Distribution and Functional Analysis of an ATP-Binding Cassette Transporter-2 in *Rhizobium leguminosarum*

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SUPERVISORY AND EXAMINING COMMITTEE

Bijaya Aryal, candidate for the degree of Master of Science in Biology**,** has presented a thesis titled, ***Distribution and Functional Analysis of an ATP-Binding Cassette Transporter-2 in Rhizobium legumionsarum,*** in an oral examination held on October 1, 2012. The following committee members have found the thesis acceptable in form and content, and that the candidate demonstrated satisfactory knowledge of the subject material.

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ABSTRACT

Rhizobia are Gram negative, motile, non-sporulating soil bacteria, which interact with legume plants symbiotically to form nitrogen-fixing nodules. In the free-living state, rhizobia dwell in the rhizosphere and surrounding soil where they encounter various environmental stresses such as desiccation stress, nutrient and oxygen limitations, as well as temperature and pH fluctuations. To overcome such conditions, rhizobia adopt different survival mechanisms such as exopolysaccharide (EPS) production, swarming motility and biofilm formation. A previous study identified an ATP binding cassette transporter-2 (ABC) operon, which contains three genes, RL2975-RL2977 (here after referred as RL2975 transporter) that is involved in desiccation tolerance. However, the exact function of the transporter is not known. The study also reported that the transporter genes were present only in the *R. leguminosarum* and *R. etli* genomes sequences currently available in publicly accessible databases. Using bioinformatics, PCR and gene expression experiments, I examined the functional activities and diversity of the RL2975 transporter in *Rhizobium* species. PCR and subsequent DNA sequencing of the amplicons revealed that the transporter is predominantly constrained strains of *R. leguminosarum* but may also be found in some *R. gallicum*-like strains. Based on comparison between mutant and wild type, the transporter was not required for persistence in soil and swarming motility.

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Dedication

This thesis is dedicated to my beloved parents

Late Gopal Prasad Aryal

&

Indira Aryal

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LIST OF ABBREVIATIONS

A	Absorbance
ABC	ATP-binding cassette transporter
Ap	Ampicillin
BNF	Biological Nitrogen Fixation
Bp	base pair
Cfu	colony forming unit
EPS	exopolysachharide
Gm	gentamycin
gusA	Gene encoding *β*-glucuronidase
HMW	High molecular weight
Km	Kanamycin
LB	Luria Bertani
Min	Minutes
NBD	Nucleotide binding domain
ND	Not determined
Nm	Neomycin
OD	Optical density
OMA	Outer membrane auxiliary
PCR	Polymerase chain reaction
PNPG	P- nitrophenol-a-D-glucopyranoside
Sm	Streptomycin
Sp	Spectinomycin
Tc	Tetracycline

TMD	Transmembrane domain
Tn	Transposon
TY	Tryptone Yeast Extract media
VMM	Vincent's minimal media
X-Gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid

INTRODUCTION

Rhizobium species

Rhizobia are Gram-negative, motile and non-sporulating soil bacteria, which interact with legume plant roots symbiotically for the development of nitrogen-fixing nodules (Gruber and Galloway, 2008; Oldroyd and Downie, 2008). They are found within the α-subdivision of proteobacteria and more recently the β-proteobacteria (Chen et al., 2003; Moulin et al., 2001; Spaink et al., 1998). Since the first identification of genus *Rhizobium* (Frank 1889), studies on symbiosis, nodulation and N_2 fixation has continued with great interest (Oono and Ford, 2010; Thrall et al., 2011; Olivieri and Frank, 1994; Simms and Bever, 1998; Bever and Simms, 2000; Crespi, 2001).

Nitrogen is an integral part of all life forms. Approximately 80% of atmospheric air contains nitrogen as a stable covalent triple bonding structure difficult to utilize by most organisms (Lee and Hirsch, 2006). Rhizobia (α- and β-classes of Proteobacteria) are capable of reducing atmospheric nitrogen into a fixed form of nitrogen efficiently during rhizobia-legume symbiosis. In effective nodules, rhizobia fix nitrogen gas (N_2) from the atmosphere into ammonia (NH_3), which is utilized by plants as natural fertilizer (O'Gara and Shanmugam, 1976). On the other hand, the plants provide essential nutrients (predominantly organic acids such as dicarboxylic acids malate and succinate) to rhizobia (Lodwig and Poole, 2003). At this time, rhizobia are protected inside the nodule structure (van Rhijn and Vanderleyden, 1995). In contrast, no nitrogen is fixed in ineffective nodules, but rhizobia are still supplied with nutrients and in this, the rhizobia could be considered parasitic (Denison and Kiers, 2004).

It is estimated that about 175 million metric tons of N_2/yr comes from biological nitrogen fixation (BNF) worldwide (Peter et al., 2002). Of which, 80% comes from symbiotic plants and strains of rhizobia. Suitable legume host are essential for rhizobia to survive longer and for efficient nitrogen fixation. In the absence of the host plant, rhizobial cells exist in a free-living state in the soil. The unique abilities of rhizobia are not only restricted to fix atmospheric nitrogen in legume plants but also to utilize a wide range of organic compounds present in the soil for their mutual benefits (Johnston & Beringer, 1975). Thus, legume productivity is highly dependent on the effective mutualism between rhizobia and plants that enhances the growth and competitive ability of legumes (Vitousek and Walker, 1989; Spehn et al, 2002). Nitrogen fixation during legume-rhizobia symbiosis is therefore of great ecological and socio-economic importance due to the reduced nitrogen that is utilized as a natural fertilizer (Drinkwater et al., 1998).

Rhizobial diversity

Rhizobia are a phylogenetically diverse group of bacteria, which are currently classified into five genera within the α-proteobacteria (*Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium*, and *Mesorhizobium*) (van Berkum and Eardly et al., 1998). There are also at least two genera of β-proteobacteria (*Burkholderia* and *Ralstonia*) (Moulin et al., 2001). Previous studies suggest that *Mesorhizobium* are phylogenetically distinct and differ in genomic structure (Galibert, et al., 2001, Wood et al., 2001). While, phylogenetic analysis suggested that some of the strains of *Rhizobium* and *Sinorhizobium* appear to be closely related to each other (Pulawska et al., 2000). In a study using 16s small subunit rRNA gene phylogeny, *Rhizobium etli*, *Rhizobium tropici* and *Rhizobium galegae* formed a clade (Amarger et al., 1997).

Agrobacterium rhizogenes, a known plant pathogen, is taxonomically close to *R. tropici* and falls on the same phylogenetic branch (Young et al., 1996). However, *Rhizobium galegae* forms effective nodules with perennial forage legumes, *Galega oficinalis* and *G. orientalis*, is phylogenetically distant from all the other root-nodule bacteria except *Rhizobium* sp. OK55 (Sawada et al., 1993). Some of the genera and species of rhizobia that form root-nodules of better-known host legumes are given in Table 1.

Rhizobium-legume symbiosis

The legume-*Rhizobium* symbiosis is considered a central dogma of mutualism. In this process, legume secreted flavonoids play an important role that influences the expression of rhizobial *nod* genes that initiates legume-rhizobia symbiosis and nitrogen fixation (de Bruijn et al., 1991; Fisher and Long, 1992). The resulting symbiosis, which is a highly complex and multistep process, involves interactions between partners that communicate through molecular signalling pathways. Nod factors are recognized uniquely by legumes to promote rhizobia entry into roots actively. The legume plant appears to select rhizobia with better mutualism by limiting the oxygen supply to nodules that fix less nitrogen, and subsequently the frequency of parasitic rhizobia is reduced.

The *Rhizobium*-legume symbiosis is a complex process of infection of legume roots by rhizobia, nodule development, nodule function, and nodule senescence (Swaraj and Bishnoi, 1996; D'Haeze and Holsters, 2002). Regardless of whether the strains of rhizobia are native or introduced through inoculation, they must be adapted to the site for a successful symbiosis (Zahran 1999). Rhizobia encounter various environmental stresses such as acidity, drought, high temperatures, or other stress

conditions in different environments (Ali et al., 2009, Brockwell et al., 1995). These conditions can greatly reduce the number of rhizobia surviving in the soil. However, once adapted to the site with availability of favorable conditions, they multiply quickly in the rhizosphere and initiate partner recognition with compatible legume species (Rinaudi et al., 2010; Zahran, 1999). Rhizobia use legume root hairs as an entry point,

Table 1. Genera and species of root-nodule bacteria of better-known host legumes

Genus & species	Host legumes	Genus & species	Host legumes
Allorhizobium		***Rhizobium***	
A. undicola, *R. undicola*	*Neptunia*	*R. etli*	*Phaseolus vulgaris*
R. radiobacter	non-nodulating saprophyte, causes hairy root disease,		
R. rhizogenes		*R. galegae*	*Galega*, *Leucaena*
R. rubi, *R. vitis*		*R. gallicum*	*Phaseolus*, *Dalea*, *Onobrychis*, *Leucaena*
Azorhizobium**			
A. caulinodans	*Sesbania*	*R. giardinii*	*Phaseolus*
Bradyrhizobium		*R. hainanense*	*Stylosanthes*, *Centrosema*
		R. huautlense	*Sesbania*
B. elkanii	*Glycine*		
B. japonicum	*Glycine*	*R. indigoferae*	*Indigofera*
B. liaoningense	*Glycine*	*R.leguminosarum*	
B. yuanmingense	*Lespedeza*	bv. *trifolii*	*Trifolium*
Mesorhizobium		bv. *viciae*	*Pisum*, *Vicia*, *Phaseolus* *Lathyrus* and *Lens*
M. amorphae	*Amorpha*	bv. *phaseoli*	*Phaseolus*
M. chacoense	*Prosopis*, mesquite	*R. loessense*	*Astragalus*
M. ciceri	*Cicer*, chickpea	*R. mongolense*	*Medicago*, *Phaseolus*
M. huakuii	*Astragalus*, milkvetch	*R. sullac*	*Hedysarum*
M. loti	*Lotus*	*R. tropici*	*Phaseolus*; *Leucaena*,

M. mediterraneum	*Cicer*		*Dalea, Macroptilium*
M. plurifarium	*Acacia, Leucaena,* Ipil-ipil	***Sinorhizobium***	
		S. abri	*Abrus*
M. tianshanense	*Glycyrrhiza,* S*ophora*	*S. americanus*	*Acacia*
		S. arboris	
		S.fredii	*Glycine*
		S. indiaense	*Sesbania*
		S. kostiense	
		S. kummerowiae	*Kummerowia*
		S. medicae	*Medicago*
		S. meliloti	*Melilotus officinalis Medicago and Trigonella*
		S. morelense	*Leucaena*
		S. saheli, S. sahalense	*Sesbania*
		S. terangae	*Sesbania, Acacia*
		S. xinjiangense	*Glycine*

Note: *Other genus and species names exist in the literature. Some predate the present names: others have not been accepted as valid. **Strains which have not yet been recognized as belonging to any named species are usually identified by the host from which they were isolated, for example, *Rhizobium* sp. (Acacia) or *Bradyrhizobium* sp. (Lupinus) (Young et al., 2001)

but some strains can also enter through "cracks" or breaks in the root surface where lateral roots emerge. After entry into the root hair, an infection tunnel or infection thread is formed using several cell layers to the site where a nodule will develop. Upon suscessful entry into the plant system, rhizobia are protected at some level form outside stressful environment.

Two common hypotheses have been proposed on legume-*Rhizobium* symbiosis for the mechanism that maintains legume-rhizobium symbiosis. Firstly, the sanctions hypothesis suggests that plants police cheating rhizobia leading to reduced nodule growth, early nodule death, decreased carbon supply to nodules, or reduced oxygen

supply to nodules that fix less nitrogen (Denison, 2000). Secondly, the partner choice hypothesis proposes that the plant uses pre-nodulation signals from the rhizobia to decide whether to allow nodulation and chooses only non-cheating rhizobia strains (Kiers et al., 2003).

Mutual exchange of signal molecules between the rhizobia and the plants occurs throughout all stages of nodule formation (Werner et al., 2001). During the initiation of nodule formation on the root of legume plants, rhizobia produce signaling molecules known as nodulation (Nod) factors. Nod factors are recognized by a specific class of receptor kinases, also known as LysM domains from the host plant (Limpens et al., 2003). Although a Nod factor–independent symbiotic signaling pathway has been discovered (Ibanez and Fabra, 2011), signaling factors such as Nod and flavonoids appear as major contributors in the symbiosis process in nearly all cases. As soon as plants release chemicals, such as flavonoids, phenolics, sugars, dicarboxylic acids and amino acids into the rhizosphere, rhizobia are attracted towards the roots (Brencic and Winans, 2005). Free-living rhizobia, once activated adhere and colonize the root surface. Flavonoid secretion also induces expression of nodulation genes (e.g. *nod*, *noe*, *nol*). Curling of root hairs, development of infection threads and initial root cortex cells reinitiate mitosis leading to the formation of nodule primordia (Gage, 2004). For successful symbiosis, some kind of quorum sensing mechanism in rhizobia might play a role, which helps in the establishment of a successful relationship.

Molecular basis of host specificity

The *Rhizobium*–legume symbiosis is a highly systematic process involving several molecular factors from both partners that initiates nodulation of roots of

legumes. Partners must recognize each other for successful nitrogen-fixing nodulation. Rhizobial species vary in their host range from a narrow host range, limited to one or several closely related species of legume plants to those with much broader host range, for example, *Rhizobium leguminosarum* bv. *viciae* has a relatively narrow host range limited to species within the *viciae* which include the genera *vicea, pisum, lathyrus* and *lens* (Mutch et al., 2004). *Rhizobium* species NGR234, however, shows a broad host range and forms symbiose with species from over 112 genera of leguminous plants. Whereas, *S. meliloti* fix nitrogen in only three genera of legume plants, *Medicago, Melilotus*, and *Trigonella* (Fisher et al., 1992; Dénarié and Cullimore, 1993; Schultze et al., 1994; Spaink et al., 1995).

Several regulatory and structural nodulation genes are involved in host specificity control. Flavonoids are signal molecules secreted by plants activates root nodulation by prompting expression of *nod* genes that regulates host-specificity (Mathesius et al., 2000; Brockwell et al., 1995; Spaink and Lugtenberg, 1994). The early interaction between flavonoids and NodD regulatory protein activates *nod* gene transcription and the synthesis of Nod factor that initiates nodule primordium (Peck et al., 2006). Nodulation factors then initiate multiple responses in the host plants so that invading bacteria is perceived by the plants (Broughton et al., 2000). The whole process is a highly host-specific interaction and upon successful infection by a specific rhizobial strain, results the formation of nitrogen-fixing nodules (Jones et al., 2007).

Lectins are a diverse group of carbohydrate binding proteins that reversibly bind specific carbohydrates. They are not enzymatically active, but have ability to recognize molecules inside cells, on cell surfaces, and in physiological fluids (Gabius, 2006; Lis and Sharon, 1998). Lectins are found in most organisms, ranging from

viruses and bacteria to plants and animals. Plant lectin serves as a receptor protein for attachment to bacterial EPS and likely mediates *Rhizobium*-legume host specificity (Hirsch, 1999; Rudiger and Gabius, 2001). The control of species-specific or genotype-specific nodulation is controlled by resistance genes (Krishnan et al., 2003; Marie et al., 2003). A recent study showed that incompatible rhizobial strains can induce root hair curling but fail to nodulate host plant carrying the cognate resistance genes (Yang et al., 2010). It was speculated that the failure of infection-thread development was likely caused by defense responses triggered by the recognition of unknown rhizobial effectors by the host.

Molecular studies on legume-rhizobia symbiosis led to the identification of several nodulation-associated genes called *nod* and *nol* (Young and Johnston, 1989; Akakura and Winans. 2002; Adler et al; 1973). These genes are responsible for the synthesis of extracellular host-specific signal molecules, which eventually induce early nodulation (Truchet et al., 1991). The *nod* genes are classified into three main categories: the common *nod* genes, host-specific *nod* genes and the regulatory *nod* genes (Fox, 2005). Common *nod* genes are also called *nodABC*, which is structurally conserved and functionally interchangeable among rhizobia. The *nodABC* genes are responsible for the synthesis of the N-acylated oligosaccharide core of Nod factors (NFs), which suggests that these genes are of a monophyletic origin (Debelle, et al, 2001). Alteration in *nodABC* gene ceases its ability to initiate root nodules. Genes associated with host specificity are believed to be clustered with *nif* genes on a transmissible plasmid in *R. leguminosarum* bv. *viciae*. The exact mechanism underlying host-specific *nif* gene regulation in rhizobia host specificity is not clear. Studies by Banfalvi *et al.*, (1988) suggested that a functional *nodD* gene appears to be required for the induction of the *nodY* and *nodD* operons, which indicates that the

nodD gene product controls its own gene expression as well as other *nod* genes. The regulatory nod gene is therefore one of the major determinants of host range. Studies have shown that insertional mutagenesis in the regulatory *nodD* gene of *R. trifolii* and transfer of the *nodD*l gene of *Rhizobium* sp. NGR234 into other *Rhizobium* species result in an extension of the host range (Horvath et al., 1987; McIver et al., 1989).

Rhizosphere

The rhizosphere is one of the most biologically complex niches, which include the plant root system, mineral nutrients and soil-borne microorganisms including rhizobia (Ryan and Delhaize, 2001). Rhizobia inhabiting the rhizosphere encounter various environmental stresses such as desiccation stress, nutrient and oxygen limitation, temperature and pH fluctuation (Kent and Triplett, 2002; Deaker et al., 2004). Depending on the type of the soil environment, rhizobia can survive for a long period of time (Crozat, et al., 1982). Some strains of *S. meliloti* can survive up to 40 years in air-dried soil while strains of *R. leguminosarum* bv. *trifolii* and *R. leguminosarum* may survive for up to 10 or 14 years, respectively (Jensen, 1961). Survival of rhizobial species in the rhizosphere niche depends primarily on the ability of rhizobia to perceive environmental changes in the rhizosphere, which require an adaptive response (Stintzi and Browse, 2000; Stotz et al., 2000). Studies suggested that genotypic variations in legume plants can greatly influence the composition of the rhizosphere microbial community (Nehl et al., 1997). It is well documented that desiccation stress negatively impacts several bacterial cellular components with particular emphasis on protein denaturation, DNA degradation and loss of membrane integrity (Billi & Potts, 2002). Survival of rhizobacteria within the soil environment is greatly enhanced by increased tolerance to desiccation (Rokitko *et al.*, 2003). A recent

study by Vanderlinde et al. (2010) reported a previously uncharacterized ATP-binding cassette (ABC) transporter (RL2975–RL2977) in *R. leguminosarum* bv. *viciae* 3841 that is important for stress tolerance. Mutagenesis experiments showed that the transporter exhibited a role for *R. leguminosarum* desiccation tolerance by producing an adequate amount of EPS, presumably to maintain a hydrated state.

Rhizobia Survival and adaptation

Secretion of EPS and membrane modification are some of the examples of rhizobial adaptive responses to environmental changes (Potts, 1994). These responses depend on the type of bacteria and the surrounding environment. EPS synthesis in rhizobia may be regulated depending on whether it is in the free-living stage or during symbiosis (Becker and Pühler, 1998). Previous studies reported that regulation and biosynthesis of EPS is required for the effective nodulation in legumes such as *Medicago*, *Pisum*, *Trifolium*, *Leucaena*, and *Vicia* sp. (Becker and Puhler, 1998; Leigh and Walker, 1994). Genes such as *pssA*, *pssB* and *pssN* are involved in the regulation and biosynthesis of EPS. Transposon mutations in *pss* genes encoded by *R. leguminosarum* bv.*viciae* results in strains unable to produce acidic EPS and are unable to nodulate peas and vetch, indicating their critical role in EPS synthesis and nodulation (Borthakur et al 1988). The survival strategy in a low water stress environment may be due to EPS production. The EPS layer may maintain a hydrated microenvironment around microorganisms during desiccation (Chenu et al., 1996). A previous study demonstrated that desiccation survival of *Rhizobium* sp. required an increased EPS production (Roberson et al., 1992).

The *pssA* gene of *R. leguminosarum* may be responsible for the first glycosyl

transferase step in EPS biosynthesis (Borthakur et al., 1988, Vashina et al., 1994). The *pssB* gene is not unique to *R. leguminosarum* as the *pssB* encodes a protein homologous to members of a family of inositol monophosphateses appears to present in diverse prokaryotic and eukaryotic organisms (Janczarek and Skorupska, 2004). A previous study indicated that the *pssB-pssA* region of *R. leguminosarum* bv. *trifolii* is important for the polysaccharide synthesis. However, a different study found that *pssB* gene exhibited an important role in negative regulation of extracellular polysaccharides synthesis in *R. leguminosarum* bv. *trifolii* (Wielbo et al., 2004). In the free-living stage under phosphate sufficient conditions, expression of *pssA* increased 15-fold and *pssB* 2–3-fold (Janczarek and Skorupska, 2004, Wielbo et al., 2004). The mutation in *pssB* caused EPS overproduction, and introduction of *pssB* into the wild-type TA1 strain reduced EPS synthesis (Janczarek and Skorupska, 2001). The putative *pssN* protein is highly homologous to the family of the outer membrane auxiliary (OMA) proteins (Mazur et al., 2001). Sequence analysis identified the *pssN* protein as a member of the OMA family of proteins and a putative lipoprotein that might be involved in translocation of polysaccharides through the outer membrane (Mazur et al., 2001, Paulsen et al., 1997).

Swarming activity

Swarming motility is considered to be a social phenomenon that enables groups of bacteria to move coordinately atop solid surfaces. The differentiated swarmer cells are often hyper-flagellated and elongated. It has been observerd that the migration front is preceded by a visible layer of slime-like extracellular material (Tambalo et al., 2010; Berg, 2005; Daniels et al., 2004; Fraser et al., 1999; Burkart et al., 1998;). The differentiated swarmer cell population is embedded in an extracellular

slime layer, and the phenomenon has previously been linked with biofilm formation and virulence (Braeken et al., 2008). A previous study reported that nutrient-rich medium was essential for surface migration of *R. leguminosarum* (Tambalo et al., 2010). The authors also found that surface migration was significantly reduced in basal medium lacking a carbon source. The most flagellated and active swarmer cells are found in the periphery, however those in the interior of colony are less motile and are in a vegetative state (Horshey; 2003). The swarming phenotype has been well studied in a number of bacteria including members of *Aeromonas, Azospirillum, Bacillus, Burkholderia, Chromobacterium, Clostridium, Escherichia, Proteus, Pseudomonas, Salmonella, Serratia, Vibrio,* and *Yersinia* (Daniels et al., 2004; Sharma and Anand, 2002). Using a mutagenesis approach of targeted genes, Braeken et al., (2008) observed that the mutant strain of *R. etli* CNPAF512 were likely affected in quorum-sensing, polysaccharide composition or export, motility, and amino acid and polyamines metabolism as well as reduced symbiotic nitrogen fixation activity. It was suggested that the targeted gene was disrupted due to the transposon insertion.

Flocculation

Flocculation is a process of cells clumping (i.e. aggregation) together to form flocs. The floc forming Gram-negative bacteria produce cellulose fibrils (Deinema and Zevenhuizen, 1971). The production of cellulose fibrils causes persistent flocculation in several species of *Rhizobium*. It has been suggested that the cell-to-cell attachment in *R. leguminosarum* is due to binding of cellulose microfibrils (Napoli *et al.*, 1975; Laus *et al.*, 2005). The flocs contain a network of polysaccharide and include a portion enriched in encysted cells with thick capsules.

Flocculated cells exhibit several properties such as high cell titre, and increased adhesiveness (Neyra et al., 1999). These properties have been utilized in agricultural input delivery such as coflocculation. These authors found that the highest nodulation efficiency occurred after using coflocculated forms of *Rhizobium* and *Azospirillum* to inoculate *P. vulgaris* seeds. Factors affecting successful colonization of the roots by *Rhizobium* species are not yet fully understood, but it has been suggested that bacterial surface polysaccharides such as β-Glucans, lipopolysaccharides, and EPS, have been shown to play a role in defining plant-bacterial associations (Leigh and Coplin, 1992). Neyra et al., (1999) found that the use of flocculating *R. etli* was superior compared to the non-flocculated form in terms of nodule numbers and plant growth.

ABC transporters

The ABC superfamily is one of the most widely distributed and most ancient gene families, which includes more than 1100 member organisms representatives all phyla from prokaryotes to humans (Jones and George, 2004),. Specifically, rhizobia are enriched with ABC transporter genes. In comparison with 67 ABC transporter genes available in *E. coli* and 127 genes in *P. aeruginosa*, more than 269 ABC genes are predicted in the genome of *R. leguminosarum* followed by 240 in *Bradyrhizobium japonicum*, 216 in *Mesorhizobium loti* and 200 genes in *S. meliloti* (Galibert et al., 2001; Young et al., 2006; Kaneko et al., 2000; Kaneko et al., 2002; Blattner et al., 1997; Stover et al., 2001; Ren et al., 2005).

ABC transporters are ubiquitous proteins found in all life forms. They utilize the energy of adenosine triphosphate (ATP) to catalyze the transport of a various substrates across biological membranes (Jumpertz et al., 2009). The transporter

system can be divided into three main functional categories including importers mediating uptake of nutrients in prokaryotes, exporters mediating secretion of various molecules and non-transport-related processes. The non-transport process is a category that soluble ABC ATP-binding proteins use energy from ATP hydrolysis and are involved in translation elongation (Chakraburtty, 2001) and DNA repair (Goosen et al., 2001). Most importantly, molecules such as proteins, peptides, polysaccharides, synthesized in cytoplasm must often be exported across the membranes via ABC transporters (Schatz et al., 1990; Pugsley, 1993; Young et al., 2006). Some of the ABC transporter systems that are involved in nutrient acquisition include a solute binding protein-dependent transport systems, the tripartite ATP-independent periplasmic transport systems, peptide transport systems (Mauchline et al., 2006), dicarboxylate transport system (Yurgel et al., 2004) and monocarboxylate transport system (Hosie et al., 2002b). The core structure of an ATP-binding cassette-transporter is composed of a common minimum structure consisting of four domains: two hydrophobic integral membrane domains and two nucleotide-binding domains, referred to as ATP-binding cassettes (ABC) (Blattner et al., 1997).

Export and import mechanisms

The ATP-Binding Cassette (ABC) superfamily comprises one of the largest of all protein families with a diversity of physiological functions and several subfamilies that have been described using phylogenetic and functional classification (Saurin et al., 1999; Cardenas et al., 1996). The ABC transporters are involved in the export or import of a wide variety of substrates ranging from small ions to macromolecules (Reizer et al., 1992; Davidson et al., 2008). Hydrolysis of ATP to ADP by the highly conserved ABC motif is the most common feature of all ABC transport systems

(Higgins et al., 1992). One of the important functions of ABC import systems is to provide essential nutrients to bacteria. One of the most important roles of the import system is to import iron-siderophore complexes, free iron, iron bound complexes, mono-and oligosaccharides, organic and inorganic ions, amino acids and short peptides, metals and vitamins (Dassa et al., 2001). ABC transporters are also involved in the export of a wide variety of substrates ranging from small ions to macromolecules across the cell surface such as oligo- and polysaccharides (Cuthbertson et al., 2010).

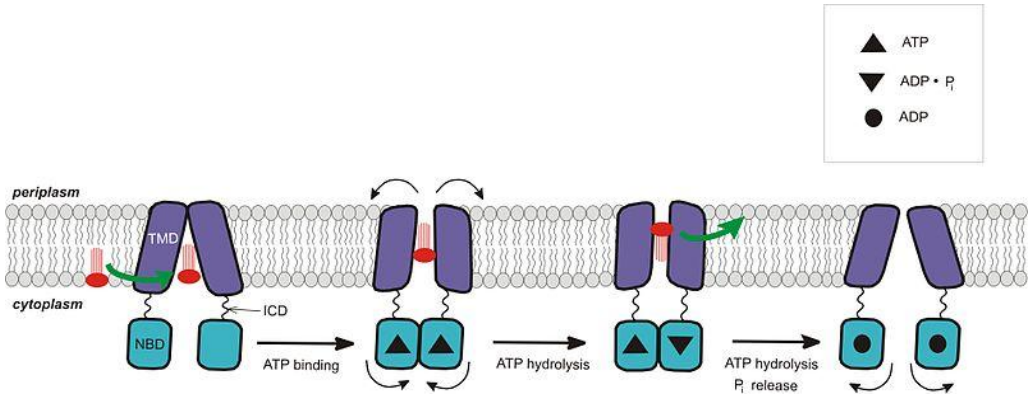

Figure 1. Proposed mechanism of transport for ABC exporters. This model was based on structural and biochemical studies on MsbA, bacterial lipid flippase (Dong et al., 2005), "Reprinted with permission from The American Association for the Advancement of Science."

The proposed substrate transport mechanism of an ABC exporter is highly dependent on a specific substrate and powered by ATP-molecules as an energy source (Dong et al., 2005), (Figure 1). Initially, the substrate transport cycle begins with specific substrate binding to an inward facing open nucleotide binding domain (NBD) of the ABC-transporter. This configuration allows ATP binding and initiates the closure of inward facing NDB. With the closure of NDB, substrate is pushed upward, which opens conformation outward. Subsequent binding of a second ATP molecule

hydrolysis makes NDB returns to the initial configuration NDB.

A recent study identified a novel ATP-binding cassette-2 (ABC) transporter that is required for the survival of *R. leguminosarum* during desiccation stress (Vanderlinde et al., 2010). The authors observed that mutants flocculated extensively. Mutation of the RL2975 transporter in *R. leguminosarum* bv.*viciae* resulted in a significant reduction of EPS production and desiccation tolerance. The mutants were also impaired in biofilm formation, which is an important survival strategy in soil bacteria. The results from this previous study support the role of the ABC transporter in the survival of the *R. leguminosarum* in the rhizosphere environment (Vanderlinde et al., 2010). The two transmembrane proteins of the ABC transporter are likely encoded by two of the three genes, RL2976 and RL2977, in an operon that includes the gene RL2975. The conserved domain of unknown function (DUF990) shares homology to a subgroup of ABC transporters classified as ABC Type 2 transporters (Reizer et al., 1992). With the exception of *Rhizobium etli* and *R. leguminosarum* bv. *trifolii*, the newly identified RL2975 transporter operon is not present in the genomes of other *Rhizobiales* sequenced to date (Vanderlinde et al., 2010).

OBJECTIVES

A recent study identified a previously uncharacterized ATP-binding cassette (ABC) transporter (RL2975–RL2977) in *R. leguminosarum* that is important for stress tolerance (Vanderlinde et al., 2010). To further characterize this transporter, the specific objectives of this study were,

1. To investigate possible functional activities of RL2975 transporter, Specifically, this objective was designed to understand impacts on regulation of EPS biosynthesis genes, swarming phenotype and identifying possible mechanisms of flocculation in the transporter mutant, and
2. To describe phylogenetic diversity of the RL2975 transporter in *Rhizobium* sp.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The bacterial strains and plasmids used are listed in Table 2 and Table 3. *Rhizobium* sp. and *Agrobacterium* sp. strains were routinely cultured in Tryptone Yeast medium (TY) (Beringer, 1974), or modified arabinose-gluconate (MAG) medium (USDA strains; van Berkum, 1990). *R. leguminosarum* strains were also grown on Tryptone-Yeast (TY) medium (Beringer, 1974) or Vincent's minimal medium (VMM) (Vincent, 1970) at 30°C, and *E. coli* strains were grown on Luria-Bertani (LB) medium (Sambrook *et al*, 1989) at 37°C. When required, *R. leguminosarum* strains were grown in the presence of antibiotics in the following concentrations: gentamicin (Gm), 30µg/mL; neomycin (Nm), 50µg/mL; streptomycin (Sm), 500µg/mL; spectinomycin (Sp), l00µg/mL; and tetracycline (Tc), 5µg/mL. *E. coli* strains were cultured in the following concentrations of antibiotics where appropriate: ampicillin (Ap), l00µg/mL; Gm, 15µg/mL; kanamycin (Km), 50µg/mL; Sp, l00µg/mL; Tc, 10µg/mL.

Bacterial Conjugation

Conjugation was performed by mixing 1 ml of overnight donor cultures of *E.coli* S17-

1 cells (OD600 ~ 1.0) with 0.5 ml of overnight cultures of recipient *R. leguminosarum* cells (OD600 ~ 0.5) in a microfuge tube. The mixture was pelleted by spinning at 9632 X g for 5 min, the supernatant removed and the pellet was resuspended in 50 µl of sterile water. The cell suspension was then spotted on TY agar or VMM agar containing 0.5 mM proline and incubated at 30°C for 24h-48h after which the cells were scraped off and resuspended in 1ml of sterile water. Conjugants were selected by plating 100 µl of cells on the appropriate selective media.

Table 2. Bacterial strains used in the study and their associated characteristics

Strain	Host plant	Species (16s rRNA type)	Location
S027B-3 Profile 4a	*Lathyrus venosus*	*R. leguminosarum* bv. *viciae*	Radisson, SK
S018A-3 Profile 3	*Vicia cracca*	*R. leguminosarum* bv. e: *viciae*	Rouleau,SK
S016B-1 Profile 10	*V. cracca*	*R. leguminosarum* bv. *viciae*	Davidson SK
S016A-3 Profile 11	*V. cracca*	*R. leguminosarum* bv. *viciae*	Davidson, SK
S018A- 1 Profile 5	*V. cracca*	*R. leguminosarum* bv. *viciae*	Rouleau, SK
S012A -2 Profile 1a	*L. venosus*	*R. leguminosarum* bv. *viciae*	Battleford, SK
S019B-5 Profile 27	*Vicia americana*	*R. gallicum*	Belgone, SK
S015A-1 Profile 12	*V. americana*	*R. leguminosarum* bv. *viciae*	Cutbank, SK
S020A-5 Profile 16a	*V. americana*	*R. leguminosarum* bv. *viciae*	Qu'Apple, SK
S0012B-3 Profile 20	*Pisum sativum*	*R. leguminosarum* bv. *viciae*	NA
S011B-1 Profile 18	*V. americana*	*R. leguminosarum* bv. *viciae*	Maymont, SK
S022A-2 Profile 2	*V. americana*	*R. leguminosarum* bv. *viciae*	LeRoss, SK
S003B-1 Profile 25	*V. americana*	*R. leguminosarum* bv. *viciae*	MacDowall, SK
S006A-2 Profile22	*L. ochroleucas*	*R. leguminosarum* bv. *viciae*	Rosthern, SK
306	*P. sativum*	*R. leguminosarum* bv. *viciae*	Los Angeles, USA
336	*P. sativum*	*R. leguminosarum* bv. *viciae*	NA
S023A-4 Profile 1	*V. americana*	*R. gallicum*	Mozart, SK
S030B-4 Profile 13b	*Lathyrus* sp.	*R. leguminosarum* bv. *viciae*	Dalmany, SK
S014B-4 Profile 6	*V. cracca*	*R. gallicum*	Outlook, SK
S010B-1 Profile	*V. americana*	*R.leguminosarum* bv. *viciae*	Borden, SK

24			
S013A-1 Profile15b	*V. americana*	*R. gallicum*	Whitecap, SK
S00BA-5 Profile 8	*V. americana*	*R. leguminosarum* bv. *viciae*	Melfort, SK
S004A-2 Profile 14	*V. cracca*	*R. gallicum*	Bigger, SK
S012A-1 Profile 9	*L. venosus*	*R. leguminosarum bv. viciae*	Battleford, SK
S010A-2 Profile 23A	*L. venosus*	*R. leguminosarum* bv. *viciae*	Borden, SK
S028A-4 Profile 21	*V. americana*	*R. leguminosarum* bv. *viciae*	Hafford, SK
S001B-3 Profile 7	*V. americana*	*R.leguminosarum* bv. *viciae*	Saskatoon, SK
S002A-4 Profile 17a	*V. americana*	*R. leguminosarum* bv. *viciae*	Hague, SK
S021A-2 Profile 25	*V. americana*	*R. gallicum*	NA
ARG22-01	NA	*R.leguminosarum* bv. *viciae*	NA
ARG48-01	NA	*R. leguminosarum* bv. *viciae*	NA
W-101-02	NA	*R. leguminosarum* bv. *viciae*	NA
W108-0119	NA	*R. leguminosarum* bv. *viciae*	NA
4292	NA	*R. leguminosarum* bv. *viciae*	NA
CG#1	*P. sativum*	*R. leguminosarum* bv. *viciae*	Calgary, AB
TP6	NA	*R. leguminosarum* bv. *viciae*	NA
TP11	NA	*R. leguminosarum* bv. *viciae*	NA
F1	*P. sativum*	*R. leguminosarum* bv. *viciae*	Regina, SK
F3	*P. sativum*	*R. leguminosarum* bv. *viciae*	Regina, SK
W14-2	*Trifolium vesiculosum*	*R. leguminosarum* bv. *trifolii*	East Texas
162Y10	NA	*R. leguminosarum* bv. *viciae*	East Texas
CFN42	NA	*R. etli*	Mexico
8401	*P. staivum*	*R. leguminosarum* bv. *phaseoli*	Britain
BAUR.1	*P. sativum*	*R. leguminosarum* bv. *viciae*	Cowichan Bay, BC
BAUR.2	*P. sativum*	*R. leguminosarum* bv. *viciae*	Cowichan Bay, BC
BAUR.3	*P. sativum*	*R. leguminosarum* bv. *viciae*	Cowichan Bay BC
BAUR.4	*P. sativum*	*R. leguminosarum* bv. *viciae*	Cowichan Bay BC
BAUR.5	*P. sativum*	*R. leguminosarum* bv. *viciae*	Cowichan Bay Bc
USDA 3394	*Galega officinalis*	*Rhizobium* sp	NA
USDA 2435	*Vicia faba*	*R. leguminosarum* bv. *viciae*	NA
USDA 2676	*Phaseolus vulgaris*	*R. tropici*	NA
USDA 2479	*V. faba*	*A. tumifaciens*	NA
USDA 2354	*V. sativa*	*R. leguminosarum* bv. *viciae*	NA
USDA 2483	*V. villosa*	*A. tumifaciens*	NA
USDA 2334	*V. americana*	*A. tumifaciens*	NA
USDA 2416	*Lathyrus ochroleucas*	*R.gallicum*	NA
USDA 2063	*Trifolium repens*	*R.leguminosarum* bv. *viciae*	NA
USDA 3040	*Lupinus albus*	*A. tumifaciens*	NA
USDA 2129	*Trifolium alexandrum*	*A. tumifaciens*	NA
USDA 2116	*Trifolium subterranean*	*R.leguminosarum* bv. *viciae*	NA
USDA 2046	*Trifolium pratense*	*R.leguminosarum* bv. *viciae*	NA

3855	*P. sativum*	*R. leguminosarum* bv. *viciae*	Britain
3841	*P. sativum*	*R. leguminosarum* bv. *viciae*	Britain
VF39	*P. sativum*	*R. leguminosarum* bv. *viciae*	Germany

Note: Typing was done using 16 rRNA gene sequencing

Assay for β-glucuronidase (*gusA*) gene activity

Reporter gene expression from free-living cells was measured using *R. leguminosarum* strains carrying the transcriptional fusions grown in the appropriate media at 30°C and harvested in late exponential phase. The *gusA* gene activities were measured as described by Jefferson et al (1986) and modified by Yost et al., (2004). Briefly, 100 µl of the cultures was added to 900µl of Z buffer containing 3mg/ml PNPG (4-nitrophenyl B-D-glucuronidase).

Table 3: Characteristics of plasmids and *R. leguminosarum* constructed strains used in this study

Strains	Relevant characteristics	Reference
Plasmids		
pEV77	pFus1par with *pssB*::*gusA* transcriptional fusion, Tc^r	Vanderlinde (unpublished data)
pEV 30	1.3 kb RL2976 and RL2977 fragment cloned from pEV25 into pJQ200SK using Sst/Xba Gm^r	Vanderlinde et al., 2009
pEV110	pFus1par with *pssN*::*gusA* transcriptional fusion, Tc^r	Vanderlinde and Yost et al., 2012
pEV109	pFus1par with *pssA*::*gusA* transcriptional fusion, Tc^r	Vanderlinde et al., 2012
R. leguminosarum		
3841	Spontaneous streptomycin-resistant derivative of *R. leguminosarum* bv. *viciae* strain 300	Glenn *et al*. 1980; Johnston & Beringer, 1975
17b	3841 ABC transporter, RL2975–RL2977 mutant, Tn5 TGN mutant Sm^r, GM^r, Nm^r	Vanderlinde et al., 2010

3841EV77	3841with *gusA-pssB* promoter fusion, Sm^r, Tc^r	This study
3841EV110	3841 with *gusA-pssN* promoter fusion, Sm^r, Tc^r	This study
3841EV109	3841 with *gusA-pssA* promoter fusion, Sm^r, Tc^r	This study
3841EV66	3841 with *gusA*-RL2975 transporter fusion, Smr, Tc^r	This study
17bEV77	17b with *gusA-pssB* promoter fusion Sm^r, Tc^r, Gm^r	This study
17bEV110	17b with *gusA-pssN* promoter fusion Sm^r, Tc^r, Gm^r	This study
17bEV 109	17b with *gusA-pssA* promoter fusion Sm^r, Tc^r, Gm^r	This study
A1060 (*celA*)	Mutant of 3841, *celA*::Tn5,Sm^r, Nm^r	Williams et al., 2008
celA:RL2975	Double mutant, c*elA* and Rl2976 transporter mutant, Sm^r, Gm^r. Nm^r,	This study

Time was monitored until a yellow color was formed in the solution. Upon color change, the reaction was stopped by adding 400 ul 1M of Na_2CO_3. Spectrophotometric measurement was performed at OD_{600} for the cellular density and at OD_{420} for the quantity of PNP produced by β-glucuronidase. *β*-glucorinidase activity was calculated in Miller units using the formula (OD_{420} X1000)/ (OD_{600} X time (min) X dilution). All the data given are the means from at least triplicate experiments.

Swarming assay

The medium used for swarm assays contained 0.01% K_2HPO_4; 0.01%NaCl; 0.02% $MgSO_4$ $7H_2O$; 0.04% KH_2Po_4, 0.4% yeast extract; 0.7% bactoagar with 0.2% glycerol as supplementary carbon source (Tambalo et al., 2010). 30 mL of swarm medium was air dried in a petri dish with the lid on for 24h. The strains were grown in TY broth for 24 h and the cell density (OD_{600}) was adjusted to 1.2. A 2 µL culture

suspension was inoculated at the center of the swarm plate and the plate was wrapped with parafilm. The plates were incubated at room temperature for 3-4 weeks. The expression of the RL2975 transporter genes in *R.leguminosarum* swarmer cells was compared with the expression of *R. leguminosarum* vegetative cells. Pre-existing *gusA* RL2975 transporter fusion plasmid construct (3841 EV66) (Vanderlinde et al., unpublished data) was used for the experiment. The cells from the swarm plate and the vegetative plates were scraped from three different spots i.e. edge, middle and center. The cells were resupended in 1ml swarm broth and 100 µl was used for the *gusA* assay.

Double mutant (*celA* and RL2975 transporter) construction

For this study, pEV30 vector was kindly provided by Vanderlinde et al., (2010). The vector pEV30 features a 1.3 kb internal fragment from the RL2975 - RL2977 operon cloned from pEV25 into pJQ200SK. The *celA* mutant (A1060) was kindly provided by Williams et al., (2008), and was created in a RL3841 background with Tn5 mutagenesis. The double mutation of RL2975 transporter and *celA* was created by bi-parental mating of the pEV30 vector into the *celA* mutant background as described in the conjugation section of methodology and Figure 2. Putative single crossover mutants were selected on the basis of neomycin and gentamycin resistence. The putative mutant was confirmed via PCR.

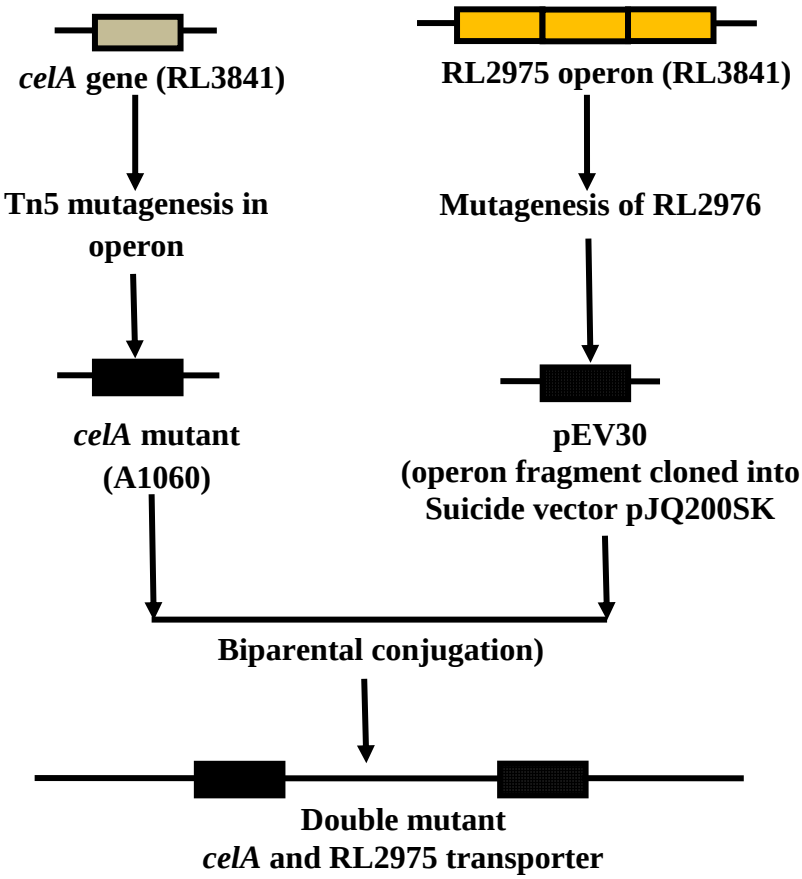

Figure 2. Double mutant construction of *celA* and RL2975 operon by conjugation between pEV30 and *celA* mutant

Double mutant (*celA* and RL2975 transporter) verification

To verify the double mutant, primers specific to the genes were designed for PCR amplification. For RL2975 transporter mutant verification, the primer pair 3841EV30F and 3841EV30R, which bind the internal fragments of RL2976 and RL2977, were used, respectively. Similarly, another set of primers, T3F, 3841EV30R and T7R, 3841EV30F, which binds the vector and internal fragment of RL2976 and RL2977 were used, respectively. Then, PCR was performed as described in methodology using an annealing temperature of 56 ^{o}C and extension at 72 ^{o}C for 1

min. The *celA* mutant was verified using the primer set CelATn5F and CelATn5R, which bind to the *celA* gene and transposon. PCR amplification was performed as described in method and methodology section using annealing temperature at 58 ^{o}C for 1 minute.

Flocculation Assay

R. leguminosarum bv.*viciae* 3841 and mutant strains containing gene specific mutations (*celA*, RL2975 transporter, and *celA* RL2975 transporter double mutant) were grown at 30 °C in VMM broth supplemented with neomycin, gentamycin, and neomycin and gentamycin, respectively. The culture broths were kept on the bench for about 30 minutes for the bacteria to settle out of solution. Supernatant was collected and the optical density was measured at 600nm using a spectrophotometer. The bacterial cell suspensions were homogenized using a homogenizer at speed 4 for 30 seconds. Optical density was measured again after homogenization. The percent difference in optical density of flocculated cells was calculated by subtracting OD after homogenization from the initial OD.

Persistence of the RL2975 transporter mutant in soil

The *R. leguminosarum* RL2975 transporter mutant was evaluated for its ability to persist and survive in soil under laboratory conditions. A total of 10 g of soil was placed in a 30 ml capacity beaker. The soil-filled beaker was autoclaved at 121°C. 10^9 CFU/mL of rhizobia strains (WT 3841, RL2975 transporter mutant, and mixture of WT 3841 & RL2975 transporter) were inoculated in room temperature normalized soil-filled beaker. The inoculum was prepared by growing each strain in TY broth on a rotary shaker at 200 rpm at 28°C for 4

days. Bacterial inocula were mixed thoroughly into the soil with a sterile glass rod by washing with few drops of sterile water. The experiment was conducted in laboratory conditions with three replications. Samples were kept at 25°C. The rhizobia strains in the soil-filled samples were enumerated at day 0, 3, 7, 14, 21 and 28 after inoculation by preparing ten-fold serial dilutions and plating on respective media with appropriate antibiotics. After incubating the plates for 2-4 days at 30°C, colonies were counted manually from each plate separately.

***Rhizobium* sp. isolation from soil**

Soil samples were collected from a garden from Victoria B. C., Canada with pea cultivation. Pea seeds were surface sterilized in 50% sodium hypochlorite for 10 minutes then 70% ethanol for 10 minutes followed by three washes with sterile water as described by Weaver and Graham (1994). The sterilized seeds were placed on 1.25% water agar plates and allowed to germinate in the dark at room temperature for two to three days. Germinated seedlings were transferred into modified magenta jars resembling Leonard jars (Vincent 1970) filled with vermiculite and about 200 mL of Hoagland's plants medium (Hogland and Arnon, 1938). Seeds were inoculated with three soil samples in three replicates. Afterward sterile distilled water was used to water the plants. Plants were grown for about three weeks in growth chambers at 20 ^{o}C and 16 hours of light/day. Plants were pulled out from the plant pots after three weeks, and roots were rinsed thoroughly. Five nodules from each plant were picked and a surface-sterilization procedure was carried out using 95% ethanol and 1% sodium hypochlorite as described by Weaver and Graham (1994). Nodules were crushed in 100 µl of sterile water using a sterile stick. 10 µl of the nodule solution was

streaked on TY media and incubated at 30 ^{o}C. For the purified isolated colony, a single colony was streaked for the second time in TY media. The newly isolated strains were identified by PCR amplification and sequencing of the 16s rRNA gene and subsequently comparing to known sequences using a BLAST search of the GenBank nucleotide database (Zhang et al., 2000).

Bean nodulation experiment was performed determine whether *R. gallicum* like strains received from the University of Calgary were, in fact, *R. gallicum*. *P. vulgaris* seeds were prepared as described above. Seeds were inoculated with 500uL of overnight culture (OD600 ~ 0.5) with different strains of *Rhizobium* resuspended in sterile water. Then sterile distilled water was used to water the plants as needed. Plants were grown for about three weeks in growth chambers at 20 ^{o}C and 16 hours of light/day. Plants were pulled out from the plant pots after three weeks, and roots were rinsed thoroughly and examined for nodules.

DNA extraction

Total genomic DNA was extracted from bacterial cultures grown in TY or MAG broth until late exponential phase. 500 µL of cells were pelleted at 9632 X g for 10 minutes. Cells were resuspended in 82 µl of 1 mM Tris-Cl pH 8.0, 75 µg lysozyme and incubated at 37 ºC for at least 30 min. Protease K 3.2 µl (5 mg/ml) was added and the cell suspension was incubated for an additional 1.5 h at 37 ^{o}C. The cell suspension was incubated at 100 ºC for 10 min, and then the cell debris was pelleted at 15000 rpm for 2 min. The supernatant was then used as template for the PCR reactions.

PCR amplification of 16S rRNA and transporter operon

Primers were designed using Primer 3 software (Rozen and Skaletsky, 2000). The universal primers, FD1 and RD1 (Table 4) were used to amplify the 16S rRNA gene from all the strains yielding 1500bp products (Weisburg et al., 1991). PCR reactions contained: 1 U of *Taq* DNA polymerase (UBI, Calgary, Canada), 2 mM MgSO4, 0.2 mM dNTPs, 0.2 μM of each primer and 1 μL of genomic DNA. PCR amplification was performed using a Techne TC312 Thermocycler (Techne, Staffordshire, UK) and the following reaction conditions: 94 °C for 5 min; 30 cycles of 95°C for 30 s, 57°C for 55 s and 72°C for 2 min; final extension at 72°C for 5 min.

The Primers, 2976F1 forward (5′ TGGTGCATGTAGTACAAACTCAAG 3′) and 2976R1 reverse (5′ AGTCCGCCTCCGCTTAGAT 3′) were used to amplify 200 bp of the 3' end of the RL2976 and 600 bp of the 5' end of the RL2977 gene, yielding a 800 bp product. As the RL 2976 and RL 2977 contain the conserved domain, the primer location selected across the RL2976 and RL2977 genes. For the taxonomic position of the strains, the 16s rRNA gene sequencing was also used. The PCR reactions contained: 1 U of *Taq* DNA polymerase (UBI, Calgary, Canada), 2 mM MgSO4, 0.2 mM dNTPs, 0.2 μM of each primer and 1μL of genomic DNA. PCR amplification was performed using a Techne TC312 Thermocycler (Techne, Staffordshire, UK) and the following reaction conditions: 94 °C for 5 min; 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1 min; final extension at 72°C for 5 min.

Table 4: List of primers used in this study

Primer Name	Sequence (5' - 3')	Melting temperature (^{o}C)	Reference
FD1	AGAGTTTGATCCTGGCTCAG	61.0	Weisburg et al., 1991
RD1	AAGGAGGTGATCCAGCC	60.2	Weisburg et al., 1991
RL2976F1	TTCGCCTGGGTGGTCTATTA	64.2	This study
RL2976R1	GATGGAGAACAGATGGTTGGA	64.0	This study
T3	ATTAACCCTCACTAAAGGGA	57.0	McGraw et al., 1985
T7	TAATACGACTCACTATAGGG	48.0	McGraw et al., 1985
38EV30F	GAGGTCATCCGCACCTTCTA	64.1	This study
38EV30R	CTGGCAAATTCGAGTGTCAG	61.4	This study
CelATnF	TCTTTGGAGTGCAGATCGTC	64.2	This study
CelATnR	TTGCGGTAAAATGGGTAAGC	63.6	This study

RL2975 transporter and 16S rRNA sequencing

A PCR clean-up reaction was performed prior to DNA sequencing. Calf intestinal phosphatase (Promega, Madison, WI) and exonuclease (New England Biolabs, ON, Canada) were added to a final concentration of 0.01U/µL^{-1} and PCR reactions were incubated at 37 °C for 30 min to remove residual primers and dNTPS, followed by incubation at 85 °C for 15 min to inactivate the enzymes. DNA Sequencing of PCR products was performed by Eurofins MWG Operon (Huntsville, Alabama) using the same forward and reverse primers for the respective amplicons.

DNA sequence analysis and phylogeny

Prediction of transmembrane domains were made using TMPRED (Hoffman and Stoffel, 1993). Contig assembly was performed using Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, MI). Nucleotide sequences were aligned with CLUSTALX (Thompson et al., 1997). The quality of sequence was checked and edited using the

Sequencher software. For preliminary identification, 16S rRNA gene sequences were compared to the Gene Bank database by using the nucleotide BLASTn program (Altschul et al., 1997). Neighbour-joining phylogenetic trees were constructed using MEGA version 4 (Tamura et al., 2007). The phylogenetic tree branches were supported based on neighbor-joining analysis of 1000 resampled data sets. The evolutionary distances were computed using the Maximum Composite Likelihood Method (Tamura et al., 2007) and are presented as the number of base substitutions per site.

RESULTS

Functional investigations of the RL2975 transporter

Expression of EPS synthesis genes

From a previous study (Vanderlinde et. al., 2010), it was reported that the RL2975 transporter mutant produces three-fold less EPS compared to wild type. To examine whether the decrease in EPS production is due to down regulation of EPS biosynthesis genes, the strains 38EV77, 38EV109 and 38EV110 containing *pss* gene-*gusA* fusions by conjugating *R. leguminosarum* bv. *viciae* 3841 wild type with the plasmids pEV77, pEV109 and pEV110 was constructed, respectively. Similarly the vectors pEV77, pEV109 and pEV110 were conjugated into the RL2975 transporter mutant (17B) to make strains 17BEV77, 17BEV109 and 17BEV110, respectively. The promoter activity was measured in the free-living cells grown in TY and VMM media as described in the materials and methods.

The results of EPS biosynthesis gene expression in RL2975 transporter mutant

(17B) and wild type *R. leguminosarum* bv. *viciae* 3841 in TY and VMM media are provided in Table 5. The result is an average of three replicates. Results show that the specific activity of *pssA* gene was significantly higher in wild type compared to RL2975 transporter mutant in TY media. However, the *pssA* gene expression was slightly higher in the wild type compared to the mutant in VMM media. The *pssB* gene activity was not different between RL2975 transporter mutant background compared to wild type in both TY and VMM media. The expression of *pssN* gene was significantly higher ($p<0.05$) in RL2975 transporter mutant background compared to wild type in TY media, whereas expression of *pssN* gene was slightly higher in mutant background compared to wild type in VMM media. However, whether this difference is of physiological significance remains to be determined. The *pssN* expression is higher in the mutant background in light of the observed reduced levels of EPS.

Table 5. Expression of *EPS* (EPS) biosynthesis genes (Miller units) in the RL2975 transporter mutant (17B) and wild type *R. leguminosarum* bv. *viciae* 3841

Gene fusion	Gene expression (Miller unit)			
	TY media		VMM media	
	Wild type	Mutant type	Wild type	Mutant type
pssA	14083 ± 476.7*	9740 ± 416.7	10937 ± 60.3	9706 ± 297.0
pssB	2280 ± 102.2	2151.17 ± 244.9	2070 ± 482.5	2533 ± 316.6
pssN	11838 ± 712.8	17452 ± 1803.4*	19374 ± 7698.1	22329 ± 2308.2

Note: * - denotes the value is significantly different at $p<0.05$.

Expression of the RL2975 transporter promoter fusion during swarming behavior

R. leguminosarum RL2975 transporter promoter fusion (38EV66) was used to examine expression of the transporter genes during growth on swarming motility in 0.7% agar medium (Figure 3). The colony diameter gradually increased during the first few days and the swarming migration was observed after 4-5 days of inoculation. A slime-like layer was observed preceding the migration front in swarming motility. Complete swarming motility behaviour was observed 3 weeks after inoculation. Swarmed cells produced a number of dendrite-like structures on the media compared to vegetative growth medium (1.5% agar).

Expression of the RL2975 transporter operon in *R. leguminosarum* 3841 swarmer cells was compared with non-swarming cells using the transporter promoter gene fusion (3841EV66). Gene expression was compared between cells taken from different locations of swarming and vegetative colonies such as center, middle and edge of the colony. The expression of the RL2975 transporter increased by 46% (swarm, 2906.8$\pm$1908.6 versus vegetative 1995.9$\pm$229.1) under swarming conditions compared with expression on solid medium at the edge (Table 6). However, the standard deviation for swarm edge was unusually high. The expressions in replicate 1, 2 and 3 of swarm edge plates were 5097.5, 2019.8 and 1603 Miller units, respectively. The value of 5097.5 might have come as an outlier, hence the result should be viewed with caution and future trials would be needed to ascertain the biological significance of this result. However, the expression of RL2975 transporter at the middle of the cells was not different between the two media. Similarly, the expression of the transporter at the center of the cells was also not different in the swarming and

vegetative media. This result suggests that the higher expression seems to only occur in the active front of swarmer cell activities.

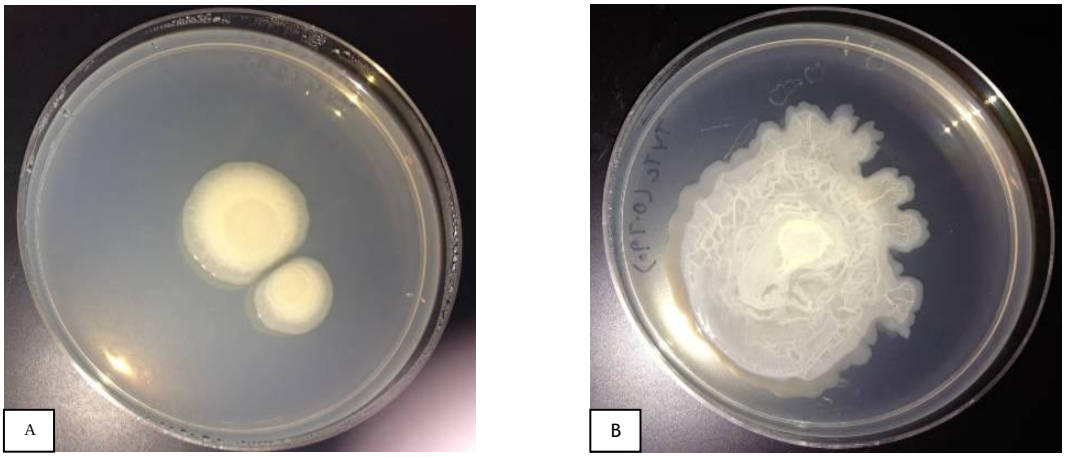

Figure 3. Swarming motility of *R. leguminosarum* RL2975 transporter promoter fusion, A- vegetative state (1.2% agar); B-swarming state (0.7% agar).

Table 6. Expression of RL2975 transporter gene promoter in vegetative and swarming cells of *R. leguminosarum* bv.*viciae* 3481.

ID	Edge	Middle	Center
Swarm	2906.8 ± 1908.6*	1805.0 ± 408.2	2565.4 ± 975.6
Vegetative	1995.9 ± 229.1	1890.0 ± 703.3	2362.0 ± 456.4

Note: Gene expression is measured as *gusA* activity and expressed in Miller Units. Values are means of three independent replicates and standard deviation. * - significant

Swarming phenotype of RL3841 RL2975 transporter mutant

It has been demonstrated from a previous study (Vanderlinde et al., 2010) that the RL2975 transporter mutant produces decreased levels of EPS. It was speculated

that swarming motility might be related to the level of EPS production. I examined the swarming phenotype in RL3841, and the RL2975 transporter mutant. There was no notable difference in swarming phenotype between the RL2975 transporter mutant and the wild type (Figure 4). The mutant and the wild type exhibited similar swarming patterns.

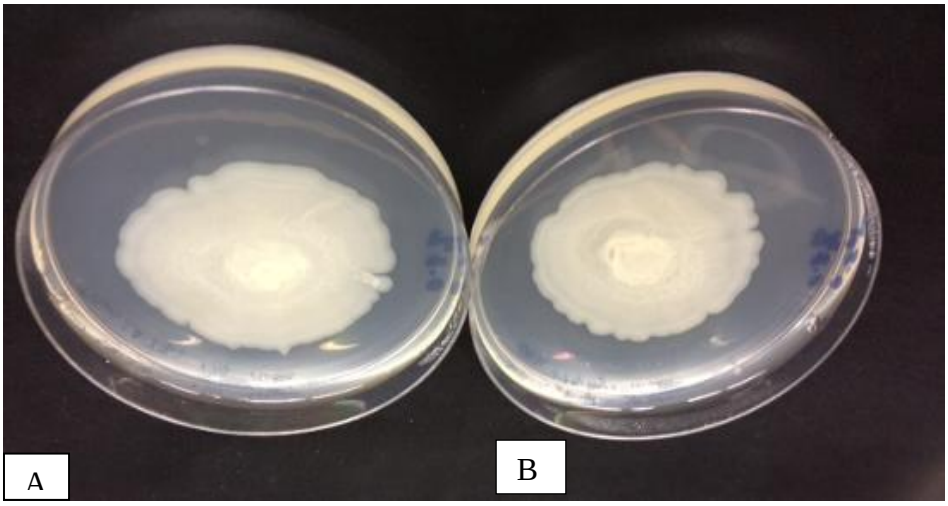

Figure 4. Swarming phenotypes of (a*) R. leguminosarum* 3841wildtype and (b) 3841 RL2975 transporter operon mutant.

Double mutant (*celA* and RL 2975 transporter) verification

The double mutant (*celA* and RL2975 transporter) was constructed by mutating the RL2975 transporter mutant in the *celA* mutant background. The putative double mutant produced expected band sizes for each set of primers. PCR amplification produced a 750 bp amplicon with the following primers, T3 and 3841EV30R (Figure 5). Similarly, primers 3841EV30F and T7 produced a 750 bp amplicon and the third pair primer; 3841EV30F and 3841EV30R produced a 1500 bp amplicon. The positive control, pEV30, also produced the expected band sizes. The wild type as a negative control did not produce bands.

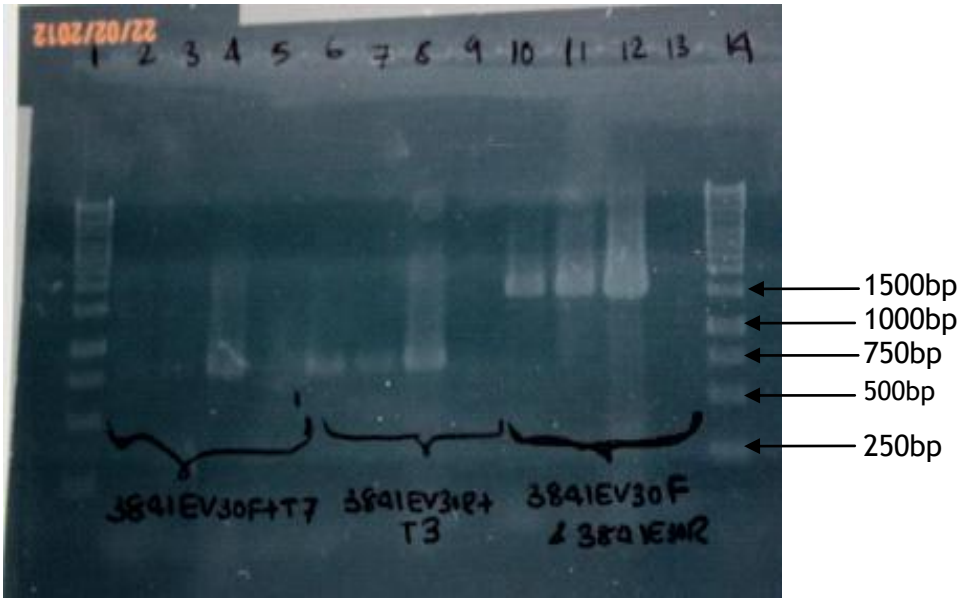

Figure 5. PCR amplicon to amplify RL2975 transporter mutant on *celA* and RL2975 transporter mutant. DNA template from two colonies of double mutant was used for PCR amplification. Lane 2 and 3-*celA* and RL2975 transporter with primer (3841EV30F+T7), lane 6 and 7- *celA* and RL2975 transporter with primers (3841EV30+T3), lane 10 and 11- *celA* and RL2975 transporter with primer (3841EV30F+3841EV30R), Lanes 4, 8 and 12 - positive control pEV30, Lanes 5, 9 and13- negative control, *R. leguminosarum* bv. *viciae* 3841, Lane 1 and 14 - molecular markers

Primers, celATnF and celATnR, were designed to confirm the *celA* mutant (A1060). The primer binding sites are shown in Figure 6. The *celA* mutant produced band size as expected 1000bp as seen in the Figure 7.

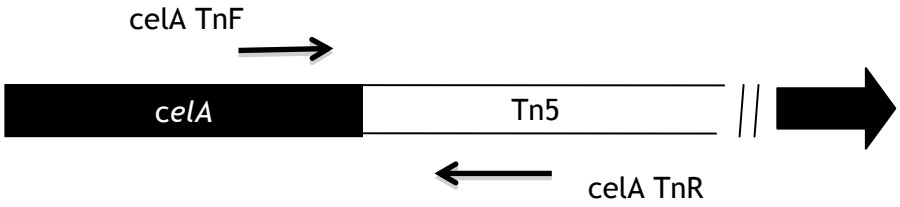

Figure 6. Schematic diagram of the *celA* gene with Tn5 transposon. Arrow indicates the primers binding sites.

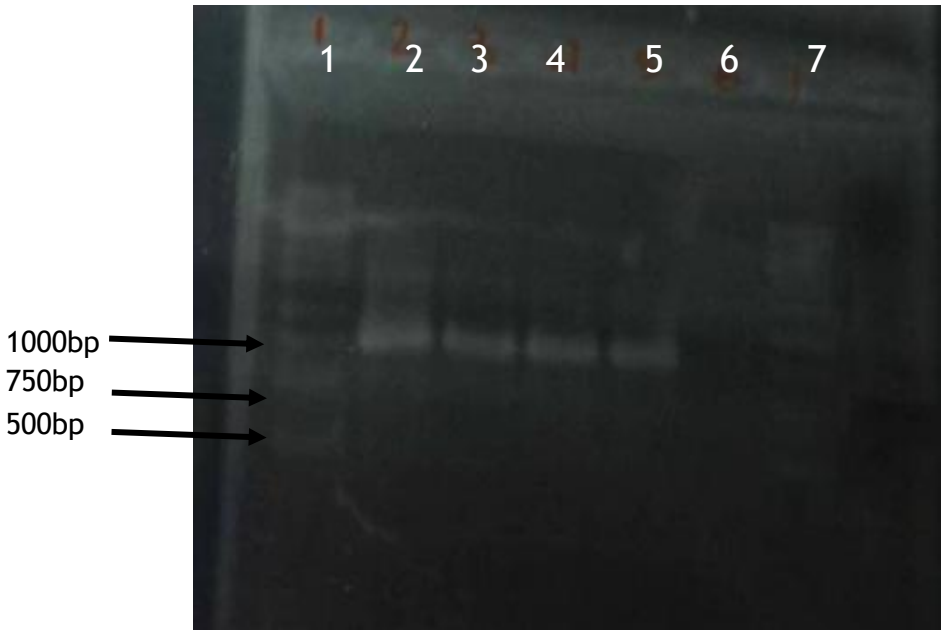

Figure 7. PCR amplicon of the double mutant (*celA* and RL2975 transporter) DNA sample isolated from two colonies to amplify *celA* mutant using the following primer sets; CelATnF and CelATnR, Lane 1 and 7 : molecular marker; lanes 2 and 3: *celA* mutant (colony #1), lanes 4 and 5: *celA* mutant (colony #2), lane 6: Negative control *R. leguminosarum* bv. *viciae* 3841

Flocculation assay

Cellulose fibrils are considered as a factor contributing to cell flocculation. It has been demonstrated that the RL2975 transporter mutant, which is EPS deficient, flocculates in VMM media (Vanderlinde et al., 2009). To further understand the role of cellulose fibrils in flocculation, the RL2975 transporter was mutated in a *celA* mutant background to construct the double mutant (*celA* and RL2975 transporter mutant). Then the transporter mutant and transporter mutant with cellulose-deficient mutant were used in a flocculation assay as described in the method and methodology section. The flocculation assay showed that the RL2975 transporter mutant (17B) flocculated in VMM media, whereas the double mutant, *celA* mutant (A1060) and wild type *R. leguminosarum* 3841 did not flocculate in VMM media (Figure. 8).

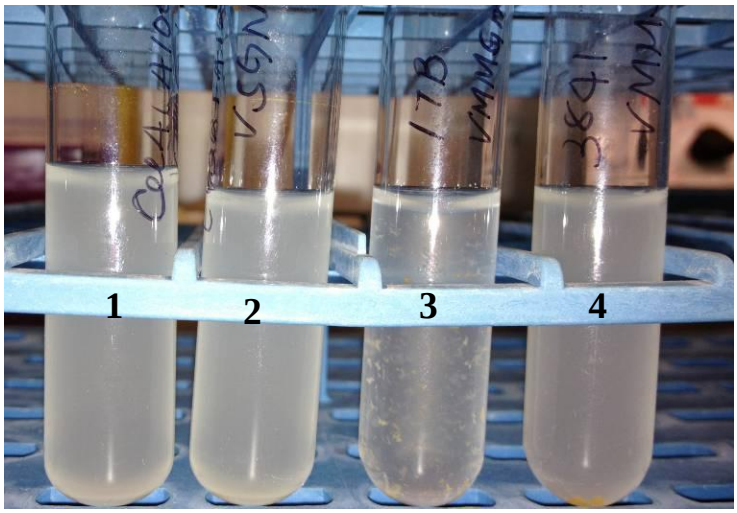

Figure 8. Independent trial of flocculation assay on *R. leguminosarum* double mutant (*celA* and RL2975 transporter) and single mutant (RL2975 transporter) grown on VMM media.1-*celA* mutant; 2-Double mutant; 3- Transporter mutant; 4- Wild type.

Cell flocculation was quantified by measuring the optical density after the cells were allowed to settle from solution followed by a second OD measurement after

homogenization of the cells. The culture broths were kept on the bench for about 30 minutes for the bacteria to settle from solution before taking the initial OD. The final homogenization step released the cells from the flocs so they remained suspended in solution for the final OD measurement; therefore a higher change in % indicates a larger initial floc in the tube. RL2975 transporter mutant showed a quantitative flocculation of 43.6% and 32.5% in two different independent trials, respectively, whereas, other mutants, *celA* and double mutants (*celA* and RL2975 transporter), did not show substantial flocculation in the two independent trials (Table 7). The RL2975 transporter mutant did not grow in the third replication; therefore data from the 3rd replicate were not presented.

Table 7. Independent trials of optical density measurement during flocculation assay on *R. leguminosarum* double mutant (*celA* and RL2975 transporter) and single mutant (RL2975 transporter) grown on VMM media

Strain	Replicate 1		% Change in OD	Replicate 2		% Change in OD
	Initial OD	Final OD		Initial OD	Final OD	
celA mutant	0.895	1.048	14.6%	0.955	1.035	7.7%
Double mutant	1.049	1.11	5.5%	1.051	1.116	5.8%
Transporter mutant	0.5	0.886	43.6%	0.62	0.919	32.5%
Wild type	0.988	1.068	7.5%	0.975	1.054	7.5%

Persistence of rhizobia

R. leguminosarum bv. *viciae* 3841 RL2975 transporter mutant (17B) produces three-fold less EPS compared to wild type (Vanderlinde et al., 2010) and does not form biofilms. Robertson and Firestone (1992) found that reduced desiccation tolerance and defective biofilm formation are associated with the alteration of

secreted polysaccharides in bacteria. It appears that EPS is important for desiccation tolerance in *R. leguminosarum* (Vanderlinde et al., 2010). In the current study, the role of RL2975 transporter in *R. leguminosarum* bv. *viciae* 3841 persistence in soil was examined. The experiment was conducted for 28 days under laboratory conditions. The inoculated soil was kept moist adding 1ml of sterile water at seven day intervals. The results showed that the *R. leguminosarum* bv. *viciae* 3841 RL2975 transporter mutant and wild type persisted equally for 28 days. There was no difference between initial inoculum levels and the concentration recovered on 3, 7, 14, 21 and 28 days. An increase in cell number was observed in both strains on the third day of inoculation (Figure 9). This observation may suggest that rhizobia were in log phase until day 3. After day 21, the growth of the mutant appears to decrease and the growth of the wild type strain increases. After 28 days no substantial difference was observed on the cell numbers of both wild and the RL2975 transporter mutant.

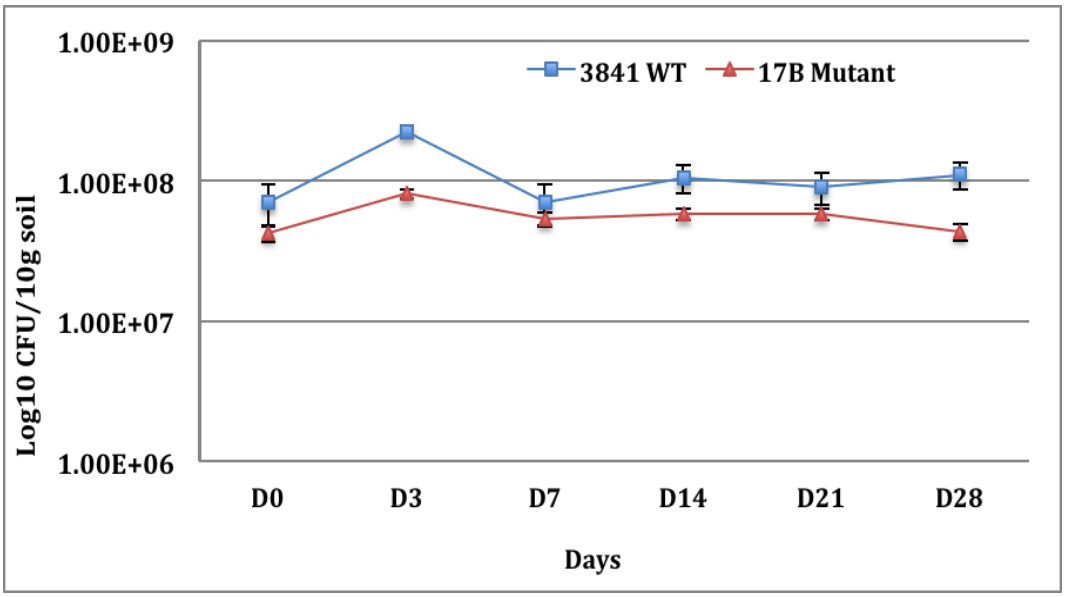

Figure 9. *R. leguminosarum* bv. *viciae* 3841 persistence in soil

Competition between *R. leguminosarum* bv. *viciae* 3841 and RL2975 transporter mutant 17B

The competitive ability of *R. leguminosarum* bv. *viciae* 3841wild type against its RL2975 transporter mutant was examined by colony enumeration up to day 28 after the initial inoculation. Results show that *R. leguminosarum* bv. *viciae* 3841wild type and *R. leguminosarum* bv. *viciae* 3841 RL2975 transporter mutants were equally competitive up to 28 days (Figure 10).

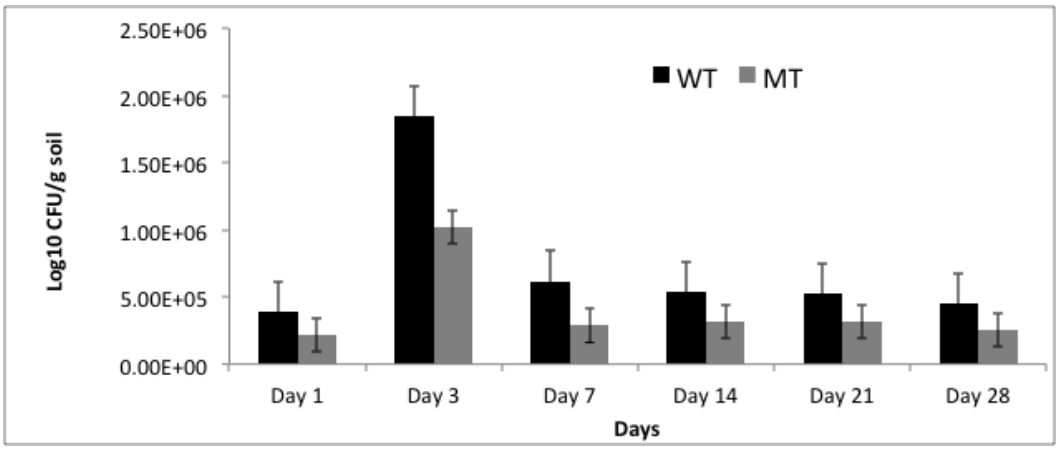

Figure 10. Competition between of *R. leguminosarum* bv. *viciae* 3841, A- Wild type; B- RL2975 transporter mutant

***Rhizobium* sp. isolation from the soil**

Five *Rhizobium* isolates, BAUR.1, BAUR.2, BAUR.3, BAUR.4 and BAUR.5, were isolated from garden soil with a history of pea cultivation after inoculation in peas to enrich for *R. leguminosarum. Rhizobium* isolation from the soil was performed to increase the number of strains for testing for transporter presence. 16S rRNA PCR produced one band of size approximately 1500 bp for each isolate. The isolates were identified as *R. leguminosarum* bv. *viciae* with Gene Bank database using the nucleotide BLAST program.

Phaseolus vulgaris plants nodulation

Bean nodulation experiment showed that the predicted *R. gallicum* strains nodulated bean plants. The plants were healthy and green (Figure 11). Nodule examination revealed that the nodules were fully-grown and of comparable size compared to positive control, *R. etli* (Figure 12) whereas the negative control, *R. leguminosarum* bv. *viciae* 3841 did not form nodules as expected (Table 8). The experiment was performed in three replicates. The negative control *R. leguminosarum* did not produce any nodules as expected whereas the positive control *R. etli* CFN42 produced 30 large pink nodules.

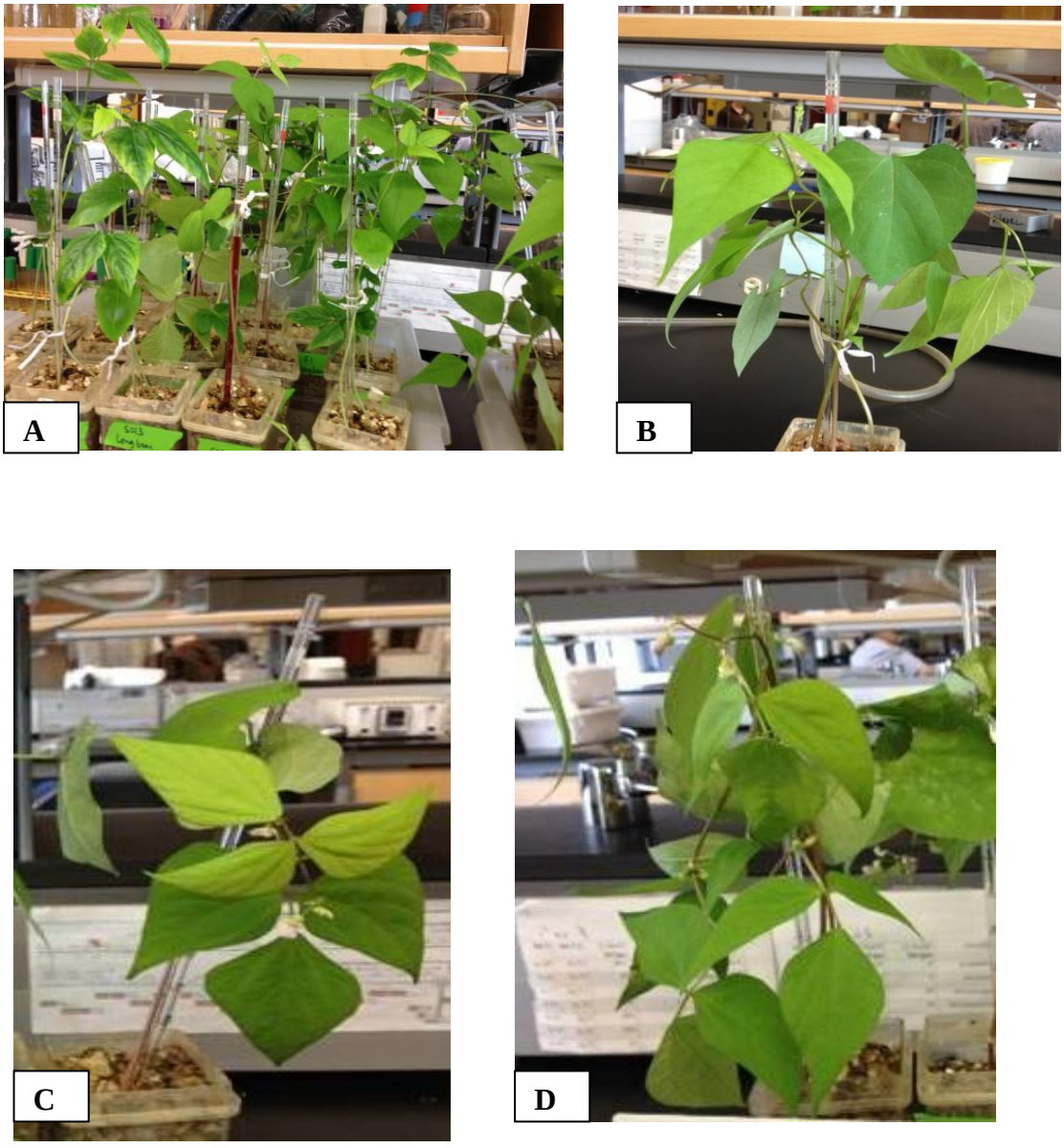

Figure 11. *P. vulgaris* plants inoculation with *Rhizobium* species (positive control *R. etli* CFN 42; negative control *R. leguminosarum* bv. *viciae* 3841). A- *R. gallicum*, B- positive control, *R. etli* CFN42, C- uninoculated and D- negative control, *R. leguminosarum* bv. *viciae* 3841

The nodules were round in shape and pink in color. The un-inoculated plants did not have any nodules. Other strains such as S019B-5-27, S004A-2-14, S014B-4-6, and S021A-2-25 colonize beans with lots of small to big nodules. Strain S013A-1-

15b had very small 5-6 nodules in one replicate but no nodules in other two replicates. Strain S023A-4-1 produced

very small 5 nodules in one replicate, 5 big and 15 white small nodules in second replicate and no nodules in third replicate.

PCR amplification of 16s rRNA and RL2975 transporter operon gene

Out of 61 different strains of *Rhizobium*, all of the strains were positive for 16s rRNA gene amplification. The PCR amplification of 16s rRNA produced a 1.5 kb fragment as expected and they produced single band for all of those strains (Figure 13). The strain *R. leguminosarum* 336 and *R. leguminosarum* 248 did not amplify for the first attempt, respectively, however the amplification was successful in subsequent PCR reaction (data not shown).

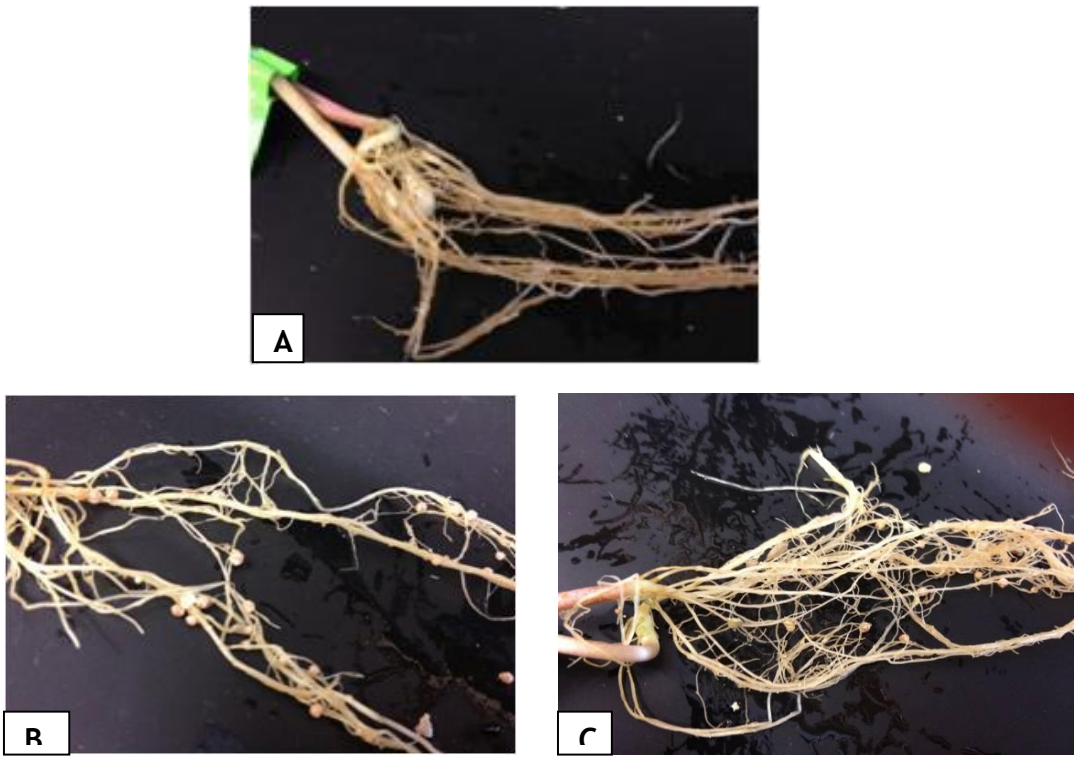

Figure 12. *P. vulgaris* root nodulation by *Rhizobium* species: A- *R. leguminosarum* bv. *viciae* 3841 (negative control), B - *R. etli* (positive control), C – *R. gallicum*

Table 8. Legume nodulation by rhizobia and nodule characteristics

Strain	Peas (*P. sativum*)*	Vetch (*V. villosa*)*	Vetch (*V. cracca*)*	Bean (*P. vulgaris*)		
				Replicate 1	Replicate 2	Replicate 3
S019B-5-27	Several small, white nodules	Lots of white nodules (these were not fully developed nodules, but almost like tiny bumps on the root	Lots of white nodules (these were not fully developed nodules, but almost like tiny bumps on the root	Small, white nodules 15-20	No nodules	5-6 big, a lot of small nodules
S004A-2-14	N/A	N/A	N/A	Small and big 15-20 nodules	5-6 big, 15-16 small nodules	Small and big 20 nodules
S013A-1-15b	Several small, white nodules. (There were 2 red nodules, which were able to grow on TYSm)	Small, pink nodules	Lots of white nodules (these were not fully developed nodules, but almost like tiny bumps on the root	Very small 5-6 nodules	No nodule	No nodules
S014B-4-6	Several small, white nodules. (There were 2 red nodules, which were able to grow on TYSm)	Lots of small, red nodules	Lots of small, white nodules	Big and small lots of nodules	Big and small 10-15 nodules	No nodules
S021A-2-25	Lots of light pink nodules	Small, white nodules	Lots of white nodules (these were not fully developed nodules, but almost like tiny bumps on the root)	One big nodule	5-7 big, 15-20 small nodules	10 big, 20-30 small nodules
S023A-4-1	N/A	N/A	N/A	5 very small	No nodule	5 big, 15 white

				white nodules		small nodules
F1	N/A	N/A	N/A	Very small white 5-6 nodules	5 big, 9-10 small nodules	Small white 3 nodules
R.leguminosarum 3841				No nodules		
R. etli				Big 30 nodules		
Blank				No nodules		

*Data obtained from Dr. Michael Hyne's lab, University of Calgary, AB, Canada

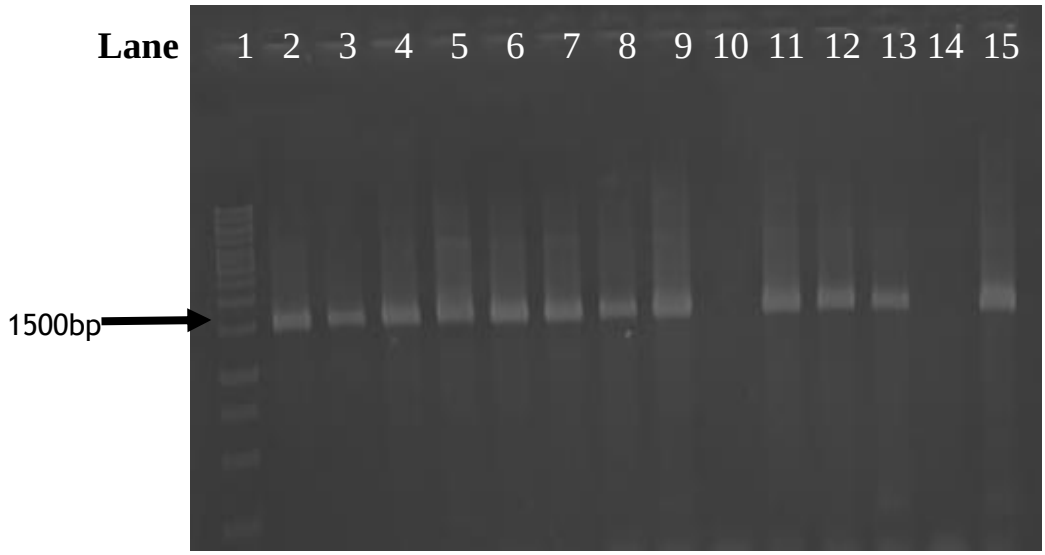

Figure 13. PCR amplification of 16S rRNA gene of *Rhizobium* sp. produced by universal primers, FD1 & RD1; Lanes: 1, molecular marker; lanes 2 – *R.legumnosarum* VF39, 3-*S. meliloti*, 4 - *R. leguminosarum*. 4292, 5- *R. leguminosarum* CG#1, 6 - *R. leguminosarum* TP11, 7 - *R. leguminosarum* TP6, 8- *R. leguminosarum* F1, 9 - *R. leguminosarum* F3, 10- *R. leguminosarum* 336, 11- *R. leguminosarum* W10102, 12 - *R. leguminosarum* S004A-2-14, 13- *R. leguminosarum* ARG48-01, 10 & 14- *R. leguminosarum* 336 and *R. leguminosarum,* 248 amplification failed, respectively, and 15- *R. leguminosarum* W1080119

The PCR amplification of a conserved region of RL2975 transporter operon

produced an approximately 800bp fragment. The primer-binding site in the operon is shown in Figure 14. Of the 61 strains tested, 51 strains were positive for RL2975 transporter. All strains that were positive for transporter produced a single band (Figure 15).

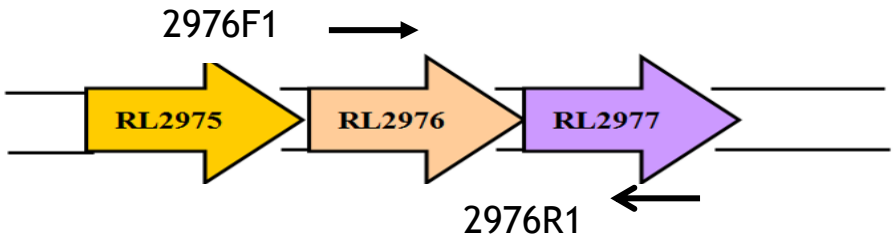

Figure 14. Schematic diagram of RL2975 transporter operon in *R. leguminosarum* bv. *viciae* 3841; small black arrow shows the primer binding sites.

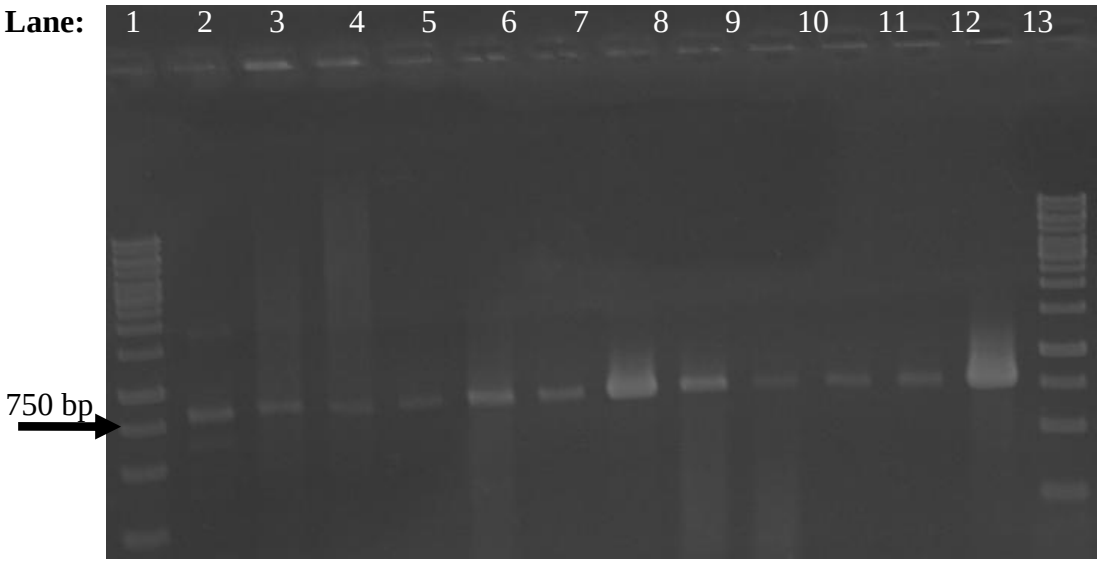

Figure 15. PCR amplification of *Rhizobium* species using RL2975 transporter primers; 2976F1 & 2976R1, Lanes: 1 & 14, molecular marker; 2- *R. gallicum* S019B-5-27, 3-*R. gallicum* S013A-3-13B, 4-*R. gallicum* S004A-2-14, 4-*R. gallicum* S021A-2-25, 6-*R. etli* CFN42, 7- *R. leguminosarum* TP6, 8- *R. leguminosarum* BAUR.3, 9-*R. leguminosarum* BAUR.4, 10- *R. leguminosarum* bv. *viciae* BAUR.1, 11- *R. leguminosarum* bv. *viciae* BAUR.5, 12-*R. leguminosarum* BAUR.5, 13- *R. leguminosarum* VF39

Table 9. Number of strains positive for RL2975 transporter based on PCR amplification in different species of *Rhizobium* and *Agrobacterium*.

Strain name	Number of strains	Number of strains positive for RL2975 transporter
R. leguminosarum	47	45
A. tumefaciens	6	0
R. gallicum	7	4
R. etli	2	2
R. tropici	1	0

Table 9 summarizes 16s rDNA sequencing for the preliminary identification of the different strains *Rhizobium* and the PCR results for the RL2975 transporter presence. Based on the 16S rDNA sequencing results, out of 63 strains, 47 strains were *R. leguminosarum*, 6 strains were *A. tumefaciens*, 7 strains were *R. gallicum*, two strains of *R. etli* and a strain of *R. tropici*. A total of 61 strains of rhizobia were tested for the presence of RL2975 transporters. 45 of 47 strains of *R. leguminosarum* were positive for the transporter. Two strains, BAUR.2 and USDA 2046, did not amplify in the PCR reaction. No transporter was present in *A. tumefaciens* identified strains. Four of 7 strains of *R. gallicum* and 2 of 2 strains of *R. etli* were positive for the transporter, respectively. Three of seven strains of *R. gallicum*, USDA2416, S014B-4-6 and S023A-4-1, did not amplify in PCR reaction. The *R. tropici* DNA did not produce a RL2975 transporter PCR amplicon.

Bioinformatic analysis of RL2975 transporter

The deduced sequence of the RL2975 transporter was obtained from the sequenced genome of *R. leguminosarum* bv. *viciae* 3841 (Young et al., 2006). The

transporter operon encodes three genes, RL2975-2977, whose sequences are reported homologues in VF39 SM (Vanderlinde et al., 2010). The RL2975 is annotated as a gene encoding putative ATP binding component of ABC transporter in the *R. leguminosarum* bv. *viciae* 3841 in rhizobase. The nucleotide sequence of RL2975 gene is 1001 bp and predicted to be 330 amino acids. According to TMRED program, RL2975 contains one transmembrane domain. RL2976 is annotated as a gene encoding transmembrane protein of ABC transporter in the *R. leguminosarum* bv. *viciae* 3841 in rhizobase. The nucleotide sequence of RL2975 gene is 801 bp and predicted to be 266 amino acids. According to TMRED program, RL2976 contains five transmembrane domains. The RL2977 is annotated as a gene encoding transmembrane protein of ABC transporter in the *R. leguminosarum* bv. *viciae* 3841. The nucleotide sequence of RL2975 gene is 848 bp and predicted amino acid sequence length is 282. According to TMRED program, RL2977 contains six transmembrane domains. The operon RL 2975-RL2977 is highly conserved within the *R. leguminosarum*. To determine operon distribution, I compared the nucleotide sequence of the entire operon to the genome sequences currently available in the Rhizodb and US Department of Energy Joint Genome Institute database (http://img.jgi.doe.gov/cgi-bin/geba/main.cgi). Presently the operon is confined to the genomes of *R. leguminosarum* and *R. etli* strains. Of the 69 strains, 9 strains of *R. leguminosarum* and a strain of *R. etli* were positive for the operon genes (Table 10).

Sequence analysis and RL2976 transporter phylogeny

The evolutionary history was inferred using the Neighbor-Joining Method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates (Tamura et al.,

2004) is taken to represent the evolutionary history of the taxa analyzed (Tamura et al., 2004). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Tamura et al., 2004). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Table 10. List of strains positive for RL2975 transporter operon gene in different rhizobial genomes sequenced to date.

Genome Name	RL2975	RL2976	RL2977
Azorhizobium doebereinerae UFLA1-100	-	-	-
B. elkanii USDA 3254	-	-	-
B. elkanii USDA 3259	-	-	-
B. elkanii USDA 94	-	-	-
B. elkanii WSM1741	-	-	-
B. elkanii WSM2783	-	-	-
B. japonicum USDA 122	-	-	-
B. japonicum USDA 135	-	-	-
B. japonicum USDA 38	-	-	-
B. japonicum USDA 4	-	-	-
B. japonicum USDA 6	-	-	-
B. japonicum WSM1743	-	-	-
B. japonicum WSM2793	-	-	-
Bradyrhizobium. sp. ARR65	-	-	-
Bradyrhizobium. sp. EC3.3	-	-	-
Bradyrhizobium. sp. TV2a.2	-	-	-
Bradyrhizobium. sp. USDA 3384	-	-	-
Bradyrhizobium. sp. WSM2254	-	-	-
Bradyrhizobium. sp. WSM3983	-	-	-
Burkholderia mimosarum LMG 23256	-	-	-
B. mimosarum STM3621	-	-	-
Burkholderia sp. UYPR1.413	-	-	-
Burkholderia sp. WSM2230	-	-	-
Burkholderia sp. WSM2232	-	-	-
Burkholderia sp. WSM3556	-	-	-

Cupriavidus sp. UYPR2.512	-	-	-
C. taiwanensis STM6018	-	-	-
C. taiwanensis STM6070	-	-	-
M. ciceri CMG6	-	-	-
M. ciceri WSM4083	-	-	-
M. loti CJ3sym	-	-	-
M. loti R7A	-	-	-
M. loti R88b	-	-	-
M. loti USDA 3471	-	-	-
M. loti WSM1293	-	-	-
M. sp. WSM2561	-	-	-
M. sp. WSM3224	-	-	-
M. sp. WSM3626	-	-	-
R. etli CIAT652	-	-	-
R. giardinii bv. *giardinii* H152T	-	-	-
R. mongolense USDA 1844	-	-	-
R. sp. OR 191	-	-	-
R. sp. STM6155	-	-	-
R. sullae WSM1592	-	-	-
S. arboris LMG 14919	-	-	-
S. medicae Di28	-	-	-
S. medicae WSM1115	-	-	-
S. medicae WSM1369	-	-	-
S. medicae WSM244	-	-	-
S. meliloti CIAM1775	-	-	-
S. meliloti GVPV12	-	-	-
S. meliloti Mlalz-1	-	-	-
S. meliloti MVII-I	-	-	-
S. meliloti RRl128	-	-	-
S. meliloti WSM1022	-	-	-
S. meliloti WSM4191	-	-	-
S. sp. TW10	-	-	-
S. terangae WSM1721	-	-	-
R. leguminosarum bv. *viciae* UPM1131	+	+	+
R. leguminosarum bv. *viciae* UPM1137	+	+	+
R. leguminosarum bv. *viciae* VF39	+	+	+
R. leguminosarum bv. *phaseoli* FA23	+	+	+
R. leguminosarum bv. *trifolii* WSM 2304	+	+	+
R. etli CFN42	+	+	+
R. leguminosarum bv. *viciae* 3841	+	+	+
R. leguminosarum bv. *viciae* GB30	+	+	+
R. leguminosarum bv. *viciae* 248	+	+	+
R. leguminosarum bv. *viciae* VF39	+	+	+

Symbol: + and - indicate positive and negative for RL2975 transporter operon gene,

respectively.

16S rRNA phylogeny

Figure 16 shows that a phylogenetic (neighbour-joining) tree showing the relationships between 61 rhizobia and a reference strain *R. leguminosarum* bv. *viciae* 3841 based on 1500 bp aligned 16S rRNA sequences. *Rickettsia bellii* was used as an out group reference. The 16S rRNA gene sequence similarities among the strains were more than 96%. In the first branch, different strains of *R. leguminosarum* with a reference strain *R. leguminosarum* 3841 clustered together with the bootstrap support of 70%. Within this branch there are two smaller sub-branches. The first sub-branch contains two strains of

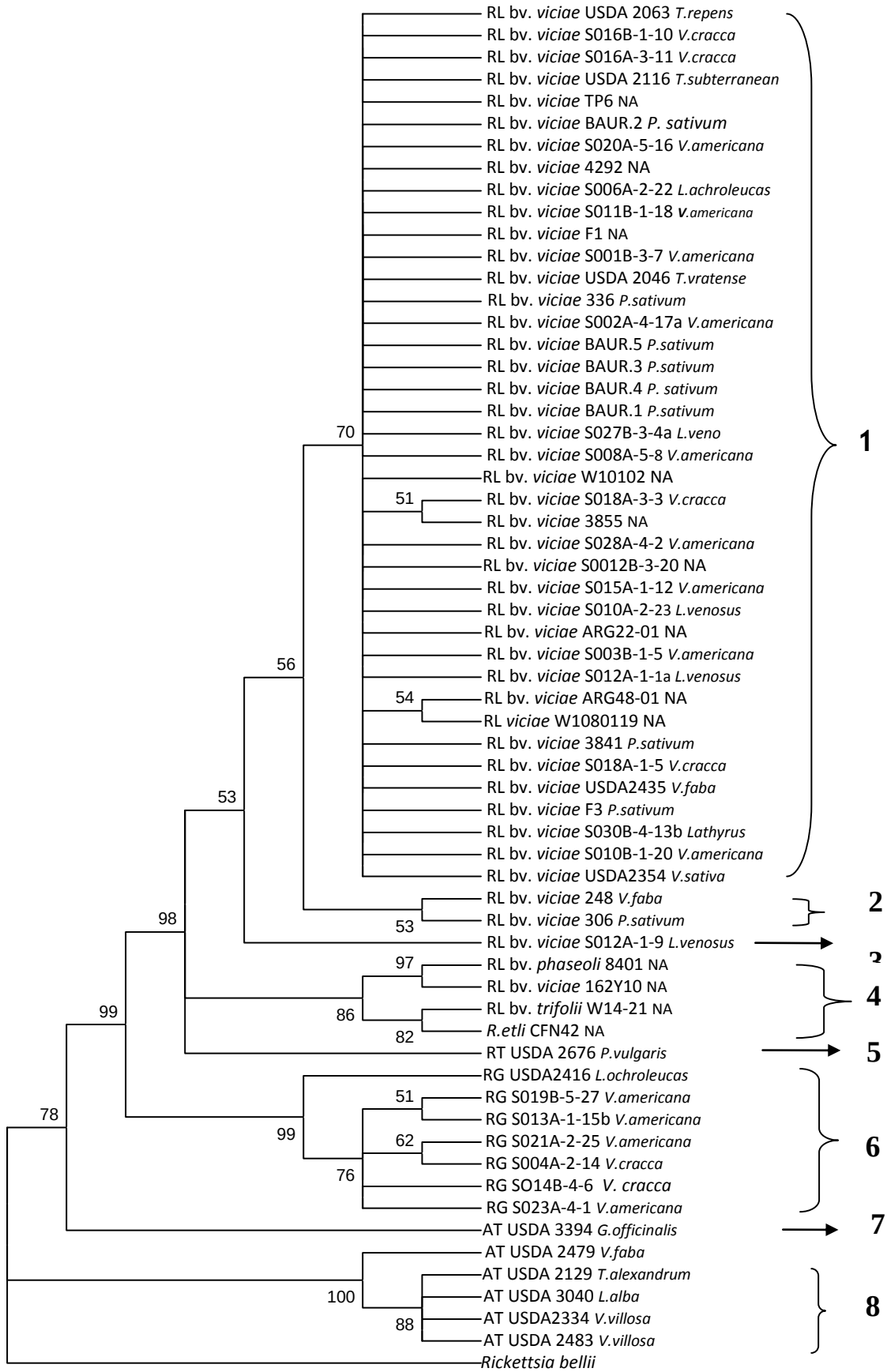
RL bv. viciae USDA 2063 T.repens
RL bv. viciae S016B-1-10 V.cracca
RL bv. viciae S016A-3-11 V.cracca
RL bv. viciae USDA 2116 T.subterranean
RL bv. viciae TP6 NA
RL bv. viciae BAUR.2 P. sativum
RL bv. viciae S020A-5-16 V.americana
RL bv. viciae 4292 NA
RL bv. viciae S006A-2-22 L.achroleucas
RL bv. viciae S011B-1-18 v.americana
RL bv. viciae F1 NA
RL bv. viciae S001B-3-7 V.americana
RL bv. viciae USDA 2046 T.vratense
RL bv. viciae 336 P.sativum
RL bv. viciae S002A-4-17a V.americana
RL bv. viciae BAUR.5 P.sativum
RL bv. viciae BAUR.3 P.sativum
RL bv. viciae BAUR.4 P. sativum
RL bv. viciae BAUR.1 P.sativum
RL bv. viciae S027B-3-4a L.veno
RL bv. viciae S008A-5-8 V.americana
RL bv. viciae W10102 NA
RL bv. viciae S018A-3-3 V.cracca
RL bv. viciae 3855 NA
RL bv. viciae S028A-4-2 V.americana
RL bv. viciae S0012B-3-20 NA
RL bv. viciae S015A-1-12 V.americana
RL bv. viciae S010A-2-23 L.venosus
RL bv. viciae ARG22-01 NA
RL bv. viciae S003B-1-5 V.americana
RL bv. viciae S012A-1-1a L.venosus
RL bv. viciae ARG48-01 NA
RL viciae W1080119 NA
RL bv. viciae 3841 P.sativum
RL bv. viciae S018A-1-5 V.cracca
RL bv. viciae USDA2435 V.faba
RL bv. viciae F3 P.sativum
RL bv. viciae S030B-4-13b Lathyrus
RL bv. viciae S010B-1-20 V.americana
RL bv. viciae USDA2354 V.sativa
RL bv. viciae 248 V.faba
RL bv. viciae 306 P.sativum
RL bv. viciae S012A-1-9 L.venosus
RL bv. phaseoli 8401 NA
RL bv. viciae 162Y10 NA
RL bv. trifolii W14-21 NA
R.etli CFN42 NA
RT USDA 2676 P.vulgaris
RG USDA2416 L.ochroleucas
RG S019B-5-27 V.americana
RG S013A-1-15b V.americana
RG S021A-2-25 V.americana
RG S004A-2-14 V.cracca
RG SO14B-4-6 V. cracca
RG S023A-4-1 V.americana
AT USDA 3394 G.officinalis
AT USDA 2479 V.faba
AT USDA 2129 T.alexandrum
AT USDA 3040 L.alba
AT USDA2334 V.villosa
AT USDA 2483 V.villosa
Rickettsia bellii
70
51
56
54
53
98
53
97
86
82
99
78
99
51
62
76
100
88
1
2
3
4
5
6
7
8

Figure 16. Phylogenetic trees based on 16S rRNA gene of *R. leguminosarum, R. etli, R. gallicum, R. tropici* and *Agrobacterium* sp. The branching pattern was produced by the neighbour-joining method. Bootstrap method used as a test of phylogeny using 1000 number of resamplings. Numbers at nodes indicate levels of bootstrap support. Bootstrap values below 40% are not shown (Tamura et al., 2004). Symbols: RL- *R. leguminosarum*, RE-*R. etli*, RG-*R. gallicum,* RT-*R. tropici*, AT- *A. tumefaciens*. Host plant's name is given after the strain identification from which the strains were isolated initially. NA-host plant not available.

R. leguminosarum S018A-3-3and 3855 with bootstrap support of 51%. The second sub-branch contains *R. leguminosarum* strains W10810119 and ARG 48-01 with bootstrap support of 54%. The second phylogenetic branch contains only two strains of *R. leguminosarum* 248 and 306 with bootstrap support of 56% whereas branch three contains a strain of *R. leguminosarum* S012A-1-5 with bootstrap support of 53%. *R. etli* CFN 42 clustered together with other *R. etli* and two other *R. leguminosarum* strains with bootstrap support of 86%. This branch also contains two sub-groups within it. The first sub-branch, which contains *R. leguminosarum* bv. *phaseoli 8401 and R. leguminosarum* bv. *viciae* 162Y10, with bootstrap support of 97%, the second sub-branch has *R. leguminosarum* bv. *trifolii* W14-2 with *R. etli* CFN42 with bootstrap support of 82%. The phylogenetic branch five has a member of *R. tropici* with bootstrap support of 98%.

Phylogenetic branch six consists of all of the *R. gallicum* used in this study. This branch is separated from rest of the *R. leguminosarum* with high bootstrap support of 99%. Branch six comprised of five sub-branches. The first sub-branch of this branch contains *R. gallicum* USDA 2416 strain with bootstrap support of 99%. The second sub-branch consists of two strains of *R. gallicum*; S019B-5-27 and S013A-1-15b with bootstrap support of 51%. The third sub branch and fourth sub-

branch contain S021A-2-25 and S004A-2-14, respectively. Lastly, the fifth sub-branch seperated form other sub-branches with bootstrap support of 76%. This sub-branch contains SO14B-4-6 and SO23A-4-1 strains of *R. gallicum*.

The phylogenetic branch seven contains a strain, USDA3394, of *Rhizobium* sp. with bootstrap support of 78%, whereas the rest of the *A.tumefaciens* strains USDA2479, USDA2129, USDA3040, USDA 2334, and USDA 2483 clustered together in the 8th branch with high bootstrap support of 78%. Within this branch, strain USDA2479 is separated from the rest of the *A. tumefaciens* with 100% bootstrap support.

RL2975 transporter phylogeny

Phylogenetic (neighbour-joining) tree showing the relationships between 51 strains of rhizobia based on 800 bp aligned conserved region of RL2975 transporter gene sequence (Figure. 17). Among the strains used are 45 strains of *R. leguminosarum* bv. *viciae*, 1 *R. leguminosarum* bv. *trifolii*, 4 *R. gallicum*, 2 *R. etli* and a reference strain, *R. leguminosarum* bv. *viciae* 3841. Amplification of RL2975 transporter gene was successful for all isolates except two *R. leguminosarum* bv. *viciae* strains USDA2046 and BAUR.2 and three strains, *R. gallicum* USDA 2416, S014B-4-6 and S023A-4-1. Sequence similarities among RL2975 transporter PCR amplicon were shown to be above 94%. The RL2975 transporter gene sequences of members of the genus *Rhizobium* formed seven major branches, of which branches 1-5 contained more than 80% of the isolates included in the study (Figure 17). The phylogenetic branch 1 comprised of six sub-branches separated from each other with bootstrap value of 76%. These sub-branches contain all strains of *R. leguminosarum* except a strain of *R. phaseoli* in branch 1a. The sub-branch 1a is the largest within

branch 1.Two strains of *R. leguminosarum*, BAUR.4 and BAUR.1 belong to sub-branch 1b supported with 65% bootstrap value. The sub-branch 1c contains a strain of *R. leguminosarum* USDA 2116. The sub-branch 1d (strain USDA 2063) and 1e (strain S020A-5-16a) contains strains of *R. leguminosarum.* The sub-branch 1f composed of two strains of *R. leguminosarum* (strains S003B-1-25 and S006A-2-22) supported by high bootstrap value of 99%.

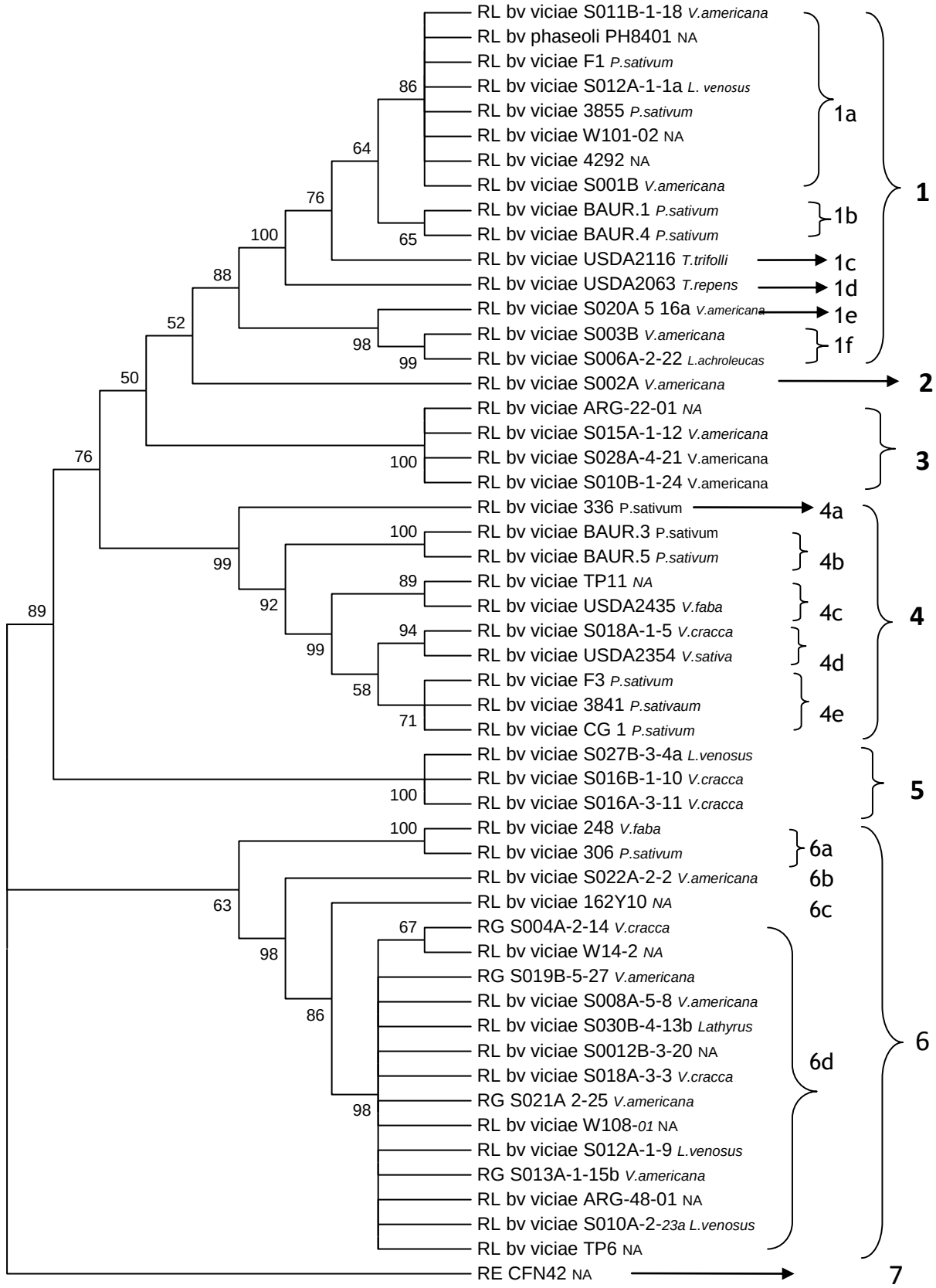

Figure 17. Phylogenetic trees based on the RL2975 transporter sequence of strains of *R. leguminosarum, R. etli* and *R. gallicum* from the PCR amplicon. The branching pattern was

produced by the neighbour-joining method. Bootstrap method used as a test of phylogeny using 1000 number of resamplings. Numbers at nodes indicate levels of bootstrap support. Bootstrap values below 40% are not shown (Tamura et al., 2004). Symbols: RL- *R. leguminosarum*, RE-*R. etli*, RG-*R. gallicum*. Host plant`s name is given after the strain identification from which the strains were isolated initially. NA-host plant not available

The phylogenetic branch 2 contains a strain of *R. leguminosarum* (S002A-4-17a). The branch 3 contains four strains of *R. legiminosarum* ARG -22-01, S015A-1-12, S028A-4-21and S010B-1-24 connected with high bootstrap support of 100%. This branch separated from branch 1 and 2 with bootstrap support of 50%.

The branch 4 contains five sub-branches within it. The sub-branch 4a contains a single strain of *R. leguminosarum* 336.The sub-branch 4b contains two strains of *R. leguminosarum* BAUR.3 and BAUR .5 grouped together with 100% bootstrap value. The sub-branch 4c contains two strains of *R. leguminosarum* TP11 and USDA 2435 with bootstrap support of 89%. The sub-branch 4d contains two strains of *R. leguminosarum* S018A-1-5 and USDA 2354 with bootstrap support of 94%. The sub branch 4e has three strains of *R. leguminosarum* F3 and CG1 including the reference strain *R. leguminosarum* 3841. Members in this sub-branch are similar at bootstrap value of 71%. The branch 5 contains three strains of *R. leguminosarum* S027B-3-4a, S016B-1-10 and S016A-3-11 with high bootstrap support of 100%. The phylogenetic branch 6 is the largest branch in this tree containing about 33% (18/54) of strains used in the phylogeny analysis. The sub-group 6a includes two strains, *R. leguminosarum* bv.*viciae* 306 and *R. leguminosarum* bv. *viciae* 248 connected together with bootstrap support of 100%. The sub-branch 6b and 6c contains a strain of *R. leguminosarum* bv.*viciae* S022A-2-2 and 162Y10, respectively. The sub-group 6d is the largest in this branch, which contains 10 strains of *R. leguminosarum* and 4

strains of *R. gallicum* (strains S004A-2-14, S019B-5-27, S021A-2-25 and S013A-1-15B). The first sub-branch within 6d contain a strain of *R. gallicum* (strain S004A-2-14) grouped together with *R. leguminosarum* bv.v*iciae* (strain W14-2) supported by bootstrap value of 67%. The branch 7 contains a strain of *R. etli* (strain CFN42), which is separated from the rest of the strains in this phylogeny with bootstrap support of 89%.

The multiple sequence alignment of a portion of the conserved region of representative strains from RL2975 transporter phylogeny tree is provided in Figure 18.

CLUSTAL 2.1 multiple sequence alignment

```
R.L._bv_viciae-3841     -TTCGCCTGGG-TGGTCTATTATCCCAATGCGGTCTATCTCGGCAGGCTT 48
R.L._bv_viciae_USDA     -------GGGG-TGGTCTATTATCCCA-TGCGGTCTATCTCGGCAGGCTT 41
R.L._bv_viciae-F1       ---CGCCTGGG-TGGTCTATTATCCCAATGCGGTCTATCTCGGCAGGCTT 46
R.L._bv_viciae_S015     TTTCGCCCGGG-TGGTCTATTATCCCA-TGCGGTCTATCTCGGCAGGCTT 48
R.L._bv_viciae_S002     ----------------TATTATCCCAATGCCGTCTATCTCGGCAGGCTT 33
R.L._bv_viciae-S027     -------------------------------------CTCGGCAGGCTT 12
R.gallicum_S019B-5-27   -TTCGCCTGGGGTGGTCTATTATCCGAATGCCGTCTATCTCGGCAGGCTG 49
R.etli_CFN42            TTTCGCCTGGG-TGGTCTATTATCCCAATGCGGTCTATCTCGGCAGGCTC 49
                                    **********

R.L._bv_viciae-3841     TCCACGGCCGATGTCTGGCTCCATCTCGGCCTGGGCCTTGCCTGGGCCGC 98
R.L._bv_viciae_USDA     TCCACGGCCGATGTCTGGCTCCATCTCGGCCTGGGCCTTGCCTGGGCCGC 91
R.L._bv_viciae-F1       TCCATGGCCGATGTCTGGCTTCATTTCGGCCTGGGCCTTGCCTGGGCCGC 96
R.L._bv_viciae_S015     TCCACGGTCGATGTGTGGCTTCATTTCGGCCTGGGCCTTGCCTGGGCCGC 98
R.L._bv_viciae_S002     TCTACCGCCGATGTCTGGCTCCATCTCGGCCTGGGCCTTGCCTGGGCCGC 83
R.L._bv_viciae-S027     TCCACCGCCGATGTCTGGCTCCATTTCGGCCTGGGTATTGCCTGGGCCGC 62
R.gallicum_S019B-5-27   TCCGCGGCCGATGTCTGGCTGCATCTCGGCCTGGGCCTTGCCTGGGCCGC 99
R.etli_CFN42            TCGGTCGCCGACACCTGGCTTCATCTCGGCCTCGGCCTCGGCTGGGCCGG 99
                  **   * ***   ***** *** ******* **  * * ********

R.L._bv_viciae-3841     GTTGTTCCTTGCCGGCGTCCTCTGGCTGTGGCGCTGTGCCTCCGGCCGCA 148
R.L._bv_viciae_USDA     GTTGTTCCTTGCCGGCGTCCTCTGGCTGTGGCGCTGTGCCTCCGGCCGCA 141
R.L._bv_viciae-F1       GTTGTTCCTTGCCGGCGTCCTCTGGCTGTGGCGCCGGGCCTCCGGCCGCA 146
R.L._bv_viciae_S015     GTTGTTCCTTGCCGGCGTCCTCTGGCTGTGGCGCCGGGCCTCCGGCCGCA 148
R.L._bv_viciae_S002     GTTGTTCCTTGCCGGCGTCCTCTGGCTGTGGCGCCGGGCCTCCGGCCGCA 133
R.L._bv_viciae-S027     GTTGTTCCTTGCCGGCGTCCTCTGGCTGTGGCGCCGGGCCTCCAGCCGCA 112
R.gallicum_S019B-5-27   GCTGTTCCTCGCCGGCGTCCTCTGGCTGTGGCGCTGCGCCTCCACCCGCA 149
R.etli_CFN42            GCTGTTCCTTTTGGGCGTGCTCCGGCTCTGGCGCCTGGCGTCGCGCCGCA 149
                  * ********    ***** *** ***** *******   ** **   *****

R.L._bv_viciae-3841     TCACCGTGCAGGGAGGTTGATCGTGCTTGTTCATCACCTGCGGGTCCTTC 198
R.L._bv_viciae_USDA     TCACCGTGCAGGGAGGTTGATCGTGCTTGTTCATCACCTGCGGGTCCTTC 191
R.L._bv_viciae-F1       TCACCGTGCAGGGAGGTTGATCGTGCTCATACATCACCTGCGCGTCCTTC 196
R.L._bv_viciae_S015     TCACCGTGCAGGGAGGTTGATCGTGCTCATTCATCACCTGCGCGTCCTTC 198
R.L._bv_viciae_S002     TCACCGTGCAGGGAGGTTGATCGTGCTCATTCATCACCTGCGCGTCCTTC 183
R.L._bv_viciae-S027     TCACCGTGCAGGGAGGTTGATCGTGCTCATTCATCACCTGCGCGTCCTTC 162
R.gallicum_S019B-5-27   TCACCGTGCAGGGAGGTTGATCGTGCTTATTCATCACCTTCGCGTCCTGC 199
R.etli_CFN42            TCACCGTCCAGGGAGGCTGATCGTGTTCCTTCATCACCTCCGCGTCATTC 199
                  ******* ******** ******** *  * ******** ** *** * *

R.L._bv_viciae-3841     CGCTGCTGGTCCGGATGCATGTCCGCTCCAAGATGGAATATCGCGGCGCT 248
R.L._bv_viciae_USDA     CGCTGTTGGTCCGGATGCATGTCCGCTCCAAGATGGAATATCGCGGCGCT 241
R.L._bv_viciae-F1       CGCTGCTGGTCCGGATGCATGTCCGCTCCCAGATGGAATATCGCGGCGCC 246
R.L._bv_viciae_S015     CACTGCTGGTCCGGATGCATGTCCGCTCCCAGATGGAGTATCGCGGCGCC 248
R.L._bv_viciae_S002     CGCTGCTGGTCCGGATGCATGTCCGCTCCCAGATGGAATATCGCGGCGCT 233
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R.L._bv_viciae-S027     CGCTGCTGGTCCGGATGCATGTCCGCTCCCAGATGGAATATCGCGGCGCT 212
R.gallicum_S019B-5-27   CGCTGCTGGTGCGGATGCATGTCCGCTCCCAGATGGAATATCGCGGCGCT 249
R.etli_CFN42            CGCTCCTGGTGCGCATGCATGTGCGCGCACAAATGGAATATCGCGGCGCC 249
                        * **  **** ** ******** *** *  * ***** ***********

R.L._bv_viciae-3841     TTCTGGCTCGACCGTCTTGCGCAGATCCTTTCCTATGGCAGCGTCTTCGC 298
R.L._bv_viciae_USDA     TTCTGGCTCGACCGTCTTGCGCAGATCCTTTCCTATGGCAGCGTCTTCGC 291
R.L._bv_viciae-F1       TTCTGGCTCGACCGTCTTGCGCAGATCCTTTCCTATGGCAGCGTCTTCGC 296
R.L._bv_viciae_S015     TTCTGGCTCGACCGTCTTGCGCAGATCCTTTCCTATGGCAGCGTCTTCGC 298
R.L._bv_viciae_S002     TTCTGGCTCGACCGTCTTGCGCAGATCCTTTCCTATGGCAGCGTCTTCGC 283
R.L._bv_viciae-S027     TTCTGGCTCGACCGTCTTGCGCAGATCCTTTCCTATGGCAGCGTCTTCGC 262
R.gallicum_S019B-5-27   TTCTGGCTCGACCGTCTTGCGCAGATCCTTTCCTATGGCAGCGTCTTCGC 299
R.etli_CFN42            TTCTGGCTCGACCGGTTTGCGCAGATCCTCTCCTATGGCAGCGTCTTTGC 299
                        ***************  ************** ****************** **

R.L._bv_viciae-3841     CACCATCGGCATCCTGCTTGCCCGCTTCGACACCCTCGGCGGCTGGAGCT 348
R.L._bv_viciae_USDA     CACCATCGGCATCCTGCTTGCCCGCTTCGACACCCTCGGCGGCTGGAGCT 341
R.L._bv_viciae-F1       CACCATCGGCATCCTGCTCGCCCGCTTCGCCACGCTTGGCGGCTGGACCT 346
R.L._bv_viciae_S015     CACCATCGGCATCCTGCTCGCCCGCTTCGACACGCTTGGCGGCTGGACCT 348
R.L._bv_viciae_S002     CACCATCGGCATCCTGCTCGCCCGCTTCGACACGCTCGGCGGCTGGACCT 333
R.L._bv_viciae-S027     CACCATCGGAATCCTGCTCGCCCGCTTCGACACACTCGGCGGCTGGACCT 312
R.G_S019B-5-27          CACCATCGGCATCCTGCTCGCCCGCTTCGATACGCTTGGCGGCTGGACCT 349
R.etli_CFN42            CACCATCGGCATCCTGCTTGCCCGCTTCCAGACGCTTGGCGGCTGGACCT 349
                        ********* ******** *********   ** ** ********** **

R.L._bv_viciae-3841     GGCCGGAGCTTGCGCTGCTGTTCAGCTTCCAGCTGCTCGCTTATTCGCTC 398
R.L._bv_viciae_USDA     GGCCGGAGCTTGCGCTGCTGTTCAGCTTCCAGCTGCTTGCTTATTCGCTC 391
R.L._bv_viciae-F1       GGCCGGAGCTTGCGCTGCTGTTCAGCTTCCAGTTGCTGGCTTATTCGCTC 396
R.L._bv_viciae_S015     GGCCGGAGCTTGCGCTGCTGTTCAGCTTCCAGCTGCTGGCTTATTCGCTC 398
R.L._bv_viciae_S002     GGCCGGAGCTTGCCCTGCTGTTCAGCTTCCAGCTGCTGGCTTATTCGCTC 383
R.L._bv_viciae-S027     GGCCGGAACTTGCCCTGCTGTTCAGCTTCCAGCTGCTGGCTTATTCGCTC 362
R.G_S019B-5-27          GGCCGGAACTGGCGCTGCTGTTCAGCTTCCAGCTGCTGGCTTACTCGCTC 399
R.etli_CFN42            GGCCGGAACTGGCGCTGCTCTTCAGCTTCCAGCTGCTCGCCTATTCGCTG 399
                        ******* ** ** ***** ************ **** ** ** *****

R.L._bv_viciae-3841     GGCGCTGCGATGAGCTTCGTGCAGTTGCGCGAGCTCGAAGAACTGGTGCG 448
R.L._bv_viciae_USDA     GGCGCTGCGATGAGCTTCGTGCAGTTGCGCGAGCTCGAAGAACTGGTGCG 441
R.L._bv_viciae-F1       GGCGCCGCGATGAGTTTCGTGCAACTGCGCGATCTCGAAGAACTGGTGCG 446
R.L._bv_viciae_S015     GGCGCCGCGATGAGTTTCGTGCAACTGCGCGATCTCGAAGAACTGGTGCG 448
R.L._bv_viciae_S002     GGCGCCGCGATGAGCTTCGTGCAATTGCGCGATCTCGAAGAACTGGTGCG 433
R.L._bv_viciae-S027     GGCGCCGCGATGAGCTTCGTGCAATTGCGCGACCTCGAAGAACTGGTGCG 412
R.gallicum_S019B-5-27   GGCGCCGCGATGAGCTTCGTGCAATTGCGCGACCTCGAAGAACTGGTGCG 449
R.etli_CFN42            GGTGCGGCGATGAGCTTCGTGCAATTGCGCGATCTCGAGGAGCTGGTGCG 449
                        ** ** ********* *********  ******* ***** ** *********

R.L._bv_viciae-3841     GCTCGGCACTTACGACACACTGTTGGTCAAGCCTTTCAGCCCCTGGGCCT 498
R.L._bv_viciae_USDA     GCTCGGCACTTACGACACACTGTTGGTCAAGCCTTTCAGCCCCTGGGCCT 491
R.L._bv_viciae-F1       GCTCGGCACTTTCGACACACTGTTGATCAAGCCTTTCAGCCCCTGGGCCT 496
R.L._bv_viciae_S015     GCTCGGCACTTTCGACACACTGTTGGTCAAGCCTTTCAGCCCCTGGGCCT 498
R.L._bv_viciae_S002     GCTCGGCACTTTCGACACACTGTTGGTCAAGCCTTTCAGCCCCTGGGCCT 483
R.L._bv_viciae-S027     GCTCGGCACTTTCGACACGTTGTTGGTCAAGCCTTTCAGCCCCTGGGCCT 462
R.gallicum_S019B-5-27   GCTCGGCACTTTCGACACGCTGTTGGTCAAGCCTTTCAGCCCCTGGGCCT 499
R.etli_CFN42            GCTCGGCACCTACGACACGCTGCTCATCAAGCCGTTCAGCCCCTGGGCCT 499
                         ********* * *******  ** *  ******** *****************

R.L._bv_viciae-3841     ATCTGGTCTTTTCCGGCCTTAATATCGGCTATGCCGGCCACGTCATCCTC 548
R.L._bv_viciae_USDA     ATCTGGTCTTTTCCGGCCTTAATATCGGCTATGCCGGCCACCTCATCCTC 541
R.L._bv_viciae-F1       ATCTGGTCTTTTCCGGCCTCAATATCGGCTATACCGGCCACGTCATCCTC 546
R.L._bv_viciae_S015     ATCTGGTCTTTTCCGGCCTCAATATCGGCTATACCGGCCACGTCATCCTC 548
R.L._bv_viciae_S002     ATCTGGTCTTTTCCGGCCTCAATATCGGCTATGCCGGCCACGTCATCCTC 533
R.L._bv_viciae-S027     ACCTGGTCTTTTCCGGCCTCAACATAGGCTATGCCGGCCACGTCATCCTC 512
R.G_S019B-5-27          ATCTGGTTTTTTCCGGCCTCAATATCGGCTATGCCGGCCACGTCATCCTG 549
R.etli_CFN42            ATCTGGTCTTCTCCGGCCTCAATATCGGCTATGCCGGCCATATCATCCTG 549
                        * ****** ** ********* ** ** ******* ********  ********

R.L._bv_viciae-3841     GCAGTGGCGCTGATGGCTTGGGCCGTTCTGTCCGTCGACTTCACCTGGTC 598
R.L._bv_viciae_USDA     GCAGTGGCGCTGATGGCTTGGGCCGTTCTGTCCGTCGACTTCACCTGGTC 591
R.L._bv_viciae-F1       GCGGTGGTGCTGATGGGCTGGGCGGTCCTGTCCGTCGACTTCGCCTGGTC 596
R.L._bv_viciae_S015     GCGGTGGCGCTGATGGGCTGGGCCGTTCTGTCCGTCGACTTCACCTGGTC 598
R.L._bv_viciae_S002     GCGGTGGCGCTGATGGGCTGGGCGGTCCTGTCCGTCGACTTCGCCTGGTC 583
R.L._bv_viciae-S027     GCGGTGGCGCTGATGGGCTGGGCCGTTCTGTCCGTCGACTTCGCCTGGTC 562
R.G_S019B-5-27          GCGGTGCCGCTGATGGGCTGGGCCGTCCAGTCCGTCGACTTCGCCTGGTC 599
R.etli_CFN42            GCGGTGCCGCTGCTCTGCTGGGCCGTCCTGTCGGTCGACTTCGCCTGGTC 599
                        ** ***  **** *    ****** ** * *** ********** ********
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R.L._bv_viciae-3841     GGTCTTGTCCGCGTCATTCTTCATTGCTGCGCTTATCAGCGCGACGCTGC 648
R.L._bv_viciae_USDA     GGTCTTGTCCGCGTCATTCTTCATTGCTGCGCTTATCAGCGCGACGCTGC 641
R.L._bv_viciae-F1       GGTCTGGCCCGTGTCGTTCCTCGTTGCTGCGCTCATCAGCGCAACGCTGC 646
R.L._bv_viciae_S015     GGTCTGGTCGGCGTCGTTCTTAGTTGCCGCGCTCATCAGCGCGACGCTGC 648
R.L._bv_viciae_S002     GGTCTGGTCCGCGTCGTTCTTCGTTGCCGCGCTCATCAGCGCGACGCTGC 633
R.L._bv_viciae-S027     GGTCTGGTCCGCCTCGTTCTTCGTTGCCGCGCTCCTCAGCGCGACGCTGC 612
R.G_S019B-5-27          GGTCTGGTCGGCATCGTTCCTTATTGCCGCGCTCCTCAGCGCGACGCTGC 649
R.etli_CFN42            TGCCGCGTCCGTGGCGTTCCTGCTTGCCGCAATCGTCAGCGCGACGCTGC 649
                  * *  * * *   * *** *  **** **  *  ******* *******

R.L._bv_viciae-3841     TGACGGGTGCGCTCATCACCATGATCGGCGCCACGGCATTGATCTGGGTG 698
R.L._bv_viciae_USDA     TGACGGGTGCGCTCATCACCATGATCGGCGCCACGGCATTGATCTGGGTG 691
R.L._bv_viciae-F1       TGACCGCTGCCCTTATTACCATGATCGGCGCCACGGCGCTGATCTGGGTA 696
R.L._bv_viciae_S015     TGACCGCTGCGCTCATCACCATGATTGGCGCTACGGCGCTGATCTGGGTG 698
R.L._bv_viciae_S002     TGACCGCTGCCCTCATCACCATGATCGGCGCCACGGCGCTGATCTGGGTG 683
R.L._bv_viciae-S027     TGACCGCGGCCCTCATCACCATGATCGGCGCCACGGCGCTGATCTGGGTG 662
R.gallicum_S019B-5-27   TGACCGCGGCACTCATCACCATGATCGGCGCCACGGCGCTGATCTGGGTG 699
R.etli_CFN42            TCACCGGCGCGATGATCACCATGATCGGTGCGACGGCACTGATGTGGGTG 699
                  * *** *  **  * ** ******** ** ** *****  **** *****

R.L._bv_viciae-3841     CGGTCCAACCATCTGTT-CTCGATC----- 722
R.L._bv_viciae_USDA     CGGTCCAACCATCTGTT-CTCGATCAA--- 717
R.L._bv_viciae-F1       CGCTCCAACCATCTGTT-CTCGATCA---- 721
R.L._bv_viciae_S015     CGCTCCAACCATCTGTT-T---------- 716
R.L._bv_viciae_S002     CGCTCCAAC------------------- 692
R.L._bv_viciae-S027     CGCTCCAACCATCTGTT-CTCGATCAAAAA 691
R.gallicum_S019B-5-27   CGCTCCAACCATCTGTTTCTCGAT------ 723
R.etli_CFN42            CGGTCCAACCATCTGTT-CTCGATCC---- 724
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Figure 18. Multiple sequence alignment of 800bp conserved region of representative strains from each branch of RL2975 transporter phylogeny tree using ClustalX. An * (asterisk) indicates positions which have a single, fully conserved residue.

DISCUSSION

The genome of *Rhizobium* contains a large number of genes associated with specific functions to interact with a host plant during symbiosis (Miller et al., 2007). To gain a deeper understanding of the symbiotic process it becomes essential to understand the function of the genes involved, and identify novel genes required for the process. In particular, ABC transporters in *R. leguminosarum* have been of research interest in the past decades (Ding et al., 2012, Sugiyama et al., 2007, Hosie et al., 2002). In this thesis, functional analyses of a *R. leguminosarum* ABC transporter-2 (Rl2975-Rl2977) as well as the phylogenetic distribution among strains of *Rhizobium* are discussed.

Expression of exopolysaccharide genes

The relationship between production of EPS and desiccation tolerance has been studied in the past (Bushby and Marshall, 1977; Hartel and Alexander, 1986; Roberson and Firestone, 1992; Ophir & Gutnick, 1994). One study reported that there was an inverse relationship between exopolysaccaride production and dessication survival (Hartel and Alexander, 1986) where as Vanderlinde et al., (2010) reported a positive relationship. Many researchers have speculated that bacterial EPS may provide a protective environment for its survival from desiccation by creating hydrated microenvironments. An EPS containing microenvironment holds relatively higher amount of water and dries slowly compared to its surroundings.

Vanderlinde et al., (2010) demonstrated that the RL2975 transporter mutant produced three fold less EPS when compared to wild type *R. leguminosarum* bv. *viciae* 3841. From this study, I hypothesized that the RL2975 transporter substrate may be involved in the regulating the expression of different EPS biosynthesis genes. Genes involved in the synthesis and exports of EPS are located on the chromosome of *R. leguminosarum*. The majority of these genes are grouped in a large cluster called *pss*-I (Krol et al., 2007). In this study, I compared expression of EPS biosynthesis genes, *pssA*, *pssB* and *pssN*, using *gusA* as a reporter to measure promoter activity. In this experiment, expression of EPS sysnthesis gene was predicted to be higher in wild type compare to transporter mutant due to less production of EPS. Results showed that the specific activity of *pssN* gene expression was significantly higher ($p=0.05$) in mutant with compare to wild type. However, a physiological significance remains to be determined. Even though the RL2975 transporter mutant produced less EPS, the expression of the *pssN* gene was higher which suggest a negative result. On the one

hand, *pssA* gene expression was higher in wild type (WT) compared to mutant type (MT). On the other hand, no difference was observed in *pssB* gene expression between MT and WT.

The *pssA* gene encodes an integral membrane bound protein of glycosyl –IP-transferase that transfers glucose-1-phosphate from UDP-glucose to the lipid carrier (Borthakur et al 1988; Pollock et al., 1998). The gene participates in the synthesis of octasaccharides and is located far from other *pss* genes. The *pssA* gene is a highly conserved gene present in all characterized *R. leguminosarum* and also closely related species such as *R. etli* and *R. gallicum* (van Workum, 1997, Borthakur et al., 1988, Ivashina et al., 1994, Janczarek et al., 2009). Previous studies reported that mutation in *pssA* gene appears to cease the production of EPS and initiates non- nitrogen fixing nodules on the roots of host plant (Rolf et al., 1996; van Wrkum, 1997; Janczarek et al., 2009). Other effects of a *pssA* mutation include differences in the levels of the synthesis of several proteins (Jaancjzarek et al., 2003, Guerreiro et al., 2000). Exopolysaccharide synthesis in rhizobia is regulated both in the free-living state and during symbiosis. Under phosphate sufficient conditions, expression of *pssA* increased 15-fold (Janczarek and Skorupska, 2004). In the presence of ammonia expression of *pssA* was repressed but the EPS production was enhanced (Janczarek and Skorupska, 2004, Wielbo et al., 2004). In the present study, mutation of the RL2975 transporter resulted in 3-fold reduction of EPS but it did not alter the expression of the *pssA* gene showing slightly higher expression of *pssA* gene in wild type compared to the ABC-2 transporter mutant (Table 3).

The *pssB* gene is involved in various physiological functions such as negative regulation of EPS synthesis and exportation of EPS (Janczarek et al., 1999; Ivashina et al., 1996; Kukowska et al., 2007). The *pssB* gene, which is upstream of the *pssA*

gene, encodes a protein belonging to the family of inositol monophosphatases (IMP-ases). The IMP-ases are found in both prokaryotic and eukaryotic organisms, and are responsible for the conversion of inositol monophosphate to inositol but the role of IMP-ase is unclear in rhizobia. Janczarek and Skorupska (2001) observed that a non-polar mutation, a mutation in a gene that does not influence the downstream genes in an operon, in the *pssB* gene produced increased amount of EPS and induced nodulation. The authors also found that additional copies of this gene reduced the amount of EPS and affected the symbiotic phenotype of transconjugants. Under the phosphate sufficient condition expression of *pssB* increased 2-fold. In the presence of ammonia, expression of *pssB* was repressed and the EPS production was enhanced. In our study, EPS production was reduced in RL2975 transporter mutant but the mutation of this operon did not affect the expression of *pssB* gene compared to wild type (Table 3).

Previous studies reported that the *pssN* gene is localized within the *pss* region of *R. leguminosarum* bv. *trifolii* TA1 (Mazur et al., 2001) and is considered a member of the OMA family of proteins (Paulsen et al., 1997; Marczak et al., 2006). The gene is involved in surface expression of polysaccharides and appears to perform translocation of EPS across the outer membrane (Malgorzata et al., 2006). Polymerization and the export of the EPS outside bacteria are carried out by a secretion system consisting of at least three proteins encoded by the *ppsT, pssN* and *pssP* gene of the operon (Mazur et al., 2001).

In rhizobia, EPS synthesis is a complex process and appears to be controlled at both the transcriptional and post-transcriptional level. In this study, I observed that the RL2975 transporter mutant produced lesser amounts of EPS. Vanderlinde et al., (2010) found that structures of EPS were identical between the RL2975 transporter

mutant and the wild type suggesting that the transporter does not transport a substrate required for proper assembly of the EPS. The data from this study suggests that the RL2975 transporter function is not connected to the regulation of EPS producing genes. Therefore, the mechanism for decreased production of EPS in the transporter mutant still remains unknown.

Swarming motility

Swarming is considered to be a coordinated translocation of bacterial population across semi-solid surface (Henrichsen, 1972, Kristen et al., 2008). Motility enables bacteria to colonize different environments, attach to the surfaces and form biofilms (O'Toole and Kotler, 1998). Swarming requires bacteria to coordinate their physiology via a process called quorum sensing.

In this study, three different strains of *R. leguminosarum* were used for the swarming experiment. All strains, transporter fusion, transporter mutant and the wild type, exhibited similar swarming phenotype in swarm medium (0.7% agar). All swarmer cells exhibited a minor increase in flagellation when compared to a previous study that reported hyperflagellation in *R. leguminosarum* bv. *viciae* VF39Sm (Tambalo et al., 2010). A recent study also suggested that hyperflagellation is another important feature that helps bacteria during swarming motility (Copeland et al., 2009).

In another experiment, I did not observe a visible difference in swarming phenotype between *R. leguminosarum* wild type and *R. leguminosarum* RL2975 transporter mutant. Therefore, the mutation in RL2975 transporter operon did not affect swarming motility. It was thought that the surfactant is extracellular capsular polysaccharides that accompany swarming motility in *P. mirabilis* (Rauprich et al.,

1990). However, the transporter mutant that produces less EPS compare to wild type, did not show any difference in the swarming phenotype. Thus, it appears that RL2975 transporter may not be absolutely required in swarming motility and that the decrease in EPS production in the mutant does not limit the mutant's swarming capabilities

Swarming depends on the several features such as agar concentration, incubation, temperature, cell density and nutrient rich medium. Our observation of swarming fronts preceded by a clear transparent zone was in agreement with study that reported a similar result (Tambalo et al., 2010). Previous studies found a similar observation that the clear transparent zone of extracellular matrix consists of polysaccharides, biosurfactants and peptides, which facilitate swarming motility creating the hydrated environment for the swarming cells (Julkowske et al., 2004; Sule et al., 2009). In some bacterial species, such as *P. putida* and *Salmonella*, the production of surfactant was not detected (Matillg et al., 2003; Chen et al., 2007), respectively.

The water content is critical for swarming motility. Water limitation can result in poor swarming, whereas more water result in swimming (Fauvart et al., 2012). Bacterial swarming is typically observed on a solidified medium containing 0.5%-2% agar (Verstraten et al., 2008). In particular, agar concentration between 0.5% and 1% can produce better surface migration in *R. leguminosarum* (Tambalo et al., 2010). The authors concluded that nutrient-rich medium enhances surface migration of *R. leguminosarum*. Another study also found that *E. coli* and *Salmonella* show swarming motility at agar concentration of 0.5%-0.9% (Harshey and Matshuyama, 1994). In contrast, some of the bacterial species such as *Proteus* and *Vibrio* were capable of swarming on higher concentration of agar (1.5%-2%) (Harshey, 2003; Harshey and Matssuyama, 1994).

In the study, instead of normal incubation temperature (i.e, 30 ^{o}C), room temperature was used for the swarming motility. A previous study also used low temperature for swarming in *P. putida KT2440* (Matilla et al., 2007). The authors reported that *P. putida* swarmed well between 18 and 28 ^{o}C, however they did not observe swarming at 30 ^{o}C. Similarly, the study by Tambalo et al., (2010) found that *R. leguminosarum* 3841 and *R. leguminosarum* VF39 swarmed well at 22 ^{o}C; however the bacteria failed to swarm at 30 ^{o}C.

Little is known about the gene regulation of the bacterial sub-population comprised in a swarming colony. Previous studies on swarming motility demonstrated that the swarming phenomenon relies on the expression of many genes in *Pseudomonas aeruginosa* (Potvin et al., 2003, Overhage et al., 2007; Yeung, 2009). I compared the regulation of the RL2975 transporter using the RL2975 promoter fusion of *R. leguminosarum* bv. *viciae* 3841. Results showed that expression of RL2975 transporter operon was significantly higher in the edge of the swarming cells compare to the edge of the vegetative cells. However, expression of the genes in the middle and the center of the swarming cells were not different compared to the vegetative cells. Tambalo et al., (2010) also found a similar result in flagellar gene expression of *R. leguminosarum* bv. *viciae* VF39. A recent study by Tremblay et al., (2010) reported that majority of differentially expressed genes in *P. aeruginosa* were down regulated in the tendril tips population compared to the non-swarming control and swarm center. Overhage et al., (2008) found that swarm edge cells exhibited up-regulation of genes associated with a Type III secretion system, extracellular protease and iron transport in *P. aeruginosa*. These observations suggests that fast moving swarmer cells in tendril tips require more energy than non-swarming and swarm

center cells. Furthermore there are far fewer genes differentially expressed between the swarm center and non-swarming control. This suggests that swarm center cells are metabolically closer to non-swarming condition cells than to tendril tip cells. Based on the gene regulation and mutant phenotype results, although the transporter expression maybe higher in actively swarming cells, the transporter is not absolutely required for swarming. Therefore, the physiological significance of the gene upregulation in swarming cells remains to be determined.

Flocculation of RL2975 transporter

Flocculation of bacteria can influence the plant growth and nodulation (Neyra et al., 1999, Nakata et al., 2000). Factors affecting successful colonization of the roots by *Rhizobium* sp. are not yet fully understood, but it has been suggested that bacterial surface polysaccharides such as b-Glucans, lipopolysaccharides, and EPS, and cellulose have been shown to play a role in defining plant-bacterial associations (Leigh and Coplin, 1992). Neyra et al., (1999) showed that the use of a flocculating *R. etli* strain was superior to the non-flocculated form in terms of nodule numbers and plant growth. The transporter mutant which produces less EPS flocculates in minimal media but the authors did not quantify the flocculation nor did they identify a mechanism for the flocculation phenotype (Vanderlinde et al., 2010). It was suspected that the cell-cell aggregation of the *R. leguminosarum* Rl2975 transporter mutant is due to the cell to cell binding of exposed cellulose microfibrils. In the present study, I quantified flocculation of RL2975 transporter mutant and compared it to wild type, a mutant deficient in cellulose production and the double mutant (cellulose deficient mutant with RL2975 transporter deficient mutant) (Table 8).

In the current study, the RL2975 transporter mutant flocculated up to 44% compared to before the sample homogenization, whereas other mutants, *celA* and double mutants did not show flocculation. On the other hand, it was observed that the mutant strains of *R. leguminosarum* overproducing cellulose flocculate heavily (Ausmees et al., 1999). The removal of cellulose production in the transporter mutant stopped flocculation suggesting that cellulose fibers are contributing to the flocculation. Therefore, the possible mechanism for the flocculation change in the RL2975 transporter mutant is due to decreased level of EPS production and a possible absence of intercellular shielding of the cellulose microfibril in RL2975 transporter mutant. In a study by Laus et al., (2005), the EPS-deficient bacteria showed increased flocculation compared to wild type and cellulose-deficient bacteria which suggest that EPS reduce cellulose-mediated flocculation of bacterial cells. Although the ability of the RL2975 transporter mutant to colonize root hairs is unknown, a previous study found that an EPS mutant, which has the ability to produce cellulose fibrils, may infect elongated root hairs but is defective in infection thread colonization. In vitro experiments showed that the cellulose (*celA*) mutant had ability to attach and form normal biofilms on solid surface, but it did not form a biofilm on root hairs, although attachment did occur (Williams et al., 2008). In this study, I did not observe any flocculation in the RL 2975 transporter and cellulose deficient double mutant. This observation is in agreement with a previous study that reported cellulose fibrils might cause persistent flocculation in many rhizobial strains (Ausmees et al. 1999; Smit et al., 1987, Laus et al., 2005). The authors suggested that firm attachment of cellulose fibrils in cell-to-cell contact may hamper colonization and may inhibit nodulation. Further investigation of the attachment of the transporter mutant to plant roots therefore seems warranted.

Persistence of the RL2975 transporter mutant in soil

Two important ecological aspects, persistence in soils and their competitiveness were compared between of *R. leguminosarum viciae* 3841wild-type and the transporter mutant. Our study suggests that the RL2975 transporter mutants and the wild types were equally competitive at the laboratory conditions. Furthermore the persistence result showed that both, mutant and wild type, continued to persist in soil up to 28 days with minor growth variations. I did not observe any impacts of mutation in ABC-2 transporter in persistence of rhizobia in soil. The present tudy was conducted in the lab-controlled conditions, future experiments should include a field-based approach that could also include trials that go on beyond 28 days, up to 6 months or 1 year.

Phylogeny analysis

Several strains of rhizobia including a reference strain were used in the current study to infer the phylogenetic context of the RL2975 ABC-2 transporter. Phylogenetic inference using DNA sequences has been commonly used to describe phylogeny position among bacterial strains of interest. Comparative analysis of 16s rRNA sequences have been used in the past for the investigation of rhizobia taxonomy (de Lajudie et al., 1994, Jarvis et al., 1997, Young et al., 2001).

The 16s rRNA sequences were aligned using ClustalX before constructing a phylogenetic tree based on neighbour-joining method (Figure 15). A total of nine branches were assigned based on their neighbour joining relationship supported by high bootstrapping value. Most of the *R. leguminosarum* strains are distributed in branch 1. The branches 2 and 3 are represented mostly by *R. leguminosarum*. I

observed that the branch 4 is heterogenous and it contained strains of *R. leguminosarum* and *R. etli*. The branch 5 is represented by *R. tropici*. Branch 6 represented by *R. gallicum*, which contains one USDA strain with other strains. Branch 7 contains all the *A.tumefaciens* strains used in this study. Two strains CG#1 and S022A-2-22 which were included in the transporter phylogeny analysis were not included in the 16s rRNA analysis because of unsuccessful sequencing results.

Based on sequence of the 800bp- region of RL2975 transporter, the 54-rhizobial strains formed 7 branches with high bootstrap support. The branches are heterogenous with respect to host plants from which the strains were originally isolated. Similarly the branches are heterogenous with respect to the location from where the strains were obtained. From the transporter phylogenetic tree, it is evident that the RL2975 transporter is widely distributed among the strains of *R. leguminosarum* and is diverse among strains used in the analysis. Furthermore, the present study also gave a new insight that the RL2975 transporter is also present in species other than *R. leguminosarum* and *R. etli*. The results showed that the ABC-2 transporter is present in some (4/6) of the strains of *R. gallicum*. However, the *R. gallicum* strains, which were positive for the RL2975 transporter, did not form a separate cluster but scattered along with *R. leguminosarum* strains. Therefore, from the transporter phylogeny tree, it is speculated that the *R. gallicum* may have acquired these genes by horizontal gene transfer.

The phylogeny tree generated by 16S rRNA sequence is not similar to the RL2975 transporter tree because in the 16s rRNA tree different strains of rhizobia separated in different branches on basis of species of rhizobia. All of the strains formed in branch 1 are *R. leguminosarum*. Few of the *R. leguminosarum* strains clustered in second and third branches. Similarly, all of the *R. gallicum* species

clustered together in the same branch. *Agrobacterium*, which is distantly related to *R. leguminosarum* strains, formed the separate branch in the tree. In contrast, each branch in the transporter phylogeny tree grouped different species of rhizobia. It appears from the phylogeny analysis of the 16s rRNA sequencing and the phylogeny tree may be used for preliminary identification of the strains. In this study, I used 5 different species of *Rhizobium*. Among the five, three species of *R. etli*, *R. leguminosarum* and *R. gallicum* are positive for RL2975 transporter. Due to resource limitations, I did not use different species of *Rhizobium* for RL2975 transporter phylogeny. However, I looked at the genomes of several sequenced species of rhizobia and the result confirmed that RL2975 transporter was very restricted to only a few species of rhizobia. Therefore, a further investigation is warranted to examine transporter diversity in greater varieties of rhizobia species. In addition to PCR and phylogeny analysis, I also compared the entire operon nucleotide sequences of the transporter to the genome sequences of *Rhizobium* sp currently avialable in the public database RhizoDb and RNB (Department of Energy Joint Genome Institute database (http://img.jgi.doe.gov/cgi-bin/geba/main.cgi). Presently the operon is only confined to the *R. leguminosarum* and some strains of the *R. etli*.

Finally, a large discrepancy was observed between the 16S rRNA and the RL2975 transporter phylogeny tree, which suggest that the transporter sequence does not support with the divergence of 16S RNA sequence. It appears from the transporter phylogeny tree that the RL2975 transporter is taxonomically heterogeneous within the *R. leguminosarum* and closely related species such as *R. etli* and *R. gallicum*. Additionally, from the transporter phylogeny it is revealed that the transporter is found in all *R. leguminosarum* strains used in this study and across the biovars too. It is speculated that the operon is important for serving a unique aspect of *R.*

leguminosarum physiology that is not found in the physiology of other rhizobials. The operon has been conserved in all the *R. leguminosarum* strains and some of the closest relatives like *R. etli* and *R. gallicum* like strains showing that the operon is constrained to the narrow species range but not connected to the particular specific host range. Therefore the physiological importance may be related to a free-living condition that is common to the *leguminosarum, etli* and *gallicum* species.

SUMMARY

Rhizobia are known to contain a large number of ATP-binding cassette (ABC) transporters in genome (Young et al., 2006). One of transporters recently studied was RL2975, which contains three genes, RL2975, RL2976 and RL2977 in *R. leguminosarum* bv. *viciae* (Vanderlinde et al., 2010). The authors found that RL2975 transporter mutant produced 3-fold less EPS compared to wild type and appeared to be associated with dessication tolerance but the exact function of the operon was not known.It was also found that the operon was only present in isolates of *R. leguminosarum* and *R. etli* sequenced to date.To further understand the role of RL2975 transporter in regulation of EPS synthesis gene, swarming, flocculation and persistence in soil were studied in this study .The result in the regulation of EPS biosynthesis genes suggested that the mutation of RL2975 transporter did not alter the expression of EPS production genes. This result may suggest that the RL2975 transporter operon was not required in the regulation of these genes. Swarming phenotype on transporter mutant suggests that the transporter may be up-regulated during swarming but is not absolutely required in swarming motility.

The RL2975 transporter mutant flocculated highly whereas mutants, *celA* and double mutants (RL2975 transporter and *celA*) did not show flocculation in minimal media suggesting that EPS prevented cell-to-cell aggregation suggesting the persence

of cellulose fibrils. RL2975 transporter mutant persistence in soil did not reveal specific functions of the transporter, and further research is required to ascertain its functional role in *R. leguminosarum* physiology.

Finally, this study was conducted to examine phylogenetic distribution of RL2975 transporter in *Rhizobium* sp. Results suggested that the transporter is constrained within a subset of rhizobia but is widely distributed and diverse among strains of *R. leguminosarum* and is found in some *R. gallicum*-like strains.

REFERENCES

Adler, J. (1973). A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by *Escherichia coli*. *Journal of General Microbiology.* **74**, 77-91.

Akakura, R. & Winans, S. C. (2002). Constitutive Mutations of the OccR Regulatory Protein Affect DNA Bending in Response to Metabolites Released from Plant Tumors. *Journal of Biological Chemistry* **277**, 5866-5874.

Ali, S. F., Rawat, L. S., Meghvansi, M. K. & Mahna, S. K. (2009). Selection of stress-tolerant rhizobial isolates of wild legumes growing in dry regions of Rajasthan. *ARPN Journal of Agricultural and Biological Science* **4**, 13 - 18.

Altschul, S. R., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389-3402.

Amarger, N., Macheret, V. & Laguerre, G. (1997). *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov., from *Phaseolus vulgaris* nodules. *International Journal of Systematic Bacteriology* **47**, 996-1006.

Banfalvi, Z., Nieuwkoop, A., Schell, M., Besl, L. & Stacey, G. (1988). Regulation of nod gene expression in *Bradyrhizobium japonicum*. *Molecular genetics and genomics* **214**, 420-424.

Becker, A. & Puhler, A. (1998). Production of exopolysaccharides. in The Rhizobiaceae: molecular biology of model plant-associated bacteria, eds Spaink H. P., Kondorosi A., Hooykaas P. J. J. (Kluwer Academic Publishers, Dordrecht, The Netherlands),. pp 97–118.

Berg, H. C. (2005). Swarming Motility: It Better Be Wet. *Current Biology* **15**, R599-R600.

Beringer, J. E. (1974). R factor transfer in *Rhizobium leguminosarum*. *Journal of General Microbiology* **84**, I 88-I 98.

Bever, J. D. & Simms, E. L. (2000). Evolution of nitrogen fixation in spatially structured populations of *Rhizobium*. *Heredity (Edinb)* **85**, 366-372.

Billi, D. and Potts, M. (2002). Life and death of dried prokaryotes. *Research in Microbiology* **153**, 7-12.

Blattner, F. R., Plunkett, G., Bloch, C. A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K., Mayhew, G. F., Gregor, J., Davis, N. W., Kirkpatrick, H. A., Goeden, M. A., Rose, D. J., Mau, B. & Shao, Y. (1997). The complete genome sequence of *Escherichia coli* K-12. *Science* **277**, 1453-1462.

Borthakur, D., Barker, R. F., Latchford, J. W., Rossen, L. & Johnston, A. W. B. (1988). Analysis of *pss* genes of *Rhizobium leguminosarum* required for exopolysaccharide synthesis and nodulation of peas: their primary structure and their interaction with psi and other nodulation genes. In *Molecular and General Genetics* 155-162: Springer Berlin / Heidelberg.

Braeken, K., Daniels, R., Vos, K., Fauvart, M., Bachaspatimayum, D., Vanderleyden, J. & Michiels, J. (2008). Genetic determinants of swarming in *Rhizobium etli*. *Microbial Ecology* **55**, 54-64.

Brencic, A. & Winans, S. C. (2005). Detection of and response to signals involved in host-microbe interactions by Plant-associated bacteria. *Microbiology and Molecular Biology Reviews* **69**, 155-194.

Brockwell, J., Bottomley, P. J. & Thies, J. E. (1995). Manipulation of rhizobia microflora for improving legume productivity and soil fertility: A critical assessment. In *Plant and Soil*, pp. 143-180: Springer Netherlands.

Broughton, W. J., Jabbouri, S. d. & Perret, X. (2000). Keys to Symbiotic Harmony. *Journal of Bacteriology* **182**, 5641-5652.

Burkart, M., Toguchi, A. & Harshey, R. M. (1998). The chemotaxis system, but not chemotaxis, is essential for swarming motility in *Escherichia coli*. *Proceedings of the National Academy of Sciences* **95**, 2568-2573.

Bushby, H. V. A. & Marshall, K. C. (1977). Water status of rhizobia in relation to their susceptibility to desiccation and to their protection by montmorillonite. *Journal of General Microbiology* **99**, 19-27.

Cardenas, L., Dominguez, J., Santana, O. & Quinto, C. (1996). The role of the nodI and nodJ genes in the transport of Nod metabolites in *Rhizobium etli*. *Gene* **173**, 183-187.

Carter, J. M., Tieman, J. S. & Gibson, A. H. (1995). Competitiveness and persistence of strains of rhizobia for faba bean in acid and alkaline soils. *Soil Biology and Biochemistry* **27**, 617-623.

Chakraburtty, K. (2001). Translational regulation by ABC systems. *Research in Microbiology.* **15**, 391-399.

Chen, W. M., Moulin, L. & Bontemps, C. (2003). Legume symbiotic nitrogen fixation by α-Proteobacteria is widespread in nature. *Journal of Bacteriology* **185**, 7266–7272.

Chen, B. G., Turner, L. & Berg, H. C. (2007). The wetting agent required for swarming in *Salmonella enterica* Serovar Typhimurium Is Not a Surfactant. *Journal of Bacteriology* **189**, 8750-8753.

Cheng, J., Guffanti, A. A. & Krulwich, T. A. (1997). A two-gene ABC-type transport system that extrudes Na+ in *Bacillus subtilis* is induced by ethanol or protonophore. *Molecular Microbiology* **23**, 1107-1120.

Chenu, C. & Roberson, E. B. (1996). Diffusion of glucose in microbial extracellular polysaccharide as affected by water potential. *Soil Biology & Biochemistry* **28**, 877-884.

Copeland, M. F. & Weibel, D. B. (2009). Bacterial swarming: a model system for studying dynamic self-assembly. *Soft Matter* **5**, 1174–1187.

Crespi, B. J. (2001). The evolution of social behavior in microorganisms. *Trends in Ecology & Evolution* **16**, 178-183.

Crozat, Y., Cleyet-Marel, J. C., Giraud, J. J. & Obaton, M. (1982). Survival rates of *Rhizobium japonicum* populations introduced into different soils. *Soil Biology and Biochemistry* **14**, 401-405.

Cuthbertson , L., Kos, V. & Whitfield, C. (2010). ABC transporters involved in export of cell surface glycoconjugates. *Microbiology and Molecular Biology Reviews* **74**, 341-362.

Daniels, R., Vanderleyden, J. & Michiels, J. (2004). Quorum sensing and swarming migration in bacteria. *FEMS Microbiology Reviews* **28**, 261-289.

Dassa, E. & Bouige, P. (2001). The ABC of ABCs: a phylogenetic and functional classification of ABC systems in living organisms. *Research in Microbiology* **152**, 211-229.

Davidson, A. L., Dassa, E., Orelle, C. & Chen, J. (2008). Structure, Function, and Evolution of Bacterial ATP-Binding Cassette Systems. *Microbiology and Molecular Biology Reviews* **72**, 317-364.

de Bruijn, F. J., Rossbach, S., Schneider, M., Ratet, P., Messmer, S., Szeto, W. W., Ausubel, F. M. & Schell, J. (1989). *Rhizobium meliloti* 1021 has three

differentially regulated loci involved in glutamine biosynthesis, none of which is essential for symbiotic nitrogen fixation. *Journal of Bacteriology* **171**, 1673-1682.

de Lajudie, P., Willems, A., Pot, B., Dewettinck, D., Maestrojuan, G., Neyra, M., Collins, M. D., Dreyfus, B., Kersters, K. & Gillis, M. (1994). Polyphasic taxonomy of rhizobia: emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. *International Journal of Systematic Bacteriology* **44**, 715-733.

Deaker, R., Roughley, R. J. & Kennedy, I. R. (2004). Legume seed inoculation technology: a review. *Soil Biology and Biochemistry Nitrogen Fixation in Australian Agricultural Systems: 13th Australian Nitrogen Fixation Conference* **36**, 1275-1288.

Debellé, F., Moulin, L., Mangin, B., Dénarié, J. & Boivin, C. (2001). Nod genes and Nod signals and the evolution of the *Rhizobium* legume symbiosis. *Acta Biochimica Polonica* **48**, 359-365.

Deinema, M. H. & Zevenhuizen., L. P. T. M. (1971). Formation of cellulose fibrils by Gram negative bacteria and their role in bacterial flocculation. *Arch Mikrobiol* **78**.

Denarie, J. & Cullimore, J. (1993). Lipo-oligosaccharide nodulation factors: A new class of signaling molecules mediating recognition and morphogenesis. *Cell* **74**, 951-954.

Denison, R. F. T. K., E. (2000). Legume sanctions and the evolution of symbiotic cooperation by rhizobia. *American Naturalist* **156**, 567–576.

Denison, R. F. & Toby Kiers, E. (2004). Why are most rhizobia beneficial to their plant hosts, rather than parasitic? *Microbes and Infection* **6**, 1235-1239.

D'Haeze, W. & Holsters, M. (2002). Nod factor structures, responses, and perception during initiation of nodule development. *Glycobiology* **12**, 79R - 105R.

Ding, H., Yip, C. B., Geddes, B. A., Oresnik, I. J. & Hynes, M. F. (2012). Glycerol utilization by *Rhizobium leguminosarum* requires an ABC transporter and affects competition for nodulation16. *Microbiology* **158**, 1369-1378.

Dong, J., Yang, G. & Mchaourab, H. S. (2005). Structural basis of energy transduction in the transport cycle of MsbA. *Science* **308**, 1023-1028.

Drinkwater, L. E., Wagoner, P. & Sarrantonio, M. (1998). Legume-based cropping systems have reduced carbon and nitrogen losses. *Nature* **396**, 262-265.

Fauvart, M., Phillips, P., Bachaspatimayum, D., Verstraeten, N., Fransaer, J., Michielsa, J. & Vermant, J. (2012). Surface tension gradient control of bacterial swarming in colonies of *Pseudomonas aeruginosa*. *Soft Matter* **8**, 70.

Fisher, R. F. & Long, S. R. (1992). *Rhizobium*-plant signal exchange. *Nature* **357**, 655-660.

Fox, M. A. (2005). Adaptation of *Rhizobium* to environmental stress. PhD Thesis. *University of Reading*.

Frank, A. B. (1890). *Uber die Pilzsymbiose der Leguminosen*: P. Parey.

Fraser, G. M. & Hughes, C. (1999). Swarming motility. *Current Opinion in Microbiology* **2**, 630-635.

Gabius, H. J. (2006). Cell surface glycans: the why and how of their functionality as biochemical signals in lectin-mediated information transfer. *Critical Reviews in Immunology.* **26**, 43-79.

Gage, D. J. (2004). Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiology and Molecular Biology Reviews* **68**, 280-300.

Galibert, F., Finan, T. M., Long, S. R., PÃuhler, A., Abola, P., Ampe, F. D. R., Barloy-Hubler, F. D. R., Barnett, M. J., Becker, A., Boistard, P., Bothe, G., Boutry, M., Bowser, L., Buhrmester, J., Cadieu, E., Capela, D., Chain, P., Cowie, A., Davis, R. W., Dreano, S. P., Federspiel, N. A., Fisher, R. F., Gloux, S. P., Godrie, T. R. S., Goffeau, A., Golding, B., Gouzy, J. R. m., Gurjal, M., Hernandez-Lucas, I., Hong, A., Huizar, L., Hyman, R. W., Jones, T., Kahn, D., Kahn, M. L., Kalman, S., Keating, D. H., Kiss, E., Komp, C., Lelaure, V. R., Masuy, D., Palm, C., Peck, M. C., Pohl, T. M., Portetelle, D., Purnelle, B. N. D., Ramsperger, U., Surzycki, R., Thebault, P., Vandenbol, M., Vorholter, F.-J., Weidner, S., Wells, D. H., Wong, K., Yeh, K.-C. & Batut, J. (2001). The composite genome of the legume symbiont *Sinorhizobium meliloti*. *Science* **293**, 668-672.

Glenn, A. R., Poole, P. S. & Hudman, J. F. (1980). Succinate uptake by free-living and bacteroid forms of *Rhizobium leguminosarum*. *Journal of General Microbiology* **119**, 267-271.

Goosen, N. & Moolenaar, G. F. (2001). Role of ATP hydrolysis by UvrA and UvrB during nucleotide excision repair. *Research in Microbiology.* **15**, 2401-2409.

Gruber, N. & Galloway, J. N. (2008). An Earth-system perspective of the global nitrogen cycle. *Nature* **451**, 293-296.

Guerreiro, N., Ksenzenko, V. N., Djordjevic, M. A., Ivashina, T. V. & Rolfe, B. G. (2000). Elevated levels of synthesis of over 20 proteins results after mutation of the *Rhizobium leguminosarum* exopolysaccharide synthesis gene pssA. *Journal of Bacteriology* **182**, 4521-4532.

Harshey, R. M. (2003). Bacterial motility on a surface: many ways to a common goal. *Annual Review of Microbiology.* **57**, 249-273.

Hartel, P. G. & Alexander, M. (1986). Role of extracellular polysaccharide production and clays in the desiccation tolerance of cowpea *Bradyrhizobia*. *Soil*

Science Society of America Journal **50**, 1193–1198.

Henrichsen, J. (1972). Bacterial surface translocation: a survey and a classification. *Bacteriological Reviews* **36** 478-503

Higgins, C. F. (1992). ABC Transporters: From Microorganisms to Man. *Annual Review of Cell Biology* **8**, 67-113.

Hirsch, A. (1999). Role of lectins (and rhizobial exopolysaccharides) in legume nodulation. *Current Opinion in Plant Biology.* **2**, 320-326.

Hoagland, D. & Arno, n. D. (1938). The water culture method for growing plants without soil. *Bulletin of California Agricultural State* **346**.

Holland, I. B. & A. Blight, M. (1999). ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans. *Journal of Molecular Biology* **293**, 381-399.

Horvath, B., Bachem, C. W. B., Schell, I. & Kondorosi, A. (1987). Host-specific regulation of nodulation genes in *Rhizobium* is mediated by a plant signal, interacting with the nodD gene product. *European Molecular Biology Organization Journal* **6**:841-848.

Hosie, A. H. F., Allaway, D. & Poole, P. S. (2002). A monocarboxylate permease of *Rhizobium leguminosarum* is the first member of a new subfamily of transporters. *Journal of Bacteriology* **184**, 5436-5448.

Hosie, A. H. F., Allaway, D., Galloway, C. S., Dunsby, H. A. & Poole, P. S. (2002). *Rhizobium leguminosarum* has a second general amino acid permease with unusually broad substrate specificity and high similarity to branched-chain amino acid transporters (Bra/LIV) of the ABC family. *Journal of Bacteriology* **184**, 4071-4080.

Ibanez, F. & Fabra, A. (2011). Rhizobial Nod factors are required for cortical cell division in the nodule morphogenetic programme of the Aeschynomeneae legume Arachis. *Plant Biology* **13**, 794-800.

Issa, S., Wood, M. & Simmonds, L. P. (1993). Active movement of chickpea and bean rhizobia in dry soil. *Soil Biology and Biochemistry* **25**, 951-958.

Issa, S., Simmonds, L. P. & Wood, M. (1993). Passive movement of chickpea and bean rhizobia through soils. *Soil Biology and Biochemistry* **25**, 959-965.

Ivashina, T. V., Khmelnitsky, M. I., Shiyapnikov, M. G., Kanapin, A. A. & Ksenzenko, V. N. (1994). The *pss4* gene from *Rhizobium leguminosarum* bv *viciae* VF39: cloning, sequence and the possible role in polysaccharide production and nodule formation. *Gene* **150**, 111-116.

Janczarek, M. & Skorupska, A. (2001). The *Rhizobium leguminosarum* bv. *trifolii pssB* gene product is an inositol monophosphatase that influences exopolysaccharide synthesis. In *Archives of Microbiology*, pp. 143-151: Springer Berlin / Heidelberg.

Janczarek, M. & Skorupska, A. (2004). Regulation of *pssA* and *pssB* gene expression in *Rhizobium leguminosarum* bv. *trifolii* in response to environmental factors. In *Antonie van Leeuwenhoek*, pp. 217-227: Springer Netherlands.

Janczarek, M. & Skorupska, A. (2009). *Rhizobium leguminosarum* bv. *trifolii* rosR gene expression is regulated by catabolic repression. *FEMS Microbiology Letters* **291**, 112-119.

Janczarek, M., Jaroszuk-Scisel J. & Skorupska, A. (2009). Multiple copies of *rosR* and *pssA* genes enhance exopolysaccharide production, symbiotic competitiveness and clover nodulation in *Rhizobium leguminosarum* bv. *trifolii*. In *Antonie van Leeuwenhoek*, pp. 471-486: Springer Netherlands.

Jarvis, B. D. W., MacLean, T. S., Robertson, I. G. C. & Fanning, G. R. (1977). Phenetic similarity and DNA base sequence homology of root nodule bacteria from New Zealand native legumes and *Rhizobium* strains from agricultural plants. *New Zealand Journal of Agricultural Research* **20**, 235-248.

Jefferson, R. A., Burgess, S. M. & Hirsh, D. (1986). Betaglucuronidase from *Escherichia coli* as a gene-fusion marker. *Proceedings of the National Academy of Sciences* **83**, 8447–8451.

Jensen, H. (1961). Survival of *Rhizobium meliloti* in soil culture. *Nature* **192**, 682-683.

Jones PM, George AM (2004). "The ABC transporter structure and mechanism: perspectives on recent research". *Cell Mol Life Sci.* **61** (6): 682–99

Johnston, A. W. B. & Beringer, J. E. (1975). Identification of the *Rhizobium* strains in pea root nodules using genetic markers. *Journal of General Microbiology* **87**, 343-350.

Jones, P. M. & George, A. M. (2004). The ABC transporter structure and mechanism: perspectives on recent research. In *Cellular and Molecular Life Sciences*, pp. 682-699:

Jones, K. M., Kobayashi, H., Davies, B. W., Taga, M. E. & Walker, G. C. (2007). How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. **5**, 619-633.

Julkowska, D., Obuchowski, M., Holland, B. & Se´ror, S. J. (2004). Branched swarming patterns on a synthetic medium formed by wild-type *Bacillus subtilis* strain 3610: Detection of different cellular morphologies and constellations of cells as the complex architecture develops. *Microbiology* **150**, 1839–1849.

Jumpertz et al. (2009). ABC Transporters: A smart example of molecular machineries. in: abc transporters in microorganisms. *Ponte-Sucre, A (Ed.). Caister Academic Press, Norfolk, UK*.

Kaneko, T., Nakamura, Y., Sato, S., Minamisawa, K., Uchiumi, T., Sasamoto, S., Watanabe, A., Idesawa, K., Iriguchi, M., Kawashima, K., Kohara, M., Matsumoto, M., Shimpo, S., Tsuruoka, H., Wada, T., Yamada, M. & Tabata, S. (2002). Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110 (Supplement). *DNA Research* **9**, 225-256.

Kaneko, T., Nakamura, Y., Sato, S., Asamizu, E., Kato, T., Sasamoto, S., Watanabe, A., Idesawa, K., Ishikawa, A., Kawashima, K., Kimura, T., Kishida, Y., Kiyokawa, C., Kohara, M., Matsumoto, M., Matsuno, A., Mochizuki, Y., Nakayama, S., Nakazaki, N., Shimpo, S., Sugimoto, M., Takeuchi, C., Yamada, M. & Tabata, S. (2000). complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. *DNA Research* **7**, 331-338.

Kaur, P., Rao, D. K. & Gandlur, S. M. (2005). Biochemical characterization of domains in the membrane subunit DrrB that interact with the ABC subunit DrrA: identification of a conserved motif. *Biochemistry* **44**, 2661-2670.

Kent, A. D. & Triplett, E. W. (2002). Microbial communities and their interactions in soil and rhizosphere ecosystems. *Annual Review of Microbiology* **56**, 211-236.

Kiers, E. T., Rousseau, R. A., West, S. A. & Denison, R. F. (2003). Host sanctions and the legume-*rhizobium* mutualism. **425**, 78-81.

Krishnan, H. B., Lorio, J., Kim, W. S., Jiang, G., Kim, K. Y., DeBoer, M. & Pueppke, S. G. (2003). Extracellular proteins involved in soybean cultivar-specific nodulation are associated with pilus-like surface appendages and exported by a type iii protein secretion system in *Sinorhizobium fredii* USDA257. *Molecular Plant-Microbe Interactions* **16**, 617-625.

Kristien, B., Ruth, D., Karen, V., Maarten, F., Debkumari, B., Jos, V. & Jan, M. (2008). Genetic determinants of swarming in *Rhizobium etli*. *Microbial Ecology* **55**, 54–64.

Król, J., Mazur, A., Marczak, M. & Skorupska, A. (2007). Syntenic rearrangements of the surface polysaccharide biosynthesis genes in *Rhizobium leguminosarum*. *Genomics* **89**, 237-247.

Krulwich, T. A., Ito, M. & Guffanti, A. A. (2001). The Na+-dependence of alkaliphily in *Bacillus*. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1505**, 158-168.

Kwon, S.-W., Park, J.-Y., Kim, J.-S., Kang, J.-W., Cho, Y.-H., Lim, C.-K., Parker, M. A. & Lee, G.-B. (2005). Phylogenetic analysis of the genera *Bradyrhizobium, Mesorhizobium, Rhizobium* and *Sinorhizobium* on the basis of 16S rRNA gene and internally transcribed spacer region sequences. *International Journal of Systematic and Evolutionary Microbiology* **55** 263-270

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, E., Wallace, I. M. & Wilm, A. o. a. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947-2948.

Laus, M. C., van Brussel, A. A. N. & Kijne, J. W. (2005). Role of cellulose fibrils and exopolysaccharides of *Rhizobium leguminosarum* in attachment to and infection of vicia sativa root hairs. *Molecular Plant-Microbe Interactions*, **18**, 533-538.

Lee, A. & Hirsch, A. M. (2006). Signals and *Responses: Choreographing the Complex Interaction between Legumes and Alpha- and Beta-Rhizobia*. *Plant Signal Behav* **1**, 161-168.

Leigh, A. J. & Coplin, D. L. (1992). Exopolysaccharides in plant-bacterial interactions. *Annual Review of Microbiology* **46**, 307-346.

Leigh, J. A. & Walker, G. C. (1994). Exopolysaccharides of *Rhizobium*: synthesis, regulation and symbiotic function. *Trends in Genetics* **10**, 63-67.

Limpens, E., Franken, C., Smit, P., Willemse, J., Bisseling, T. & Geurts, R. (2003). LysM domain receptor kinases regulating rhizobial nod factor-induced infection. *Science* **302**, 630-633.

Lindstrom M. K. (1989). *Rhizobium galegae*, a new species of legume root nodule bacteria. *International Journal of Systematic Bacteriology* **39**, 365-367.

Lindstrom, M. K., Van Berkum, P., Gillis, M., Mz, E., Novikova, N. & Jarvis, B. (1995). Report from the roundtable on *Rhizobium* taxonomy. In nitrogen fixation : fundamentals and applications, edited by LA. Tikhonovich, N. A. Provorov, V. I. Romanov & W. E. Newton. Dordrecht, The Netherlands : Kluwer., 807-810.

Lis, H. & Sharon, N. (1998). Lectins: carbohydrate-specific proteins that mediate cellular recognition. *Chemistry Review* **98**, 637-674.

Lodwig, E. & Poole, P. (2003). Metabolism of *Rhizobium* Bacteroids. *Critical Reviews in Plant Sciences* **22**, 37-78.

Loh, J. T., Yuen-Tsai, J. P.-Y., Stacey, M. G., Lohar, D., Welborn, A. & Stacey, G. (2001). Population density-dependent regulation of the *Bradyrhizobium japonicum* nodulation genes. *Molecular Microbiology* **42**, 37-46.

Malgorzata, M., Andrzej, M., Jaroslaw, E. K., Wieslaw, I. G. & Anna, S. (2006). Lipoprotein *PssN* of *Rhizobium leguminosarum* bv. *trifolii*: subcellular localization and possible involvement in exopolysaccharide export. *Journal of Bacteriology.* **188**, 6943–6952.

Marchler-Bauer, A., Anderson, J. B., Chitsaz, F., Derbyshire, M. K., DeWeese-Scott, C., Fong, J. H., Geer, L. Y., Geer, R. C., Gonzales, N. R., Gwadz, M., He, S., Hurwitz, D. I., Jackson, J. D., Ke, Z., Lanczycki, C. J., Liebert, C. A., Liu, C.,

Lu, F., Lu, S., Marchler, G. H., Mullokandov, M., Song, J. S., Tasneem, A., Thanki, N., Yamashita, R. A., Zhang, D., Zhang, N. & Bryant, S. H. (2009). CDD: specific functional annotation with the Conserved Domain Database. *Nucleic Acids Research* **37**, D205-D210.

Marczak, M., Mazur, A., Król, J. E., Gruszecki, W. I. & Skorupska, A. (2006). Lipoprotein *PssN* of *Rhizobium leguminosarum* bv. *trifolii*: Subcellular localization and possible involvment in exopolysaccharide export. *Journal Bacteriology.* **188**, 6943-6952.

Marie, C., Deakin, W. J., Viprey, V., KopciÃ±ska, J., Golinowski, W., Krishnan, H. B., Perret, X. & Broughton, W. J. (2003). Characterization of Nops, nodulation outer proteins, secreted via the type iii secretion system of NGR234. *Molecular Plant-Microbe Interactions*
MPMI **16**, 743-751.

Mathesius, U., Weinman, J. J., Rolfe, B. G. & Djordjevic, M. A. (2000). Rhizobia can induce nodules in white clover by "hijacking" mature cortical cells activated during lateral root development. *Molecular Plant-Microbe Interactions***, 13, 170-182.**

Matilla, M., Espinosa-Urgel, M., Rodriguez-Herva, J., Ramos, J. & Ramos-Gonzalez, M. (2007). Genomic analysis reveals the major driving forces of bacterial life in the rhizosphere. *Genome Biology* **8**, R179.

Matsuyama, T., Tarshey, R. M. & Matsushita, M. (1993). Self-similar colony morphogenesis by bacteria as the experimental model of fractal growth by a cell population. *Fractals* **01**, 302-311.

Mauchline, T. H., Fowler, J. E., East, A. K., Sartor, A. L., Zaheer, R., Hosie, A. H. F., Poole, P. S. & Finan, T. M. (2006). Mapping the *Sinorhizobium meliloti* 1021 solute-binding protein-dependent transportome. *Proceedings of the National Academy of Sciences* **103**, 17933-17938.

Mazur, A., Krol, J. E. & Skorupska, A. (2001). Isolation and sequencing of *Rhizobium leguminosarum* bv. t*rifolii PssN*, *PssO* and *PssP* genes encoding the proteins involved in polymerization and translocation of exopolysaccharide. *Mitochondrial DNA* **12**, 1-12.

McGraw, N. J., Bailey, J. N., Cleaves, G. R., Dembinski, D. R., Gocke, C. R., Joliffe, L. K., MacWright, R. S. & McAllister, W. T. (1985). Sequence and analysis of the gene for bacteriophage T3 RNA polymerase. *Nuclic Acids Researcg* **13**, 6753-6766.

McIver, J., Djordjevic, M. A., Weinman, J. J., Bender, G. L. & Rolfe , B. G. (1989). Extension o f host range o f *Rhizobium leguminosarum* bv. *trifolii* caused by point mutations in nodD that result in alterations in regulatory function and recognition of inducer molecules. *Molecular Plant-Microbe Interactions* **2**, 97-91 06.

Mergaert, P., Uchiumi, T., Alunni, B. t., Evanno, G. n. l., Cheron, A. l., Catrice, O., Mausset, A.-E., Barloy-Hubler, F. d. r., Galibert, F., Kondorosi, A. & Kondorosi, E. (2006). Eukaryotic control on bacterial cell cycle and differentiation in the *Rhizobium* legume symbiosis. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 5230-5235.

Miller, L. D., Yost, C. K., Hynes, M. F. & Alexandre, G. (2007). The major chemotaxis gene cluster of *Rhizobium leguminosarum* bv. *viciae* is essential for competitive nodulation. *Molecular Microbiology* **63**, 348-362.

Moënne-Loccoz Y., S. D., Krause S. E., Weaver R. W (1994). Plasmid profiles of rhizobia used in inoculants and isolated from clover fields. *Agronomy Journal* **86**, 117-121.

Moulin, L., Munive, A., Dreyfus, B. & Boivin-Masson, C. (2001). Nodulation of legumes by members of the β-subclass of Proteobacteria. *Nature* **411**, 948-950.

Mutch, L. A. & Young, J. P. W. (2004). Diversity and specificity of *Rhizobium leguminosarum* biovar *viciae* on wild and cultivated legumes. *Molecular Ecology* **13**, 2435-2444.

Nakata, K., Harada, N., Sumitomo, K. & Yoneda, K. (2000). Enhancement of plant stem growth by flocculation of the antibiotic-producing bacterium, *Pseudomonas fluorescens* S272, on the roots. *Bioscience, Biotechnology, and Biochemistry* **64**, 459-465.

Napoli, C., Dazzo, F. & Hubbell, D. (1975). Production of cellulose microfibrils in *Rhizobium*. *Applied Microbiol*ogy **30**, 123-131.

Nehl, D. B., Allen, S. J. & Brown, J. F. (1997). Deleterious rhizosphere bacteria: An integrating perspective. *Applied Soil Ecology* **5**, 1-20.

Neuwald, A., Aravind, L., Spouge, J. & Koonin, E. (1999). AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Research* **9**, 27-43.

Neves, M. C. P. & Rumjanek, N. G. (1997). Diversity and adaptability of soybean and cowpea rhizobia in tropical soils. *Soil Biology and Biochemistry International Symposium - Sustainable Agriculture for the Tropics: The Role of Biological Nitrogen Fixation* **29**, 889-895.

Neyra, C., Atkinson, L., Olubayi, O., Sadasivan, D. & Zappi, E. (1999). Noval microbial technologies for the enhancement of plant growth and biocontrol of fungal diseases in crops. *Cahiers Options Mediterraneennes* **31**, 447-456.

O'Gara, F. & Shanmugam, K. T. (1976). Regulation of nitrogen fixation by Rhizobia export of fixed N2 as NH4+. *Biochimica et Biophysica Acta (BBA) - General Subjects* **437**, 313-321.

Oldroyd, G. E. D. & Downie, J. A. (2008). Coordinating nodule morphogenesis with rhizobial infection in legumes. *Annual Review of Plant Biology* **59**, 519-546.

Olivieri, I. & Frank, S. A. (1994). The evolution of nodulation in *Rhizobium*: altruism in the rhizosphere. *Journal of Heredity* **85** 46-47

Oono, R. & Denison, R. F. (2010). Comparing symbiotic efficiency between swollen versus nonswollen rhizobial bacteroids. *Plant Physiology* **154**, 1541-1548.

Ophir, T. & Gutnick, D. L. (1994). A role for exopolysaccharides in the protection of microorganisms from desiccation. *Applied and Environmental Microbiology* **60** 740-745

O'Toole, G. A. & Kolter, R. (1998). Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Molecular Microbiology* **30**, 295-304.

Overhage, J., Lewenza, S., Marr, A. K. & Hancock, R. E. (2007). Identification of genes involved in swarming motility using a *Pseudomonas aeruginosa* PAO1 mini-Tn5-lux mutant library. *Journal of Bacteriology* **189**, 2164–2169.

Overhage, J., Bains, M., Brazas, M. D. & Hancock, R. E. W. (2008). Swarming of *Pseudomonas aeruginosa* is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. *Journal of Bacteriology* **190**, 2671-2679.

Paulsen, I. T., Beness, A. M. & Saier, M. H. (1997). Computer-based analyses of the protein constituents of transport systems catalysing export of complex carbohydrates in bacteria. *Microbiology* **143**, 2685-2699.

Peck, M. C., Fisher, R. F. & Long, S. R. (2006). Diverse flavonoids stimulate NodD1 binding to nod gene promoters in *Sinorhizobium meliloti*. *Journal Bacteriology* **188**, 5417–5427.

Peter, V. M., K. Cassman, C. Cleveland, T. Crews, B.F. Christopher, B.N. Grimm, W.R. Howarth, R. Marinov, L. Martinelli, B. Rastetter and I. J. Sprent. (2002). Towards an ecological understanding of biological nitrogen fixation. Biogeochemistry,
57: 1-45.

Polgar, O. & SE., B. (2005). ABC transporters in the balance: is there a role in multidrug resistance? *Biochem Soc. Trans* **33**, 241-245.

Pollock, T. J., van Workum, W., Thorne, L., Mikolajczak, M. J., Yamazaki, M., Kijne, J. W. & Armentrout, R. W. (1988). Assignment of biochemical functions to glycosyl transferase genes which are essential for biosynthesis of exopolysaccharides in Sphingomonas strain S88 and *Rhizobium leguminosarum*. *Journal of Bacteriology* **180**, 586-593.

Postma, J., Hok-A-Hin, C. H. & Oude Voshaar, J. H. (1990). Influence of the inoculum density on the growth and survival of *Rhizobium leguminosarum* biovar *trifolii* introduced into sterile and non-sterile loamy sand and silt loam. *FEMS Microbiology Letters* **73**, 49-57.

Potts, R. (1998). Environmental hypotheses of hominin evolution. *American Journal of Physical Anthropology* **Suppl 27**, 93-136.

Potvin, E., Lehoux, D. E., Kukavica-Ibrulj, I., Richard, K. L., Sanschagrin, F. o., Lau, G. W. & Levesque, R. C. (2003). In vivo functional genomics of *Pseudomonas aeruginosa* for high-throughput screening of new virulence factors and antibacterial targets. *Environmental Microbiology* **5**, 1294-1308.

Pugsley, A. P. (1993). The complete general secretory pathway in gram-negative bacteria. . *Microbiological Reviews* **57** 50-108

Pulawska, J., Maes, M., WILLEMS, A. & Sobiczewski, P. (2000). Phylogenetic analysis of 23S rRNA gene sequences of *Agrobacterium, Rhizobium* and *Sinorhizobium* strains. *Systematic and Applied Microbiology* **23**, 238-244.

Rauprich, O., Matsushita, M., Weijer, C. J., Siegert, F., Esipov, S. E. & Shapiro, J. A. (1996). Periodic phenomena in *Proteus mirabilis* swarm colony development. *Journal of Bacteriology* **178**, 6525–6538.

Reizer, J., Reizer, A. & Saier, J. R. (1992). A new subfamily of bacterial ABC-type transport systems catalyzing export of drugs and carbohydrates. *Protein Science* **1**, 1326–1332.

Ren, Q. & Paulsen, I. T. (2005). Comparative analyses of fundamental differences in membrane transport capabilities in prokaryotes and eukaryotes. *PLOS Computational Biology*

1(3), e27.

Rinaudi, L. V. & Giordano, W. (2010). An integrated view of biofilm formation in rhizobia. *FEMS Microbiology Letters* **304**, 1-11.

Roberson, E. B. & Firestone, M. K. (1992). Relationship between desiccation and exopolysaccharide production in a soil *Pseudomonas* sp. *Applied and Environmental Microbiology* **58** 1284-1291

Rokitko PV, Romanovskaya VA, Malashenko YR, et al. (2003) Soil drying as a model for the action of stress factors on natural bacterial populations. Microbiology **72**:756–761.

Rolfe, B., Carlson, R., Ridge, R., Dazzo, F., Mateos, P. & Pankhurst, C. (1996/01/01). Defective Infection and nodulation of clovers by exopolysaccharide mutants of *Rhizobium leguminosarum* bv. *trifolii*. *Functional Plant Biology* **23**, 285-303.

Rudiger, H. & Gabius, H. J. (2001). Plant lectins: occurrence, biochemistry, functions and applications. *Glycoconjugate Journal* **18**, 589–613.

Ryan, P., Delhaize, E. & Jones, D. (2001). Function and mechanism of organic anion exudation from plant roots. *Annual Review of Plant Physiology and Plant Molecular Biology* **52**, 527-560.

Sadykov, M., Ivashina, T., Kanapin, A., Shliapnikov, M. & Ksenzenko, V. (1998). Structure-functional organization of exopolysaccharide biosynthetic genes in *Rhizobium leguminosarum* bv. *viciae* VF39. *Mololecular Biology (Mosk).* **32**, 797-804.

Saha, B., Choudhuri, S., Bhakta, R. B., Joyoti Basu, Manikuntala Kundu & Chakrabarti, P. (2002). Overexpression and functional characterization of an ABC (ATP-binding cassette) transporter encoded by the genes drrA and drrB of *Mycobacterium tuberculosis*. *Biochemistry Journal* **367**, 279-285.

Saurin, W., Hofnung, M. & Dassa, E. (1999). Getting in or out: early segregation between importers and exporters in the evolution of ATP-binding cassette (ABC) transporters. In *Journal of Molecular Evolution*, pp. 22-41: Springer New York.

Sawada, H., Oyaizu, H. & Matsumoto, S. (1993). Proposal for rejection of *Agrobacterium tumefaciens* and revised descriptions for the genus *Agrobacterium* and for *Agrobacterium radiobacter* and *Agrobacterium rhizogenes*. *International Journal of SystamaticBacteriology* **43**, 694-702.

Schatz, P. J. & Beckwith, J. (1990). Genetic analysis of protein export in *Escherichia Coli*. *Annual Review of Genetics* **24**, 215-248.

Schultze, M., Kondorosi, E., Ratet, P., Buire, M. & Kondorosi, A. (1994). Cell and molecular biology of *Rhizobium*-plant. International review of cytology. pp. 1-75. Edited by K. W. J. a. J. Jarvik: Academic Press.

Schwiebert, E. M. (1999). ABC transporter-facilitated ATP conductive transport *American Journal of Physiology - Cell Physiology* **276** C1-C8

Sharma, M. & Anand, S. (2002). Swarming: a coordinated bacterial activity. *Current Science* **83**, 707-715.

Simms, E. L. & Bever, J. D. (1998). Evolutionary dynamics of rhizopine within spatially structured *Rhizobium* populations. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **265**, 1713-1719.

Smit, G., Kijne, J. W. & Lugtenberg, B. J. J. (1987). Involvement of both cellulose fibrils and a Ca2+-dependent adhesin in the attachment of *Rhizobium leguminosarum* to pea root hair tips. *Journal of Bacteriology* **169**.

Spaink, H. P. (1994). The molecular basis of the host specificity of the *Rhizobium* bacteria. In *Antonie van Leeuwenhoek*, pp 81-98: Springer Netherlands.

Spaink, H. P. (1995). The molecular basis of infection and nodulation by rhizobia: The ins and outs of sympathogenesis. *Annual Review of Phytopathology* **33**, 345-368.

Spaink, H. P., Kondorosi, A. & Hooykaas, P. J. J. (1998). (eds). The rhizobiaceae: molecular biology of model plant-associated bacteria. Dordrecht, Boston: Kluwer Academic.

Spehn, E. M., Scherer-Lorenzen, M., Schmid, B., Hector, A., Caldeira, M. C., Dimitrakopoulos, P. G., Finn, J. A., Jumpponen, A., O'Donnovan, G., Pereira, J. S., Schulze, E.-D., Troumbis, A. Y. & Korner, C. (2002). The role of legumes as a component of biodiversity in a cross-European study of grassland biomass nitrogen. *Oikos* **98**, 205-218.

Steve, R. & Helen, J. S. (2000). Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ,. 365-386.

Stintzi, A. & Browse, J. (2000). The Arabidopsis male-sterile mutant, opr3, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. *Proceedings of the National Academy of Sciences* **97**, 10625-10630.

Stotz, H. U., Pittendrigh, B. R., Kroymann, J. r., Weniger, K., Fritsche, J., Bauke, A. & Mitchell-Olds, T. (2000). induced plant defense responses against chewing insects. ethylene signaling reduces resistance of arabidopsis against egyptian cotton worm but not Diamondback Moth. *Plant Physiology* **124**, 1007-1018.

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S. L., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K.-S., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E. W., Lory, S. & Olson, M. V. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. **406**, 959-964.

Sugiyama, A., Shitan, N. & Yazaki, K. (2007). Inolvement of a soybean ATP-binding cassette-type transporter in the secretion of genistein, a signal flavonoid in legume-*Rhizobium* symbiosis. *Plant Physiology* **144**, 2000–2008.

Sule S, Cursino L, Zheng D, HC, H. & TJ, B. (2009). Surface motility and associated surfactant production in *Agrobacterium vitis*. *Letters in Applied Microbiology* **49**, 596–601.

Swaraj, K. & Bishnoi, N. (1996). Physiological and biochemical basis of nodule senescence in legumes: a review. *Plant Physiology and Biochemistry* **23**, 105-116.

Takashi, O. (1988). Competitive modulation ability of *Bradyrhizobium japonicum* strains incubated in soil. *Soil Biology and Biochemistry* **20**, 315-318.

Tambalo, D. D., Yost, C. K. & Hynes, M. F. (2010). Characterization of swarming motility in *Rhizobium leguminosarum* bv. *viciae*. *FEMS Microbiology Letters* **307**, 165-174.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**, 1596-1599.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1977). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**, 4876-4882.

Thrall, P. H., Laine, A.-L., Broadhurst, L. M., Bagnall, D. J. & Brockwell, J. (2011). Symbiotic effectiveness of rhizobial mutualists varies in interactions with native australian legume genera. *PLoS ONE* **6**, e23545 EP -.

Tremblay, J. & Deziel, E. (2010). Gene expression in *Pseudomonas aeruginosa* swarming motility. *BMC Genomics* **11**, 587.

Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., de Billy, F., Promé, J.-C. & Dénarié, J. (1991). Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. *Nature* **351**, 670–673.

van Berkum, P. (1990). Evidence for a third uptake hydrogenase phenotype among the soybean *Bradyrhizobia*. *Applied and environmental microbiology.* **56**, 3835-3841.

van Berkum, P., and B. D. Eardly. (1998). Molecular evolutionary systematicsof the Rhizobiaceae, p. 1-24. In H. P. Spaink, A. Kondorosi, and P. J. J. Hooykaas (ed.), The rhizobiaceae. Molecular biology of plant-associated bacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.

van Rhijn, P. & Vanderleyden, J. (1995). The *Rhizobium*–plant symbiosis. *Microbiological Reviews* **59**, 124-142.

van Workum, W. A. T., Cremers, H. C. J. C., Wijfjes, A. H. M., van der Kolk, C., Wijffelman, C. A. & Kijne, J. W. (1997). Cloning and characterization of four genes of *Rhizobium leguminosarum* bv. *trifolii* involved in exopolysaccharide production and nodulation. *Molecular Plant-Microbe Interactions,* **10**, 290-301.

Vanderlinde, E. M. & Yost, C. K. (2012). Genetic analysis reveals links between lipid A structure and expression of the outer membrane protein gene, ropB, in *Rhizobium leguminosarum*. *FEMS Microbiology Letters*, [Epub ahead of print]

Vanderlinde, E. M., Harrison, J. J., Muszynski, A., Carlson, R. W., Turner, R. J. & Yost, C. K. (2010). Identification of a novel ABC transporter required for desiccation tolerance, and biofilm formation in *Rhizobium leguminosarum* bv. *viciae* 3841. *FEMS Microbiology Ecology* **71**, 327-340.

Verstraeten, N., Braeken, K., Debkumari, B., Fauvart, M., Fransaer, J., Vermant, J. & Michiels, J. (2008). Living on a surface: swarming and biofilm formation. *Trends in microbiology* **16**, 496-506.

Vincent, J. M. (1970). A manual for the practical study of the root-nodule bacteria. *Oxford: Blackwell Scientific*.

Vitousek, P. M., K Cassman, C Cleveland, T Crews, C B Field, N B Grimm, R W Howarth, et al. (2002). Towards an ecological understanding of biological nitrogen fixation. *Biogeochemistry* **57**, 1-45.

Vitousek, P. M. & Walker, L. R. (1989). Biological Invasion by *Myrica Faya* in Hawaii: Plant Demography, Nitrogen Fixation, Ecosystem Effects. *Ecological Monographs* **59**, 247-265.

Weaver, R. W., and P. H. Graham. (1994). Legume nodule symbionts, In Weaver, Angle, S., Bottomley, P., Bezdicek, D., Smith, S., Tabatabai, A., and Wollum, A

(eds.),. *Methods of soil analysis, Part 2, Am. Soc. of Agron., Madison, Wisconsin.*, p. 199-222.

Wei, Y., Guffanti, A. & Krulwich, T. (1999). Sequence analysis and functional studies of a chromosomal region of alkaliphilic *Bacillus firmus* OF4 encoding an ABC-type transporter with similarity of sequence and Na+ exclusion capacity to the *Bacillus subtilis* NatAB transporter. *Extremophiles* **3**, 113-120.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173** 697-703

Werner, T. Å., Motyka, V. C., Strnad, M. & Schmulling, T. (2001). Regulation of plant growth by cytokinin. *Proceedings of the National Academy of Sciences* **98**, 10487-10492.

Wielbo, J., Mazur, A., Krol, J. A. E., Marczak, M. G. & Skorupska, A. (2004). Environmental modulation of the *pssTNOP* gene expression in *Rhizobium leguminosarum* bv. *trifolii*. *Canadian Journal of Microbiology* *Can. J. Microbiol.* **50**, 201-211.

Wielbo, J., Mazur, A., Krol, J. A. E., Marczak, M. g. & Skorupska, A. (2004). Environmental modulation of the *pssTNOP* gene expression in *Rhizobium leguminosarum* bv. *trifolii*. *Canadian Journal of Microbiology*, *Canadian Journal Microbiology.* **50**, 201-211.

Williams, A., Wilkinson, A., Krehenbrink, M., Russo, D. M., Zorreguieta, A. & Downie, J. A. (2008). Glucomannan-mediated attachment of *Rhizobium leguminosarum* to pea root hairs is required for competitive nodule infection. *Journal of Bacteriology.* **190**, 4706–4715.

Wood, D. W., Setubal, J. C., Kaul, R., Monks, D. E., Kitajima, J. P., Okura, V. K., Zhou, Y., Chen, L., Wood, G. E., Almeida, N. F., Woo, L., Chen, Y., Paulsen,

I. T., Eisen, J. A., Karp, P. D., Bovee, D., Chapman, P., Clendenning, J., Deatherage, G., Gillet, W., Grant, C., Kutyavin, T., Levy, R., Li, M.-J., McClelland, E., Palmieri, A., Raymond, C., Rouse, G., Saenphimmachak, C., Wu, Z., Romero, P., Gordon, D., Zhang, S., Yoo, H., Tao, Y., Biddle, P., Jung, M., Krespan, W., Perry, M., Gordon-Kamm, B., Liao, L., Kim, S., Hendrick, C., Zhao, Z.-Y., Dolan, M., Chumley, F., Tingey, S. V., Tomb, J.-F., Gordon, M. P., Olson, M. V. & Nester, E. W. (2001). The Genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. *Science* **294**, 2317-2323.

Yang, S., Tang, F., Gao, M., Krishnan, H. & Zhu, H. (2010). R gene-controlled host specificity in the legume-rhizobia symbiosis. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 18735-18740.

Yeung, A. T. Y., Torfs, E. C. W., Jamshidi, F., Bains, M., Wiegand, I., Hancock, R. E. W. & Overhage, J. (2009). Swarming of *Pseudomonas aeruginosa* is controlled by a broad spectrum of transcriptional regulators, including MetR. *Journal of Bacteriology*, 5592-5602.

Yost, C. K., Rochepeau, P. & Hynes, M. F. (1998). *Rhizobium leguminosarum* contains a group of genes that appear to code for methyl-accepting chemotaxis proteins. *Microbiology* **144**, 1945-1956.

Young, J. P. W. H., K. (1996). Diversity and phylogeny of rhizobia. *New Phytologist* **33**, 87-94.

Young, J. P. W. & Johnston, A. W. B. (1989). The evolution of specificity in the legume-*Rhizobium* symbiosis. *Trends in Ecology & Evolution* **4**, 341–349.

Young, J. M., Kuykendall, L. D., Martinez-Romero, E., Kerr, A. & Sawada, H. (2001). A revision of *Rhizobium Frank* 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium Conn* 1942 and *Allorhizobium undicola* de Lajudie et al. 1998 as new combinations: *Rhizobium*

radiobacter, R. rhizogenes, R. rubi, R. undicola and *R. vitis. International Journal of Systematic and Evolutionary Microbiology* **51** 89-103

Young, J. P., Crossman, L., Johnston, A., Thomson, N., Ghazoui, Z., Hull, K., Wexler, M., Curson, A., Todd, J., Poole, P., Mauchline, T., East, A., Quail, M., Churcher, C., Arrowsmith, C., Cherevach, I., Chillingworth, T., Clarke, K., Cronin, A., Davis, P., Fraser, A., Hance, Z., Hauser, H., Jagels, K., Moule, S., Mungall, K., Norbertczak, H., Rabbinowitsch, E., Sanders, M., Simmonds, M., Whitehead, S. & Parkhill, J. (2006). The genome of *Rhizobium leguminosarum* has recognizable core and accessory components. *Genome Biology* **7**, R34.

Yurgel, S. N. & Kahn, M. L. (2004). Dicarboxylate transport by rhizobia. *FEMS Microbiology Reviews* **28**, 489-501.

Zahran, H. H. (1999). *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate *Microbiology and Molecular Biology Reviews* **63** 968-989

Zhang, Z., Schwartz, S., Wagner, L. & Miller, W. (2000). A greedy algorithm for aligning DNA sequences. *Journal of Computational Biology* **7**, 203-214.