.3. **Restriction Enzyme Digestion**

Restriction enzyme digests were performed using 1 µL of the restriction enzymes used in the appropriate buffer with ~1 µg of DNA in a 50 µL volume and were centrifuged at 13,000 xg for 30s and incubated at 37°C for 1 h.

2.5.4. **Agarose Gel Electrophoresis**

Aliquots (5 µL) of DNA samples were mixed with 3 µL of bromophenol blue tracking dye and loaded onto a gel made 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 5 µL of 1 mg/mL ethidium bromide stock. Gels were electrophoresed at 90 V for 1 hour and were visualised and captured on an El Logic 200 Gel Documentation system (Kodak Ltd.)

2.5.5. **Polymerase Chain Reaction (PCR)**

PCR reactions were performed using the Phusion High-Fidelity PCR kit (Finnzymes). Standard 100 µL reactions were used for cloning and sequencing and 25 µL reactions were used for screening. Reaction mixtures contained 20% v/v 5x Phusion HF buffer, 3% v/v DMSO, 2% v/v of supplied dNTPs mixture, 2 nM/mL of each appropriate primer (20 nM/mL stock) (Table 2.4), 3% v/v DMSO, 0.5-1.0 µL DNA template suspension, 0.5U Phusion polymerase and 71% v/v dH₂O.

The standard Hybaid PCR express thermal cycler settings used are as follows
1 cycle at 98°C for 1 min
30 cycles at 98°C for 10s
30 cycles at 57°C for 15s
30 cycles at 72°C for 20s
1 cycle at 72°C for 5 min
2.5.6. DNA Sequencing

Plasmid clones were sequenced by mixing the plasmid sample with the appropriate primers and sending them to the Allan Wilson Centre Genome Service (Massey University, Palmerston North, NZ) to be sequenced with an ABI3730 Genetic Analyzer (Applied Biosystems).

2.6. Cloning of DNA Fragments

2.6.1. Preparation of insert and plasmid DNA

One µg of plasmid and insert DNA samples were digested with 0.5-1 µL of the desired restriction enzyme(s) at 37°C for 1 hour. 2 µL of alkaline phosphatase (Roche Cat. 713023) was added to the digested vector DNA to prevent self ligation and incubated at 37°C for a further 45 mins. The digested plasmid vector was loaded onto an agarose gel for extraction. Bands excised from gels were extracted using the pure link PCR product purification kit following the instructions of the manufacturer (Thermofisher Cat. K310001).

2.6.2. Ligations

To ligate insert DNA into the plasmid vectors 2 µL of Roche 10x ligation buffer and 1 µL of ligase were used in 20 µL volumes containing 50 ng digested plasmid vector and 150 ng digested insert and the mixture was incubated at 12°C overnight. In cases where DNA concentrations were low, 5 µL of the vector and 12 µL of the insert were used. The ligation mixtures were precipitated by adding 50 µL of 100% ethanol, 1 µL pellet paint (Novagen) and 2 µL 3M sodium acetate and centrifuging the mixtures at 13,000 rpm for 10 minutes. The supernatant was discarded and 100 µL of 70% ethanol was added to wash the pellet, followed by centrifugation for another 5 mins at 13,000 rpm. The supernatant was discarded and the pellet was air dried at 37°C for 15 mins. The pellet was resuspended in 5 µL of dH2O and used in the electroporation procedure.

2.6.3. Preparation of Electrocompetent Cells

Five mL LB broth cultures of E. coli S17 cells were incubated and 150 µL was used to inoculate a 30 mL LB broth containing appropriate antibiotics. The 30 mL culture was incubated with agitation at 160-200 rpm for 3-5 hours until the OD600 was 0.7-0.9 using sterile LB as a blank measurement. The cells were pelleted in a 50 mL centrifuge tube at
7000 rpm for 5 mins at 4°C. The supernatant was poured off and the pellet was resuspended in 30 mL of ice cold sterile 10% glycerol. The suspended cells were repelleted at 7000 rpm for 5 mins at 4°C and the supernatant was removed with a disposable pipette. The pellet was resuspended in 200 µL of ice cold sterile 10% glycerol by gentle vortexing. 40 µL aliquots of the suspension were added to 1.5 mL microcentrifuge tubes and stored at -70°C.

2.6.4. Electrotransformation

Aliquots (40-50 µL) of thawed competent E. coli cells were mixed with 1-2 µL of ligations and added to a pre-chilled 1 mm Biorad cuvette and subjected to an 1800 V pulse from the Biorad GenePulser Xcell. 1 mL of LB broth was immediately added to the electrotransformed cells and the resuspended cells were incubated at 37°C with 160-200 rpm agitation for 45 mins. 100 µL aliquots were spread onto LB agar with appropriate antibiotics.

2.6.5. Transfer of Plasmids by Conjugation

Biparental and triparental spot matings were prepared by adding 25 µL aliquots taken from a 5-mL overnight E. coli TY culture broth and a 5 mL 48 hour M. loti TY culture broth. The aliquots were coinoculated as a spot onto a TY plate and left to dry. These were incubated at 28°C and then plated on G/RDM agar plates supplemented with the appropriate antibiotics.

2.7. Mutagenesis

2.7.1. Insertion Duplication Mutagenesis (IDM)

IDM mutants were constructed through the use of the pFUS2 suicide plasmid containing a 350-bp intragenic region of the gene targeted for mutagenesis (Antoine et al., 2000). Biparental spot matings were performed between E. coli ST18 containing the pFUS2 clone and M. loti R7A strains. The matings were single-colony purified to isolate the desired mutant strains by passaging on G/RDM supplemented with Gm. Chromosomal DNA was extracted and then used in Southern hybridisations 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 (IDM) mutants were constructed by replacing the wild-type gene with a mutated construct containing an internal in-frame markerless deletion through
allelic replacement of the wild-type gene. The gene replacement occurred by homologous recombination. The constructs used were generated in previous studies (Kelly, 2012; Wightman, 2015). *E. coli* ST18 strains containing plasmid constructs based on the suicide vector pJQ200SK were mated into *M. loti* R7A via bi-parental spot-matings. Colonies were passaged four times on G/RDM containing Gm to select strains into which the plasmid had integrated via a single crossover. Dilution series of stationary phase TY broth cultures were plated on RDM containing sucrose as a sole carbon source. Sucrose-resistant strains which had lost the suicide vector and targeted gene through a second round of recombination were confirmed by PCR analysis, or Southern hybridisation.

### 2.8. Southern hybridisation

#### 2.8.1. Downward capillary blotting

To prepare the transfer of genomic DNA from 1% agarose gels to Whatman Hybond-N+ (Sigma) membranes, the gels were submerged in 0.2 M HCl for 15 min and rinsed twice in dH$_2$O. The gels were then submerged in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 15 min and then soaked in neutralisation solution (1.5 M NaOH, 0.5 M Tris [pH 8]) for 15 min. The transfer involved a downward capillary system involving construction of a stack. The base of the stack was formed by a 7 cm high stack of paper towels. Four pieces of dry Whatman filter paper (same size and shape to the gel) were stacked on top of the towels and 1 piece of wet Whatman filter paper pre-soaked in 20x SSC was added to the stack. The membrane was soaked for 5 min in dH$_2$O and then 5 min in 20x SSC before being placed on the top of the stack. The agarose gel and was then placed on top of the membrane. Finally, a further 6 pieces of Whatman filter paper that had been soaked in 20X SSC on top, 2 of which had an extension placed into a reservoir of 20x SSC. The assembled blot was left for a minimum of 4 hours to allow the transfer of DNA to occur. The DNA was then fixed to the membrane by soaking in 0.4 m NaOH for 20 min followed by rinsing in 2x SSC before air-drying.

#### 2.8.2. Hybridisation of probes to membrane-bound DNA

Membranes were placed in Hybaid bottles containing 0.125 mL per cm$^2$ of AlkPhos Direct™ hybridisation buffer (GE Healthcare). The bottle was incubated for 15 min at 55°C in a rotating Hybaid hybridisation oven.
The appropriate probe DNA (purified PCR product) was diluted to 10 ng/µL and 9 µL was added to 1 µL of 10ng/µL HindIII-digested Lambda phage DNA and boiled for 5 mins. The boiled DNA was then immediately cooled on ice for 5 min. The following reagents from the AlkPhos Direct™ labelling system kit were added to the DNA: 10 µL of reaction buffer, 2 µL of labelling reagent, 10 µL of crosslinker working solution (20% [v/v]). The mix was incubated at 37°C for 30 min and then added to the membrane. The bottle was incubated overnight at 55°C.

2.8.3. Post-hybridisation washing

Primary wash buffer was pre-heated to 55°C. The membrane was washed for 10 min in the primary wash buffer with gentle agitation. 2-5 mL of the primary wash buffer was used per cm² of membrane. This step was repeated.

The membrane was transferred to a clean container and an excess of secondary wash buffer was added. The membrane was washed for 5 min at room temperature with gentle agitation. This step was repeated.

2.8.4. Signal generation/detection

The secondary wash buffer was drained from the membrane and 30-40 µL CDP-Star detection reagent (GE Healthcare cat# RPN3682) was added per cm² of the membrane and left for 1-5 min. The excess detection reagent was then removed and the membrane was sealed in plastic and exposed to X-Ray film (Cronex, Dupont) for 2-24 hours.
3. Results

3.1. Complementation of R7A pFUS2::exoK polar mutant

In a previous study, several attempts to construct an R7A nonpolar exoK mutant had proven unsuccessful which implied that deletion of exoK may cause a lethal effect. An exoK mutant was successfully constructed using the suicide vector pFUS2 to produce the strain R7AexoK (Kelly 2012). Insertion of the suicide vector likely caused a polar effect, affecting expression of EPS biosynthetic gene located immediately downstream. The mutant had a colony morphology consistent with an EPS mutation. Moreover, an attempt to complement R7AexoK using the plasmid pSKKSG2, which contained only exoK and the preceding 387-bp region was unsuccessful (Kelly et al., 2012).

To confirm the polar phenotype of R7AexoK, a cosmid pSK11 containing a sequence of DNA including exoK and the exoLAMON gene cluster was transferred into R7AexoK by conjugation (Figure 3.1). The plasmid pSKKSG2 was also introduced into R7AexoK. pSK11 complemented the dry colony phenotype of R7AexoK back to the wild-type mucoid phenotype while pSKKSG2 appeared to partially complement the phenotype by producing small, white, slightly mucoid colonies (Figure 3.1), a result which differed from that reported previously (Kelly, 2012).
Figure 3.1: Complementation of R7AexoK polar mutant
G/RDM plates photographed 8 days after streaking with A) R7A/pIJPAR; B) R7AexoK; C) R7AexoK/pSKKSG2; D) R7AexoK/pSK11.

3.2. Construction of non-polar exoK in-frame markerless deletion mutants using the FRT/Flp system

As R7AexoK was a polar mutant, a fresh attempt to produce a non-polar exoK mutant was undertaken. The first step involved inactivating exoK using a gene replacement technique in both the R7A and R7AΔexoYF backgrounds. The R7A exoYF markerless mutant R7AΔexoYF (Wightman, 2014) does not produce any EPS as exoY catalyses the first step in EPS biosynthesis. Therefore an exoK mutant should be viable in this background. The strategy
involved replacing \textit{exoK} with a neomycin resistance cassette consisting of the \( \Omega \text{kan} \) intersposon which contains the \textit{nptII} gene flanked by FRT sites in direct orientation (\( \Omega \text{kan} \text{FRT} \)). An attempt to produce a non-polar mutant could then be performed by excising the resistance cassette using FLP recombinase, leaving an inactivated, truncated version of \textit{exoK} containing an in-frame FRT site in place of wild-type \textit{exoK} (Figure 3.2). A plasmid pSKKFRT (Kelly, 2012) in which this resistance cassette was cloned between two 1-kb DNA regions that flank the \textit{exoK} gene in the suicide vector pJQ200SK was available for use. The insert from this plasmid had also been cloned into the plasmid vector pIJ3200 to create pSKKFRT2. This plasmid was used to confirm that excision of the resistance cassette produced the desired deletion in \textit{E.coli} prior to performing the mutagenesis experiments in R7A.

**Figure 3.2: Site-specific recombination of the FRT/Flp system**

The FLP recombinase enzyme will excise all genetic material, for example an antibiotic resistance gene, between the two directly-orientated recombination target (FRT) sites by mediating site-specific recombination. A FRT scar remains after recombination. Figure repurposed from Kelly 2012.
3.2.1. Testing FLP-mediated excision in *E. coli*

To confirm that excision of the *exoKFRT* cassette would leave the expected in-frame FRT scar, an *E. coli* clone containing pSKKFRT2 was transformed with the plasmid pBH474 (which encodes the FLP recombinase) by electroporation. The cells were spread onto LB containing Tc and LB supplemented with Tc and Km. Significantly more colonies grew on the LB plates that lacked Km. Colonies from the LB-Tc plates were patch-plated to an LB plate supplemented with Tc and Km and one supplemented with Tc. Most of the patched colonies had lost Km resistance, suggesting that the resistance cassette had been excised. Plasmids were extracted from four Km<sup>+</sup> colonies and sequenced with the primers exoKRseqPCR and exoKLseqPCR. The sequences confirmed that the resistance cassette had been removed and the FRT scar that remained was in-frame between the 5’ and 3’ ends of *exoK*.

3.2.2. Reconstruction of R7AΔ*exoK* and R7AΔ*exoYFΔexoK* mutants using pSKKFRT

The plasmid pSKKFRT was introduced into R7A and the R7AΔ*exoYF* using bi-parental spot matings and the transconjugants were grown on G/RDM supplemented with Gm and Nm to select for strains in which the suicide plasmid had integrated into the genome via a single crossover. After three passages on this medium, single colonies were picked and cultured in TY broth for 72 hours. A dilution series was then performed for cultures of four isolates from both the R7A and R7AΔ*exoYF* backgrounds and plated on RDM containing 5% sucrose (Suc/RDM) and neomycin. All colonies isolated from the dilution series performed using R7A/pSKKFRT clones were Gm<sup>R</sup>/Nm<sup>R</sup>/Sucrose<sup>R</sup>, indicating that R7AΔ*exoKΩkanFRT* polar mutants were not obtained as the clones retained Gm resistance and no clones with an *exo* phenotype similar to that previously seen for the polar R7A*exoK* mutant were observed. By contrast the R7AΔ*exoYF* mutagenised with pSKKFRT dilution clones yielded a number of Gm<sup>S</sup>/Nm<sup>R</sup>/Sucrose<sup>R</sup> colonies indicative of a successful mutagenesis in this strain background. However due to the fact that the R7AΔ*exoYF* strain does not produce EPS, no change in the colony phenotype was observed. The sucrose selection procedure was repeated for the R7A/pSKKFRT clones and they were plated on both plain Suc/RDM and Suc/RDM containing neomycin. As before, only Gm<sup>R</sup>/Nm<sup>R</sup>/Sucrose<sup>R</sup> colonies were isolated on Suc/RDM supplemented with Nm; however, on Suc/RDM without Nm, half of the colonies isolated were Gm<sup>S</sup>/Nm<sup>S</sup>/Sucrose<sup>R</sup> and they retained a wild-type EPS phenotype, indicating a
likely reversion to the wild-type phenotype by loss of pSKKFRT. No colonies were found with an \textit{exo} mutant colony phenotype.

It seemed possible that \textit{exoK} might be essential in which case the mutagenesis might be successful if a second copy of \textit{exoK} was provided \textit{in trans}. The pSKKSG2 and pSK11 plasmids were mated into several of the Gm resistant clones and screened on Suc/RDM supplemented with Tc and Nm to determine if \textit{exoK} could be replaced if the \textit{exoK} gene alone (pSRKG2), or \textit{exoK} and downstream genes (pSK11) were present. It was found that Gm\textsuperscript{S}/Nm\textsuperscript{R}/Sucrose\textsuperscript{R} colonies could be isolated if either of these plasmids were present in the cells. A small, white, mucoid colony phenotype was observed in all Gm\textsuperscript{S}/Nm\textsuperscript{R}/Sucrose\textsuperscript{R} colonies obtained containing pSKKSG2. This phenotype was noted to be slightly different to that observed when R7A\textit{exoK} was complemented with pSKKG2, as the latter strain produced slightly less mucoid colonies. The wild-type EPS phenotype was observed for all Gm\textsuperscript{S}/Nm\textsuperscript{R}/Sucrose\textsuperscript{R} colonies containing the pSK11 plasmid (Figure 3.3).

![Figure 3.3: Marker exchange mutagenesis of \textit{exoK} in the presence of complementing plasmids.](image)

Either pSK11 or pSKKSG2 were introduced into R7A containing pSKKFRT integrated via a single crossover and the strains were then screened on Suc/RDM medium. Gm\textsuperscript{S}/Nm\textsuperscript{R}/Sucrose\textsuperscript{R} colonies were isolated and streaked on G/RDM and grown for 9 days. A) R7A/pIJPAR; B) R7A pSKKFRT pSK11; C) R7A pSKKFRT pSKKSG2

The potential R7A\textit{ΔexoYFΔexoKΩkanFRT}, R7A\textit{ΔexoKΩkanFRT}/pSKKG2 and R7A\textit{ΔexoKΩkanFRT}/pSK11 mutants were screened using PCR and sequencing. PCR was
performed using the primer $exoK_{FRTlocatL}$ that was located upstream of the genomic region flanking $exoK$ cloned in pSKKRFT along with $nptII_ROR$, an outward-facing primer located within the resistance cassette. A second pair, $nptII_ROL$ that annealed within the resistance cassette and $exoK_{FRTlocatR}$ which was located downstream of the region cloned in pSKKFRT, was also used.

Products were obtained for $exoK_{FRTlocatL}$ and $nptII_ROR$ and were confirmed by DNA sequencing. No products were obtained for the other primer pair although, except for one of the R7AΔexoKΩkanFRT/pSK11 strains, a faint product of the expected size of 1,900 bp was seen with $exoK_{FRTlocatR}$ and $nptII_ROL$. This sample had additional DNA fragments of other sizes when PCR was performed with $exoK_{FRTlocatR}$ and $nptII_ROL$. PCR-based screening of R7AΔexoYFΔexoKΩkanFRT clones is shown in Figure 3.4 and screening of R7AΔexoKΩkanFRT/pSKKG2 and R7AΔexoKΩkanFRT/pSK11 clones is shown in Figure 3.5.
Figure 3.4: PCR confirmation of gene replacement of *exoK* to form the 
R7AΔ*exoYFΔexoKFRT* polar mutant.

A PCR was performed on DNA extracted from putative R7AΔ*exoYFΔexoKFRT* mutant 
clones. Lane 1 λ DNA digested with HindIII and ΦX174 DNA digested with HaeIII. Lanes 2, 
4, 6, 8 and 10 were loaded with PCR reactions amplified with primers *exoKFRTlocatL* and 
*nptIIOR*, while lanes 3, 5, 7, 9 and 11 were loaded with PCR reactions amplified using 
*exoKFRTlocatR* and *nptIIROL*. 
Figure 3.5: PCR confirmation of \textit{exoK} gene replacement in the R7AΔ\textit{exoKΩkanFRT/pSK11} and R7AΔ\textit{exoKΩkanFRT/pSKKG2} isolates.

PCR was performed on DNA extracted from putative R7AΔ\textit{exoKΩkanFRT/pSK11} mutant clones. Lane 1 was loaded with λ DNA digested with HindIII and ΦX174 DNA digested with HaeIII. Lanes 2 and 3 were loaded with negative no template controls. Lanes 4, 6 and 8 contain the PCR product obtained for R7AΔ\textit{exoKΩkanFRT/pSKSG2} clones using primers \textit{exoKFRTlocatL} and \textit{nptII\textsubscript{ROR}} while Lanes 5, 7 and 9 were loaded with PCR reaction performed with \textit{exoKFRTlocatR} and \textit{nptII\textsubscript{ROL}}. Lanes 10, 12, 14, 16 and 18 were loaded with PCR reactions performs on R7AΔ\textit{exoKΩkanFRT/pSK11} using the primers \textit{exoKFRTlocatL} and \textit{nptII\textsubscript{ROR}} and lanes 11, 13, 15, 17 and 19 were loaded with PCR reactions performed with \textit{exoKFRTlocatR} and \textit{nptII\textsubscript{ROL}}. Though faint, Lane 9 shows a band of ~2000 bp as indicated by the white arrow, however additional bands are also present within the lane.

3.3. Construction of a non-polar R7AΔ\textit{exoYFΔexoK} mutant

The R7AΔ\textit{exoYFΔexoKΩkanFRT} polar mutant was used to generate a non-polar mutant by excising the \textit{ΩkanFRT} cassette \textit{in vivo}. The plasmid pBH474 plasmid which contains the FLP recombinase and \textit{sacB} genes was transferred into R7AΔ\textit{exoYFΔexoKΩkanFRT} by conjugation and the transconjugants were selected on G/RDM supplemented with Gm. Single colonies were screened by patch plating onto G/RDM supplemented with Nm and Gm or Nm alone. Nm\textsuperscript{S} clones were then selected, colony purified and grown in TY broth culture. A dilution series was spread onto Suc/RDM to select strains which had spontaneously lost
pBH474 and single colonies were isolated and again screened for Nm and Gm sensitivity. Nine Nm$^5$/Gm$^5$ sucrose resistant clones were selected and the DNA was extracted and digested with XhoI. This DNA digest was used in a Southern hybridisation (Figure 3.6). The probe used was a PCR product obtained using the primers $exoK$intprobeL and $exoK$intprobeR. This probe spans a region within $exoK$ therefore would be expected to hybridise to the wild-type, but not the $\Delta exoYF\Delta exoK$ mutant. The Southern blot showed a band of the expected size (~11kb) in the two wild-type controls, but not in any of the nine into R7A$\Delta exoYF\Delta exoK$ clones confirming the removal of the $exoK$ gene.
Figure 3.6: Southern hybridisation analysis of R7AΔexoYFΔexoK clones

Panel A: DNA extracts of two R7A wild-type controls and nine putative R7AΔexoYFΔexoK clones were digested with XhoI, run on a 1% agarose gel. Lane 1 was loaded with λ DNA digested with HindIII and ΦX174 DNA digested with HaeIII. Lanes 2 and 3 contain XhoI digested chromosomal R7A wild-type DNA and lanes 4 to 12 contain XhoI-digested chromosomal DNA from 9 potential R7AΔexoYFΔexoK mutants. Panel B: A Southern hybridisation performed using the adjacent gel, and hybridised with a probe containing λ phage DNA and a PCR product obtained from R7A DNA using the exoK primers exoKintprobeL and exoKintprobeR.
3.3.1. Complementation of R7AΔexoYFΔexoK in-frame markerless deletion mutants with exoYF

The R7AΔexoYFΔexoK double mutant and the R7AΔexoYF mutant (Wightman, 2014) were transformed with a plasmid containing the R7A exoYF genes and preceding promoter region, pFAJ::exoY-F (Wightman, 2014) and the empty pFAJ1700 vector and plated onto G/RDM, Succ/RDM and TY media. Introduction of the pFAJ::exoY-F plasmid was found to be lethal in the R7AΔexoYFΔexoK mutant, but it caused the R7AΔexoYF mutant to revert to the R7A wild-type exo phenotype as shown previously (Wightman, 2014). The pFAJ1700 empty vector had no effect either strain (Figure 3.7).

**Figure 3.7: Complementation of R7AΔexoYFΔexoK polar mutants**

The strains R7AΔexoYFΔexoK and R7AΔexoYF were transformed with the pFAJ::exoY-F and pFAJ1700 plasmids and streaked onto G/RDM, Succ/RDM and TY containing Tc. Photo taken after 8 days incubation at 28°C. A) R7AΔexoYF B) R7AΔexoYFΔexoK, (Initial inoculum and primary streak of R7AΔexoYFΔexoK/pFAJ::exoY-F clones shown in photo while the third streak of R7AΔexoYFΔexoK/pFAJ1700 empty vector clones is shown).
3.4. Construction of an R7AΔexoYFΔexoU mutant

To determine whether or not the truncated EPS subunits could be transported across the inner membrane by a secondary transport mechanism, a double knockout mutant deficient in the expression of both \textit{exoT} and \textit{exoU} needed to be constructed. A \textit{ΔexoYF} mutant was used as the starting point as the loss of \textit{exoT} in the wild-type mutant has been reported to cause severe growth defects, but this growth defect is relieved in the \textit{ΔexoYF} background. A \textit{ΔexoYFΔexoU} double mutant was constructed using the R7AΔexoYF mutant as the starting point. The \textit{exoU} gene was deleted using the pJQ200SK suicide plasmid, pSKM2U (Kelly 2012) which contained a 2-kb insert consisting of two 1-kb regions of DNA flanking the \textit{exoU} gene. The plasmid was transferred to R7AΔexoYF by conjugation and transconjugants into which pSKMU2 had integrated via single crossover were selected on G/RDM containing Gm and then colony purified three times. Single colonies were then grown in TY broth and then a dilution series was spread on Suc/RDM to select strains in which a second recombination event had led to the loss of the plasmid (which encodes \textit{sacB}) by homologous recombination. Gm\textsuperscript{S} colonies were then screened for the loss of \textit{exoU} by PCR. Primer pairs used included a pair which flanked the anticipated deletion (\textit{exoULChk} and \textit{exoURChk}) which would produce a product of reduced size in the mutant versus the wild-type and two pairs, \textit{exoULChk/exoU5’RChk} and \textit{exoU3’LChk/exoURChk}, which would produce products which spanned the left and right ends of \textit{exoU} for wild-type R7A, but would not produce products for the mutant due to loss of the \textit{exoU}. The product of the correct size of 560 bp spanning the deleted region was produced for the R7AΔexoYFΔexoU clones, however no product was acquired in the wild-type where a 1400 bp product was expected. PCR products of the expected sizes of 300 bp and 800 bp were successfully produced in the wild-type using the other two primer pairs. Unexpectedly, the R7AΔexoYFΔexoU clones produced a 600 bp DNA fragment using these primer pairs, but not a product of the size produced in the wild-type controls (Figure 3.8).

The primers complementary to the ends of the \textit{exoU} gene produced a PCR product of a size indicating the \textit{exoU} deletion construct had successfully been inserted into the cell, the DNA fragments expected in the R7A PCR products were absent from the mutants indicating the loss of the \textit{exoU} gene. The 600 bp fragments using the \textit{exoULChk/exoU5’RChk} and \textit{exoU3’LChk/exoURChk} primers were unexpected as the one of the primers in each pair used were complementary to the sequence within the deleted \textit{exoU} gene. In addition, neither of the
fragments these products matched an expected size in the R7A wild-type.

**Figure 3.8:** PCR confirmation of the R7AΔexoYFΔexoU mutant.

Lanes 1, 6 and 11 contain λ DNA digested with HindIII and ΦX174 DNA digested with HaeIII. Lanes 2-5 contain products obtained with exoULChk and exoURChk primers and DNA from: No template control (Lane 2), R7A (Lane 3), R7AΔexoYFΔexoU clones 1 and 2 (Lanes 4 and 5). Lanes 7-10 contain products obtained with the exoU3’LChk and exoULChk primers and DNA from: No template control (Lane 7), R7A (Lane 8), R7AΔexoYFΔexoU clones 1 and 2 (Lanes 9 and 10). Lane 12-15 contain products obtained with the exoU5’RChk and exoURChk primer pair with DNA from: No template control (Lane 12), R7A (Lane 13), R7AΔexoYFΔexoU clones 1 and 2 (Lanes 14-15).
3.5. Construction of R7AΔexoYFΔexoU pFUS2::exoT mutant

The R7AΔexoYFΔexoU mutant was then used to construct an R7AΔexoYFΔexoUΔexoT triple mutant. Previously an R7AΔexoYFexoT mutant had been constructed. Attempts to construct an R7AexoT mutant had proven unsuccessful, and it was assumed that this mutation which would disrupt EPS transport was potentially lethal in a wild-type background due to accumulation of EPS monomers in the cell cytosol (Wightman, 2014). We planned to complement the above triple mutant with exoYF genes and, if this complementation was unsuccessful, it would suggest that the truncated polysaccharide produced by the exoU mutant was transported by the wild-type EPS transport system. Inactivation of exoT in R7AΔexoYFΔexoU was performed by insertion duplication mutagenesis (IDM) using a pFUS::exoT suicide vector constructed previously. This plasmid contained a 350-bp region from within the exoT gene (Wightman, 2014). The plasmid was transferred into R7AΔexoYFΔexoU via bi-parental spotings. The transconjugants were single colony purified and passaged three times on G/RDM supplemented with Gm. Genomic DNA was extracted from 6 clones and digested with the restriction enzyme SalI. The digests were run on a 1% agarose gel and the gel was used in a Southern hybridisation (Figure 3.9). The probe for the Southern blot was produced by PCR using the primers exoTpFUSL and exoTpFUSR. The mutant produced a large band (9.8 kb) equivalent to the size of the R7A SalI restriction fragment (2.3 kb), plus the suicide vector (which lacks a SalI site) and a duplication of the 350-bp insert contained in the vector. One of the six clones (lane 7, Fig. 3.9B) had a double insertion, indicated by a larger band.
Figure 3.9: SalI digestion and Southern hybridisation analysis of R7AΔexoYFΔexoUpFUS2::exoT clones

A) DNA preparations from R7A wild-type and six putative R7AΔexoYFΔexoUpFUS2::exoT mutant clones were digested with SalI and run on a 1% agarose electrophoresis gel overnight at 22 V. Lane 1 contains λ DNA digested with HindIII and ΦX174 DNA digested with HaeIII. Lane 2 contains SalI-digested R7A wild-type DNA and lanes 3 to 8 contain SalI-digested DNA of the mutant clones. B) A Southern blot performed using the agarose gel shown in A) probed with a labelled PCR product that was amplified using the exoT-specific primers exoTpFUSL and exoTpFUSR.
3.6. Complementation of the R7AΔexoYFΔexoU markerless deletion mutant and R7AΔexoYFΔexoU pFUS2::exoT insertion duplication mutagenesis mutant with exoYF

A plasmid pFAJ::exoY-F (Wightman, 2014) containing the exoYF genes and promoter region was conjugated into the R7AΔexoYFΔexoU and R7AΔexoYFΔexoU pFUS2::exoT mutants. The plasmid was also transferred into R7A, R7AΔexoYF, R7AΔexoU and R7AΔexoYF pFUS2::exoT. The empty vector pFAJ1700 was also introduced into these strains as a control. The transconjugants were plated onto G/RDM, Succ/RDM or TY containing Tc or Tc and Gm as appropriate. These three types of media were used as they support different levels of EPS production, with G/RDM allowing high production of EPS, Succ/RDM allowing significantly reduced EPS and TY largely suppressing EPS production. The empty vector pFAJ1700 had no effect on the growth of any strain. Introduction of pFAJ::exoY-F into R7AexoYF caused it to revert to the wild-type exo phenotype, however introduction of pFAJ::exoY-F into R7AΔexoU and R7AΔexoYFΔexoU caused a growth defect with R7AΔexoU/pFAJ::exoY-F showing the more severe defect. The growth defect was less severe on Succ/RDM media. Introduction of pFAJ::exoY-F caused severe growth defects to both R7AΔexoYFpFUS2::exoT and R7AΔexoYFΔexoUpFUS2::exoT (Figure 3.10). These results confirm that the EPS transport mutant is severely impaired in growth if the strain makes EPS and also that the truncated EPS produced by the exoU mutant is transported by this system.
A) R7AΔexoYF

G/RDM

Succ/RDM

TY

pFAJ::exoY-F  pFAJ1700
B) R7ΔexoYFΔexoU
pFAJ::exoY-F pFAJ1700

G/RDM

Succ/RDM

TY
D) R7AΔexoYpFUS2::exoT
   pFAJ::exoY-F       pFAJ1700

   G/RDM

   Succ/RDM

   TY
Figure 3.10: Complementation of R7AΔexoYFΔexoU and R7AΔexoYFΔexoUpFUS2::exoT clones with pFAJ::exoY-F

The empty pFAJ1700 vector and the pFAJ::exoY-F clone were transformed into a selection of strains and the conjugation mixtures were plated onto G/RDM, Succ/RDM and TY containing Tc or Tc and Gm and grown for 8 days. A) R7AΔexoYF; B) R7AΔexoYFΔexoU; C) R7AΔexoU; D) R7AΔexoYFpFUS2::exoT; E) R7AΔexoYFΔexoUpFUS2::exoT
3.7. Effect of expression of exoK on the composition of R7A EPS

In previous work (Kelly, 2012) the cosmid pSKU1 which harbours the major exo gene cluster from the *M. loti* strain NZP2037 including the *exoU* and *exoK* genes was examined to determine if it could complement the R7A *exoU* mutant by plating the R7AexoU/pSKU1 strain on G/RDM. The cosmid returned the *exoU* mutant to a mucoid colony phenotype, but it was noted that after prolonged incubation the colonies developed a “watery” appearance. It was postulated that this phenotype might be caused by the presence of an increased amount of the *exoK* gene product in the cells leading to production of elevated levels of the LMW EPS.

In order to investigate this phenomenon further, the cosmid pSKU1 and an R7A cosmid containing *exoK*, pSK11 (see section 3.1) were transformed into R7A, the R7AΔndvB mutant and NZP2037 along with the empty vector pIJ3200. These resulting clones were streaked onto both G/RDM and glycerol/RDM plates. It was found that when grown on glycerol medium, the R7A/pSKU1 strain grew with an extremely thin consistency causing the colonies to eventually start to dry out. The same strain on G/RDM however, did not dry out but the consistency of the colony became thin and watery. The pSK11 plasmid has no discernible effect on R7A when the strain was plated on G/RDM, however when plated on glycerol, the colonies started to show a phenotype similar to that observed for the R7A/pSKU1 strain when grown on G/RDM, though to a lesser extent. The strain R7AΔndvB/pSKU1 showed a slight change in phenotype when grown on G/RDM, however this was mostly observable at the point where streaking of the plate commenced where it began to look dry. The phenotype this strain produced on glycerol/RDM was similar, but slightly more pronounce with the “watery” phenotype extending to the secondary streaks.

The pSK11 plasmid seemed to have no effect on R7AΔndvB when grown on either G/RDM or glycerol/RDM. The pSKU1 plasmid caused NZP2037 colonies to flatten and dry out on both carbon sources. It is interesting to note that NZP2037 does not appear to show any signs of an abnormal EPS phenotype aside from the colonies looking slightly flatter versus the empty vector controls when grown on both carbon sources (Figure 3.11).
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Figure 3.11: Effects of the pSKU1 and pSK11 cosmids on the M. loti strains R7A, NZP2037 and R7AΔndvB on G/RDM and glycerol/RDM

pSKU1 and pSK11 were transferred into R7A, R7AΔndvB and NZP2037 by conjugation and the colony phenotypes of the resulting strains were observed at six and twelve hour post-inoculation on G/RDM and glycerol/RDM plates. A) R7A. R7A/pSKU1, R7A/pSK11, B) R7AΔndvB R7AΔndvB/pSKU1 R7AΔndvB/pSK11, C) NZP2037, NZP2037/pSKU1, NZP2037/pSK11. The empty vector pIJ3200 had no effect on the growth of any of the strains (data not shown).

3.8. Induced ExoK expression with pSDZ expression vector in strains R7A, R7AΔndvB and NZP2037

To determine the effect of ectopic expression of the exoK gene on the structure of EPS, an exoK expression vector was constructed. The exoK gene was amplified by PCR and cloned adjacent to the IPTG-inducible promoter in the broad host range expression vector pSDZ. Two versions of this plasmid were constructed, one designated pSDZ::exoKnatRBS used the native exoK ribosome binding site (PCR product amplified with primers exoKpSDZLRBSnat and exoKpSDZR1) and the other, pSDZ::exoKoptRBS was amplified using primers
exoKpSDZLRBSopt and exoKpSDZR2 and utilised a synthetic optimised ribosome binding site (Dombrecht et al., 2001) incorporated into the exoKpSDZLRBSopt primer. A promoterless gfp gene is located immediately downstream of the cassette. The two plasmids were confirmed by restriction endonuclease analysis and DNA sequencing and transferred into M. loti strains R7A, R7AΔndvB and NZP2037 by conjugation. The empty vector pSDZ was also transferred into these strains to provide controls. Plasmid expression was induced on glycerol/RDM plates supplemented with IPTG and Tc. Glycerol was used as the carbon source as previous work had shown that expression from the IPTG-inducible promoter was affected by catabolic expression when glucose was used (Prof. Michael Hynes, personal communication). No phenotypic differences were observed between any strains containing the pSDZ::exoK plasmids and the same strains carrying the pSDZ empty vector.

3.8.1. Fluorescent microscopy analysis of gfp expression from pBF1 and pBF2 in R7A

One possible reason for the lack of a phenotype observed when using the pSDZ exoK clones was that the plasmids were not expressing exoK. The induction of IPTG-inducible expression was investigated by observing the expression of a gfp gene located downstream of the cloning cassette. If exoK was being expressed, then the gfp gene should be expressed as well. The R7A clones containing the pDSZ vectors were grown on glycerol/RDM plates and glycerol/RDM plates supplemented with 1 mM IPTG. Material taken from single colonies was suspended in a small volume of sterile water, spread of the surface of a slide and then air-dried and viewed under a fluorescent microscope. It was found that there was strong gfp expression in the cultures grown on glycerol/RDM media supplemented with IPTG and that cultures grown on glycerol/RDM media without IPTG showed weaker gfp fluorescence (Figure 3.12).
Figure 3.12: Fluorescent microscopy images of gfp expression in R7A containing pSDZ on Glycerol/RDM

A) R7A/pFAJ1700 grown on glycerol/RDM. B) R7A/pFAJ1700 grown on glycerol/RDM supplemented with IPTG. C) R7A/pSDZ grown on glycerol/RDM. D) R7A/pSDZ grown on glycerol/RDM supplemented with 1 mM IPTG. E) R7A/pBF1 (exoK native RBS) grown on Glycerol/RDM. F) R7A/pBF1 grown on glycerol/RDM supplemented with 1 mM IPTG. G)
R7A/pBF2 (exoK optimised RBS) grown on glycerol/RDM. H) R7A/pBF2 grown on glycerol/RDM supplemented with 1 mM IPTG.
4. Discussion

5.1. Deletion of exoK in R7A is lethal with exoYF active

The exoK gene encodes a glycanase that maybe responsible for cleaving nascent EPS into a LMW form. This LMW EPS is of particular interest as it is implicated to be a secondary signalling molecule to the plant host and the loss of the exoK gene may lead to a loss of symbiotic proficiency in the M. loti R7A strain (Becker et al., 1993a; Kawaharada et al., 2015; Urzainqui & Walker, 1992; York & Walker, 1997; York & Walker, 1998a; York & Walker, 1998b). Therefore, the first aim of this study was to ultimately construct an R7A strain lacking the exoK gene that can be used in various studies using purified EPS. This was to be done by generating an in-frame marker-less deletion of exoK in the R7A wild-type.

Attempts to generate a non-polar ΔexoK in-frame marker-less deletion mutant in the past had failed (Kelly, 2012). Because of this, the possibility of this mutation being lethal in R7A was considered and a second mutant was generated in the R7A ΔexoYF background to determine whether or not this was the case. The exoK gene has been investigated in other rhizobia, in particular the species S. meliloti and Rhizobium sp. NGR234 (Mendis et al. 2013; Staehelin et al. 2006). In these studies, ExoK deficient mutants of both species were constructed using transposon mutagenesis. In addition, Mendis et al. also constructed a mutant containing a non-polar deletion of exoK in S. meliloti by adding a constitutive promoter to express downstream genes. This mutant showed a small loss of symbiotic proficiency and a defect in nodule development. Shoots of M. truncatula and alfalfa inoculated by S. meliloti with a non-polar deletion in the exoK gene were 60-70% and 54-69% of those inoculated by the wild-type respectively (Mendis et al. 2013). It is possible that the severe growth defect seen in M. loti R7A exoK mutants did not occur in S. meliloti exoK mutants because of the expression of exsH, a second glycanase that was shown to be able to substitute exoK function (York & Walker, 1998b).

The current study continued Kelly’s attempts to produce a non-polar exoK deletion in M. loti R7A. An nptII cassette conferring NmR was inserted into the exoK gene to ensure that the reversion of mutant strains was not possible. The nptII cassette was inserted in frame with the downstream genes preventing polar deleterious effects during mutagenesis. Every attempt to remove the wild-type copy of the gene in R7A failed and all isolates able to grow on sucrose and were resistant to Gm, indicating that all strains retained the pSKKFR7 plasmid. This
demonstrated that the wild-type copy of \textit{exoK} had not been removed from the strain and that the plasmid likely had a point mutation in the \textit{sacB} gene relieving the lethal effects of sucrose on the cell. When the R7A/pSKKFRT strain was plated on the Suc/RDM media in the absence of Nm, the strains obtained could grow on media containing sucrose, but not on media containing Gm or Nm. These results indicate that both the plasmid and the copy of \textit{exoK} containing the \textit{nptII} cassette were lost. This provides evidence that a selection pressure is present to retain the wild-type \textit{exoK} gene.

In order to investigate the possibility that \textit{exoK} is an essential gene, the experiment was repeated in the R7A Δ\textit{exoYF} background. Unlike the R7A strain, when selecting for colonies on sucrose, all colonies isolated were found to be Gm\textsuperscript{S} indicating a loss of pSKKFRT, and were Nm\textsuperscript{R}. This suggests that the isolated colonies had lost the wild-type copy of the \textit{exoK} gene along with the pSKKFRT plasmid. To confirm that the copy of \textit{exoK} containing \textit{nptII} cassette had been successfully integrated into the genome, the genomic DNA was extracted and analysed by PCR and sequencing. The primers used were \textit{exoKRseqPCR} and \textit{exoKLseqPCR}. The analysis confirmed that the cassette had integrated into the genome and therefore had generated a polar R7A \textit{exoYF exoK} mutant.

In order to determine the effect of deleting the \textit{exoK} gene in a non-polar mutant, the \textit{nptII} was removed to put the genes downstream of \textit{exoK} back in frame, using the pBH474 plasmid. PCR performed on the putative mutant isolates consistently produced an expected DNA fragment using the \textit{exoKFRTlocatL} and \textit{nptII/ROR} primer pair, however difficulties arose using the \textit{exoKFRTlocatR} and \textit{nptII/ROL} primer pair. DNA fragments were obtained, however the bands were faint on an agarose gel and additional fragments of unexpected sizes were present. Despite the unexpected results with the \textit{exoKFRTlocatR} and \textit{nptII/ROL} primer pair, the Nm\textsuperscript{R} gene was lost indicating that the \textit{nptII} cassette was successfully excised from the cell and sequencing confirmed that the genes downstream of the \textit{exoK} gene were in-frame leaving a successful R7A Δ\textit{exoYFΔexoK} polar mutant.

Despite the failure of obtaining an R7A Δ\textit{exoK} mutant, the R7A Δ\textit{exoYFΔexoK} mutant was successfully acquired. The effect on growth of an \textit{exoK} deletion was then investigated by complementing the \textit{exoYF} genes in the R7AΔ\textit{exoYFΔexoK} double mutant. When attempts were made to complement the strain with a plasmid containing the \textit{exoYF} genes, no single colonies were obtained on G/RDM media. The complete lack of growth provides evidence that when the \textit{exoYF} genes are expressed, \textit{exoK} performs an essential role in \textit{M. loti}. When
grown on TY, this growth effect was relieved slightly as some growth occurred, however with severe inhibition. These results suggest that as the production of EPS is increased, the role of exoK becomes more essential for the survival of M. loti. A possibility for this lethal effect may be that the exoK gene product plays a role in the process of transporting the EPS out of the cell. It has been demonstrated by York and Walker (1998a) that the ExoK glycanase cleaves nascent EPS and fails to cleave EPS in vitro in cell free cultures. It is possible that in order to cleave EPS, ExoK must interact with the secretory proteins. If this is the case, then the deletion of exoK may have an effect on EPS secretion, leading to the accumulation of EPS in the cell membrane.

It is noted that exoK was successfully knocked out without polar effects on downstream genes in S. meliloti and NGR234, but not M. loti. In S. meliloti this may possibly be due to the presence of exsH, which encodes an additional glycanase that is present in the S. meliloti species, but not NGR234. The glycanase encoded by exsH is also able to cleave HMW EPS into LMW EPS. Hence the expression of this gene may provide redundancy to the role of ExoK and relieve the lethal effects caused by the deletion of the exoK gene in S. meliloti. The exsH gene has a homolog in M. loti R7A that shares 60% amino acid identity, however it is located within clusters of genes that are not reported to have any involvement in EPS production. A ΔexsH mutant was made in R7A by Kelly (2012) that showed no change in the EPS phenotype and did not reduce the symbiotic proficiency of the strain. It therefore seems likely that the exsH homolog may not be involved in EPS cleavage in M. loti and this may be the reason why a non-polar exoK mutant is not viable in strains of this species that produce EPS. In contrast to S. meliloti, NGR234 cleaves HMW EPS to LMW EPS by exoK through a non-redundant system and exoK mutants were reported to produce HMW EPS, but were deficient in LMW EPS production suggesting a non-polar deletion of exoK. Staehelin suggested the possibility that NGR234 ExoK may have a lower affinity for its substrate expression and NGR234 may have lower exoK expression than that of other rhizobial strains due to low levels of LMW EPS produced in the NGR234 wild-type (Staehelin et al., 2006). It is possible that the loss of exoK may not cause a lethal effect because the expression of exoK and production of LMW EPS is lower than that of other rhizobial strains. This may explain why the deletion of exoK was possible in the NGR234 strain, but not the R7A strain.
5.2. **R7A pFUS2::exoK was confirmed as a successful polar exoK mutant**

As noted above, a previous attempt was made to produce a non-polar in-frame marker-less deletion of *exoK* in *M. loti* R7A (Kelly 2012). While the non-polar mutant was never successfully constructed, a polar mutant was created by transposon mutagenesis with a colony morphology characterised as small, yellow, non-mucoid colonies. Studies have produced *exoK* mutants via transposon insertion mutagenesis in the species *S. meliloti* and *Rhizobium* sp. NGR234 (Mendis *et al.* 2013; Staehelin *et al.* 2006). Staehelin *et al.* found that the *exoK* mutant of NGR234 formed mucoid colonies, clearly distinguishable from the non-mucoid EPS deficient mutants, but was symbiotically deficient. Complementation experiments by Staehelin *et al.* revealed that the *exoK* gene alone was enough to restore the symbiotic phenotype in NGR234Ω*exoK* indicating no polar effects on downstream *exo* genes due to the deletion of *exoK*. Furthermore, the NGR234Ω*exoK* strain produced no LMW EPS suggesting another glycanase does not fulfil the role of *exsH* in NGR234. The authors concluded that LMW EPS was the symbiotically active from of EPS in NGR234.

In this study, attempts to make non-polar in-frame marker-less deletion mutants failed and clones lacking the wild-type *exoK* gene were unable to be isolated. Because *exoK* had proven to be an essential gene when the *exoYF* genes were expressed, complementation experiments were carried out to determine whether or not the expression of the *exoK* gene in the pFUS2::*exoK* mutant made by Kelly (2012) was successfully inhibited. When the R7A pFUS2::*exoK* mutant was complemented with pFAJ1700::*exoK*, it failed to revert to the wild-type phenotype, though a slight phenotypic change was observed. The partial complementation caused by the pFAJ1700::*exoK* plasmid made the colony phenotype become more mucoid and as well as changed the colony colour from yellow to white. When this strain was complemented with pSK11, a full phenotype restoration was observed. These results confirm the inactivation of the *exoK* gene in the pFUS2::*exoK* mutant.

The above results also confirm that deletion of the *exoK* gene has polar deleterious effects on the downstream genes and that the suicide plasmid had been inserted into the correct location within the genome. In particular, genes affected in this mutant include the *exoLAMON* operon, several of which are involved in the addition of glycosyl groups to the EPS chain. The deleterious effect on these would prevent the second glycosyl residue from being added.
to the backbone which would be expected to result in a soft, white, non-mucoid colony. The mutant itself was distinguishable from an exoA mutant by its yellow colouration. When the exoK gene was reintroduced into the strain, there was only a partial complementation resulting in a colony morphology similar to that of an exoA mutant, however the initial inoculum showed a noticeable mucoid characteristic not reported in the exoA mutant. This result is surprising as a partial complementation suggests polar effects on the downstream genes, but a ΔexoYFexoK mutant shows no phenotypic change when complemented with exoK. This effect caused by exoK complementation may possibly be due to a low expression of the exoLAMON genes and this may explain the yellow colour of the exoK mutant. It is possible that the exoK mutant developed by Kelly (2012) may have express the exoLAMON operon in low quantities resulting in a low production of HMW EPS. This would explain the mucoid colony morphology when complemented with exoK and may have allowed the deletion of exoK by reducing EPS production to extremely low levels. Complementing this strain with the exoLAMON operon and exoK gene would therefore restore the wild-type phenotype completely by returning EPS production to higher quantities. Complementing the exoLAMON operon alone may inhibit growth due to the loss of exoK.

5.3. **exoT is required to export truncated EPS across the cell membrane**

A ΔexoYF pFUS2::exoT mutant was produced and used to show that removing the exoT gene in the wild-type was lethal (Wightman, 2015). However, the study performed by Wightman did not include the effects of deleting exoT in the ΔexoU strain. In this study the effect of deleting exoT in this mutant was investigated by creating a ΔexoYFΔexoU pFUS2::exoT mutant. The aim was to determine whether the same transport system that transports full-length EPS also transports the pentasaccharide produced by exoU mutants.

Complementation of exoYF in the ΔexoYFΔexoU pFUS2::exoT and ΔexoYF pFUS2::exoT strains were lethal in both cases, regardless of the media the strains were grown on. Addition of the pFAJ1700::exoYF plasmid to the R7A ΔexoYFΔexoU and R7A ΔexoU strains also caused severe growth defects with the most severe being that of R7A ΔexoU which fails to grow at all on G/RDM media, though on TY and Succ/RDM the growth defect of the
complemented strains is reduced. The detrimental effect that the increased copy number of \textit{exoYF} has on the strain may be due to the truncated EPS being toxic in large amounts. Therefore, increased expression of \textit{exoYF} would upregulate truncated EPS production causing a harmful concentration of the product. When \textit{M. loti} R7A is grown on TY and Succ/RDM, its EPS production is reduced. Hence the \textit{exoU} mutants plated on these media would produce less truncated EPS. The detrimental growth effect of the mutation is therefore reduced, but is still noticeable when compared to the control.

No pFUS2::\textit{exoT} mutants grew at all when complemented with pFAJ1700::\textit{exoYF} in comparison to the poor growth of the \textit{exoYF}-complemented R7A \textit{ΔexoYFΔexoU} strain suggesting that the \textit{exoT} gene product is involved in the secretion of the truncated EPS of \textit{ΔexoU} mutants. As the result of deleting \textit{exoT} is a complete lack of growth on G/RDM, TY and Succ/RDM media, it appears that no other gene is able to substitute for the secretion of either the wild-type or the truncated EPS and \textit{exoT} is therefore an essential gene when \textit{exoYF} is expressed.

Despite the expression of \textit{exoT}, overexpression of \textit{exoYF} is still lethal in the \textit{ΔexoU} mutants while an overexpression of \textit{exoYF} in the wild-type R7A strain was not. It is possible that the ExoT flippase transport protein is less effective at transporting the truncated EPS or that the EPS side-chain may play a role in secretion. If the ExoT protein is impaired in the secretion of truncated EPS, the increased copy number of \textit{exoYF} may cause a build-up of EPS within the cell that normally would not occur. This would explain the apparent toxicity truncated EPS as the \textit{exoYF} copy number is increased.

5.4. \textit{exoK} alone does not cause the colony phenotype to produce a thin extracellular EPS

When the pSKU1 plasmid genes are expressed by R7A, the EPS becomes thin and colourless and the cells begin to separate from the EPS. This was hypothesised to be due to the overexpression of the \textit{exoK} gene cleaving the nascent EPS. We hypothesised that as ExoK cleaves the HMW EPS chains into the LMW monomers, an overexpression of the \textit{exoK} would produce a visible phenotypic change in the colony morphology that resembles the phenotypic change caused by the pSKU1 plasmid which contains genes spanning from \textit{exoB} to \textit{exoU} and includes the \textit{exo} genes K, U, X, Y, F, Q, Z and B.
To investigate this, the R7A exoK gene was inserted into the pSDZ plasmid and linked to an IPTG-inducible promoter and gfp gene. The pSDZ::exοK plasmid was then inserted into R7A. To confirm the pSDZ plasmid was being expressed, the strains were grown on Gly/RDM media supplemented with IPTG to observe the expression of gfp. The gfp was successfully expressed in Gly/RDM with stronger fluorescence in the Gly/RDM plates supplemented with IPTG, but with expression still occurring in Gly/RDM without IPTG. These results indicated that the exoK gene in the pSDZ expression vector was successfully expressed and that overexpression was induced by the addition of IPTG. Despite the apparent expression of exoK from the pSDZ::exοK plasmid, there was no phenotypic change in the strain. Similarly, R7A showed no phenotypic change when pFAJ1700::exoK was conjugated into the cell.

The clones of R7A containing the pSK11 and pSKU1 plasmids were also streaked on Gly/RDM and G/RDM. The R7A pSKU1 strain showed a phenotypic change after about four days of growth on G/RDM, not shown in the clones containing the pFAJ1700::exoK or pSDZ::exoK plasmids.

Interestingly the phenotype caused by pSKU1 was increased greatly on Gly/RDM for all strains and a phenotype caused by pSK11 that was unobservable on G/RDM was noticeable on Gly/RDM, though the change was very slight. Despite the Gly/RDM increasing the extent of the phenotypic change due to pSKU1 and pSK11, no change was observed in either pSDZ::exoK or pFAJ1700::exoK.

Due to the inability to replicate the effect caused by pSKU1 using exoK expression vectors, it is unlikely that the gene implicated in causing the thin EPS phenotype is exoK alone. It is still possible that exoK overexpression is required with additional genes on the pSKU1 plasmid, however it also may not play a role at all with the phenotype being caused by a different gene entirely. Lastly, as exoK overexpression produced no phenotypic change it is unknown if the gene product produced by the pSDZ::exoK plasmid was functional.

5.5. Conclusions

The results of this study revealed several characteristics of the functions of the genes exoK, exoU and exoT.
The first conclusion drawn was that deleting \textit{exoK} in R7A causes a severe growth defect and as such it will not be possible to create an in-frame marker-less deletion R7A Δ\textit{exoK} mutant, though a polar mutant using pFUS2 was able to be developed. It is likely that expression of the several \textit{exo} genes involved in adding residues to the EPS backbone has a deleterious effect on the growth of the strain in the absence of \textit{exoK}, possibly due to the accumulation of inner membrane-bound EPS intermediates. Because ExoK cleaves nascent EPS in \textit{S.meliloti}, it’s possible that the ExoK gene product may likely play a role in the function of the secretion system responsible for polymerising EPS intermediates and transporting EPS from the inner membrane to the outer membrane. The removal of \textit{exoK} may lead to the EPS secretion system being unable to polymerise more EPS intermediates until the octasaccharide subunits are cleaved from the secretion complex and thus intermediates produced begin to accumulate in the inner membrane. It was considered that the reason that an in-frame markerless deletion in the \textit{exoK} gene was achieved in the \textit{S.meliloti} species was due to redundancy of the \textit{exoK} gene provided by another gene, \textit{exsH} that encodes a second glycanase. It was noted that a non-polar mutant was acquired in the NGR234 strain via transposon mutagenesis despite the absence of a reported \textit{exsH} gene. This could possibly be due to a lower production of LMW EPS in the NGR234 strain and the loss of \textit{exoK} function in R7A may cause a rapid accumulation of EPS in the inner membrane.

The polar mutant produced by Kelly (2012) was confirmed to negatively affect the expression of the \textit{exoLAMON} operon downstream of \textit{exoK}. Of particular interest, an \textit{exoK} complementation caused a mucoid phenotype characteristic of EPS production. As an \textit{exoA} mutant has a non-mucoid phenotype when complemented with \textit{exoK} it appears that the \textit{exoLAMON} genes may still be expressed in low quantities resulting in slight EPS secretion and that, despite the mutant containing a polar deletion of \textit{exoK}, downstream genes may still be expressed.

The pSKU1 plasmid causes a thin, watery phenotype in the colony morphology, but this does not appear to be due to \textit{exoK} as originally hypothesised and therefore it is likely that one of the other genes on the pSKU1 plasmid is responsible. This effect of this phenotype is greatly amplified by Gly/RDM media and will likely be useful in future studies involving the cause of this phenotype.

Next, \textit{exoT} was determined to be able to transfer both the truncated form and the complete form of the EPS across the periplasm. The loss of \textit{exoT} resulted is severe growth defects,
suggesting that the secretion system is non-redundant, with no other secretion system able to transport either the wild-type EPS or the truncated form.

Lastly it was also found that the deletion of exoU causes the addition of more copies of the exoYF genes to result in increasingly severe growth defects. It is possible that the truncated EPS produced by this mutant is secreted at a slower rate than the wild-type form and that increasing the expression of exoYF may cause a higher rate of EPS production than EPS secretion.

5.6. Significance of Results

The results of this study showed that an in-frame marker-less deletion of the exoK gene in the R7A strain is unobtainable and indicates exoK performs an essential role when EPS is produced by the cell. Reduced expression of the exoLAMON operon or deletion of the exoYF genes can relieve the severe growth defect, resulting in colonies that produce very little EPS. This provides valuable insight when planning future studies on the exoK gene as deleting exoK in mutants that are defective in the expression of genes responsible for the production of EPS intermediates appear to be viable. Additionally, it was shown that exoK overexpression alone did not cause the EPS of R7A to become thin and watery compared to R7A pSKU1. This suggests that the overexpression of one or more of the other genes encoded on the pSKU1 plasmid may be responsible for this phenotypic change and that exoK may not play a role in altering this phenotype at all. This requires additional research, perhaps with an experiment involving the mutagenesis of the exo genes on the pSKU1 plasmid or the construction of a new plasmid containing the same genes as pSKU1, excluding the exoK gene.

The exoT gene was shown to be lethal in all strains containing the exoYF genes. Because exoT is implicated to be involved in the secretion of EPS, this result provides strong evidence that there is no additional secretory system that allows the secretion of EPS. As the strain deficient in both exoT and exoU expression was also inviable if exoYF was expressed, it can be presumed that the truncated form of EPS is also secreted by the same system. The truncated form may not be secreted at the same rate as the wild-type EPS however, and the side-branch may be involved in secretion.
5.7. Future Directions

The results of this study showed that \textit{exoK} alone was not the gene in pSKU1 that caused the phenotypic change in the R7A strain, however the genes responsible are still unknown and \textit{exoK} may still work in conjunction with these genes. Therefore, future research may be done on the remaining genes on the pSKU1 plasmid to determine the cause of the thin, watery colony morphology. One such experiment to confirm \textit{exoK} plays no role in this effect may be to produce an expression vector similar to pSKU1, though lacking the \textit{exoK} gene.

It was also found that complementing \textit{exoK} alone in Kelly’s polar \textit{exoK} mutant resulted in an increased mucoid effect. Notable genes located downstream of the \textit{exoK} gene include the \textit{exoLAMON} operon. As the \textit{exoLAMON} operon may still be expressed in low amounts, a possible experiment may be to add an expression inducible plasmid containing the \textit{exoLAMON} operon to determine if the growth in this mutant will be severely impaired when these genes are expressed.

As an \textit{exoK} expression vector was created during this study, ExoK may be extracted and purified in future experiments. This will allow experiments where ExoK may be added to cell cultures to determine whether an increase of LMW EPS will increase symbiotic proficiency. However, as no phenotypic change was observed, it must first be confirmed that the \textit{exoK} gene product produced by the pSDz::\textit{exoK} expression vector is functional. This may be done by comparing the LMW:HMW EPS ratio between the wild-type R7A and R7A containing the \textit{exoK} expression vector.

To determine whether \textit{exoU} mutants secrete EPS at a slower rate than the wild-type, an experiment could be performed to measure and compare extracellular EPS production in both the wild-type and mutant.
6. References


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