Utjecaj sustava izbacivanja emrBAR na simbiozu biljke Medicago truncatula i bakterije Sinorhizobium meliloti

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The influence of emrBAR efflux system on symbiosis between *Medicago truncatula* and *Sinorhizobium meliloti*

Graduation thesis

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Dušik je bitan element biomolekula i potreban je za metabolizam i razvoj svih biljaka. Simbiotske interakcije između bakterija i biljaka razvile su se kako bi se omogućila apsorpcija dušika u biljkama. Glavni cilj ovog istraživanja bio je učinak sustava izbacivanja EmrBAR na simbiozu mahunarke *Medicago truncatula* i bakterije *Sinorhizobium meliloti*. Rezultati su pokazali da su geni *emrA* i *emrR* eksprimirani u ranim fazama simbiotskih interakcija, tijekom nastajanja infekcijskih niti. Gen *emrA* je eksprimiran u infekcijskoj zoni i zoni fiksacije dušika, a gen *emrR* samo u infekcijskoj zoni korijenove kvržice. Analiza nodulacijskog fenotipa *emrBAR* mutanata i divljeg tipa u nakošenim epruvetama ispunjenim agarom sugerira da je stvaranje novih korijenovih kvržica bio odgovor na neučinkovitu fiksaciju dušika. Analiza nodulacijskog fenotipa *emrBAR* mutanata i divljeg tipa u Magenta posudama sugerira da delecija *emrBAR* utječe na rast simbiontskih biljaka smanjujući učinkovitost fiksacije dušika u korijenovim kvržicama. Ispitivanje rasta bakterija je pokazalo da 2-fenilfenol inhibira rast dvaju sojeva *emrBAR* mutanta u odnosu na divlji tip. To bi moglo ukazivati na važnost sustava izbacivanja EmrBAR u izbacivanju otrovnih tvari iz bakterijskih stanica.

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The influence of *emrBAR* efflux system on symbiosis between *Medicago truncatula* and *Sinorhizobium meliloti*

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Nitrogen is an essential part of biomolecules and is needed for the metabolism and development of all plants. Symbiotic interactions between bacteria and plants have evolved in order to enable the absorption of nitrogen to plants. The main focus of this study was the effect of the EmrBAR efflux system on the symbiosis between the legume *Medicago truncatula* and the rhizobium *Sinorhizobium meliloti*. The results showed that *emrA* and *emrR* are expressed in early symbiotic interactions, during the formation of infection threads. *EmrA* is expressed in the infection and nitrogen-fixation zones, and *emrR* only in the infection zone of the nodule. Analysis of nodulation phenotype of the *emrBAR* deletion mutants and the wild type in sloped agar tubes might suggest that the creation of new nodules was a response to inefficient nitrogen fixation. Analysis of nodulation phenotype of the *emrBAR* deletion mutants and the wild type in Magenta boxes suggests that the *emrBAR* deletion affects symbiotic plant growth by decreasing nodule efficiency. A bacterial growth assay showed that 2-phenylphenol inhibits the growth of the two *emrBAR* deletion mutant strains in comparison with the wild type. This seems to indicate the importance of the EmrBAR efflux system in the extrusion of toxic substances.

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1. INTRODUCTION

1.1 Rhizobium – legume symbiosis

Nitrogen is an essential part of biomolecules and is needed for the metabolism and development of all plants. Although the atmosphere consists largely of nitrogen (about 78%), the majority of organisms on Earth do not benefit from it. In the course of evolution no mechanisms for direct admission of gaseous nitrogen from the environment have emerged in eukaryotes or higher plants. Plants absorb nitrogen from the soil by roots in the form of ammonium (NH_4) and nitrates (HNO_3) (Santi et al., 2013). Symbiotic interactions between bacteria and plants have evolved in order to enable the absorption of nitrogen to plants. This way bacteria are provided with nutrients and plants are provided with nitrogen in the form of ammonium through nitrogen fixation – the process of fixation and conversion of gaseous nitrogen (Fujita et al., 2014.).

Not all prokaryotes are capable of biological nitrogen fixation. The conversion of atmospheric nitrogen into ammonium is limited to a small group of prokaryotes (diazotroph), which contain at least one of the three types of nitrogenases (NF, VNF ANF). (Santos 2012). Nitrogenases are highly conserved and specific bacterial enzymes for nitrogen fixation that catalyze the conversion of gaseous nitrogen (N_2) into ammonium (NH_4) (Santi et al., 2013).

Bacterial diazotrophs go into endosymbiotic interactions with both legumes and non-legumes. The symbiosis between nitrogen-fixing bacteria and legumes is associated with the interaction between Gram-negative proteobacteria from the family Rhizobiacea and plants from the family Fabaceae (Desbrosses and Stougaard, 2011). Other symbiotic partners of rhizobia are representatives of the orders Fagales, Cucurbitales and Rosales. Among the nitrogen-fixing bacteria are included other proteobacteria (*Burkholderia* sp.), actinomycetes (*Frankia* sp.) and cyanobacteria (*Nostoc* sp.) (Kneip et al., 2007).

The nitrogen fixation by the family Rhizobiacea represents 70% of the total nitrogen transformation required for the maintenance of life in the biosphere (Resendis-Antonio et al., 2011). The family Rhizobiacea includes nitrogen-fixing symbionts from the group of α- and β-proteobacteria, e.g. *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Azorhizobium* and *Bradyrhizobium*, which are collectively referred to as rhizobia

(Brencic & Winans, 2005). In total, rhizobia include 12 genera and more than 70 species within the α- and β-proteobacteria, which are also known as α-rhizobia, with representatives from the order Rhizobiales and β-rhizobia, with representatives from the order Burkholderiales (Masson-Boivin et al., 2009).

Rhizobia are closely related to the plant pathogenic *Agrobacterium* (*A. tumefaciens*, *A. rhizogenes*) (Brencic & Winans, 2005). Despite the widespread presence of ineffective rhizobia in the environment, the rhizobia-legume symbiosis is evolutionary stable. Potential mechanisms that stabilize the symbiotic interactions include the mechanisms of partner fidelity feedback, partner choice and other (Fujita et al., 2014).

The limited availability of nitrogen and the dependence of many agricultural plants (e.g. alfalfa - *Medicago sativa*) led to the emergence of a massive industry that is engaged in the production of nitrogen fertilization means. However, nitrogen fixation by rhizobia is a natural and effective strategy for the development of sustainable agricultural programs, because of the reasonable cost and environmental friendliness as opposed to existing artificially produced chemicals (Resendis-Antonio et al., 2011).

1.2 Signal exchange, flavonoids as signaling molecules in initiating the symbiosis

The interaction between bacteria and plants does not appear arbitrary and is regulated by highly specific mechanisms. The capability of rhizobia to infect specific legumes and enter into symbiosis with them is governed by both symbiotic partners through the exchange of signals. Of particular importance for the initiation of symbiosis are flavonoids which are secreted from the roots of the plant (Janczarek, 2011).

Flavonoids are biologically active secondary metabolites with low molecular weight that are produced by plants and include over 10 000 structural variants. These substances fulfill numerous functions, such as transport of auxins, shoot and root development, pollination, staining the tissue, modulation of reactive oxygen species ("ROS") and signal exchange in the rhizobia-legume interactions (Weston & Mathesius, 2013). Substances commonly referred to as flavonoids have the C6-C3-C6 skeleton, a phenylbenzopyran function and are derived from 2-phenyl-1,4-benzopyrones (Jones et al., 2007).

The basic structure is a flavanone, which consists of A and B benzene rings (Fowler & Koffas, 2009). Flavonoids have different basic structures and numerous substitution patterns, resulting in structural diversity (Weston & Mathesius, 2013).

These substances are synthesized by the phenylpropanoid or acetate-malonate metabolism pathway. Usually, flavonoids are divided into six classes, depending on the position of the ring, hydroxylation and saturation of the bonds, namely flavones, flavonols, flavanones, isoflavones, anthocyanins and catechones (Fowler & Koffas, 2009). Among flavonoids are included, for example, luteolin, quercetin, genistein, apigenin, medicarpin, and others (Weston & Mathesius, 2013).

Different flavonoids are produced in the root apex and the root cap where they are accumulated as glycosides or aglycones and are then released into the soil by excretion or tissue decomposition (Weston & Mathesius, 2013). Flavonoids are the first signals between the two symbiotic partners in the rhizobia-legume interaction. The bacterial receptor NodD recognizes highly specific flavonoids (Haag et al., 2013).

The Nod proteins are transcriptional regulators from the LysR-family and induce the expression of nodulation genes (*nod* genes). Flavonoids do not affect the binding affinity of NodD proteins for target promoters, but enhance promoter activity by modifying DNA bending on conserved *nod*-boxes. The interaction between flavonoids and NodD proteins appears to be specific. Substances that induce nodulation genes and attract rhizobia include the flavonoids apigenin and luteolin. For example, the flavonoid luteolin from *M. sativa* stimulates the binding of an active form of NodD1 to the *nod*-box promoter of *S. meliloti*, which activates the transcription of genes located downstream (Weston & Mathesius, 2013).

Due to their high chemical activity, flavonoids are toxic endogenous compounds that need to be excreted from the cytoplasm of the cells. Flavonoids act as herbal antibacterial toxins. The excretion of flavonoids from bacterial cells is managed via multidrug (MDR) efflux systems (Piddock, 2006).

1.3 Nodulation

Nod genes (*nod*, *nol*, *noe*) are essential for the development of the root nodule; a specific organ, which is formed during the interaction between rhizobia and legumes (Santos et al., 2012). Nodulation may be referred to as a result of two coordinated operations. One includes the development of nodule tissue and finally the nodulation, while the other includes the infection process which mediates bacterial colonization of the plant (Haag et al., 2013).

The products of the expression of *nod* genes activate the synthesis of Nod factors, which induce root hair curvature as well as the formation of an infection

channel and root nodule primordiums (Zhang et al., 2006). The *nodABC* genes are present in all rhizobia and are necessary for the production of the basic Nod factor. In addition, different types of rhizobia have additional, species-specific, nod genes, which direct species-specific modifications of the basic Nod factor, such as *nodH*, *nodL*, *nodP*, *nodQ*, *nodZ* and others (Brencic & Winans, 2005). Nod factors are lipochito-oligosaccharides. The basic structure of Nod factors is conserved in rhizobia and consists of a chitin backbone with two to six *N*-acetyl-D-glucosamine residues to which an *N*-linked fatty acid is attached to the terminal nonreducing sugar (Desbrosses et al., 2011; Brencic & Winans, 2005). The substituent groups (e.g., acetyl, methyl, sulphuryl groups), as well as the length and degree of saturation of the fatty acid component of Nod factors, varies between rhizobia and is an important factor of bacterial host specificity (Haag et al., 2013).

Nod factor produced by rhizobia is detected by plant Nod factor receptor-like kinases (from the LysM family), located in epidermal root hair cells. The release of Nod factor triggers a calcium-dependent signal transduction pathway, which induces oscillations in calcium concentrations in the nucleoplasm and nuclear-associated cytoplasm. Thereby, the expression of specific genes, early nodulines (*ENODs*) is induced. Simultaneously, Nod factors trigger a Ca^{2+} influx at the root hair tip, causing an increase in Ca^{2+} levels in the cytosol, depolarization of the cell membrane and the reorganization of microtubules and actin filaments in root hair cells. These processes induce developmental changes in root hair cells, resulting in the formation of root hair curls, the so-called Shepherd's crooks. The rhizobia are trapped within the curls and proliferate, resulting in elevated Nod factor concentrations. Nod factors cause extensive remodelling of root hair cells, which results in the inward growth of the root hair tip and formation of a tubular structure known as an infection thread (Haag et al, 2013).

Infection threads display polar growth, with new cell wall and membrane material being synthesized at their tip. The formation and extension of the infection thread is dependent on bacterial polysaccharides. In *S. meliloti*, succinoglycan is the most important polysaccharide for infection thread initiation and elongation. The plant is colonized by differentiating endosymbiotic rhizobia. Rhizobia actively divide in the tip of the infection thread (Haag et al., 2013), which passes through the outer cortical cells and allows the migration of bacteria to the dividing cortical cells (Xiao et al., 2014). This way a nodule primordium is formed (Masson-Boivin et al., 2009).

After leaving the infection thread, bacteria are encompassed by the plasma

membrane in an endocytosis-like process. This produces symbiosomes, where bacteria differentiate into nitrogen-fixing bacteroids that are dependent on a constant energy and carbon supply from the host plant (Masson-Boivin et al., 2009). In summary, the overview of the symbiosis is shown in Fig. 1.

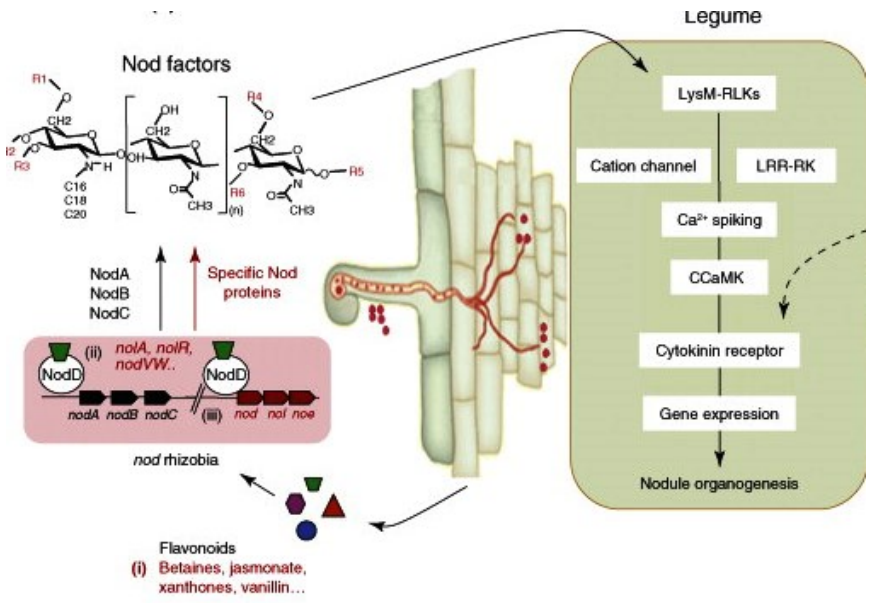

Figure 1. Overview of the rhizobia-legume symbiosis.
The expression of *nod* genes in rhizobia is induced by the secretion of flavonoids from the plant (*nodABC* and others). Nod factors formed by the bacteria trigger nodulation of the plant (taken from Masson-Boivin et al., 2009).

The central tissue of a root nodule is composed of two types of cells, namely infected cells, containing the rhizobia, and specialized non-infected cells, which are surrounded by three peripheral tissues. The non-infected tissues include the parenchyma, endodermis and the cortex (Xiao et al., 2014).

Nitrogenase activity is ensured by spatial separation of the bacteroids inside the nodule structure and special oxygen-scavenging leghemoglobin that is synthesized in the nodules (Kneip et al., 2007). The main function of leghemoglobin is controlling the concentration and diffusion of oxygen in bacteroides. Thereby, the oxygen concentration around the bacteroides is extremely low. In such conditions, proteins FixL and NifA, necessary for nitrogen fixation, are activated (Brencic & Winans, 2005).

The nodule may be determinate or indeterminate. Determinate nodules lose the meristem at early stages of development or have a temporary meristem, e.g. in *Glycine max*. Infected cells differentiate synchronically in determinate nodules and contain a

homogeneous population of bacterial nitrogen-fixing cells (Masson-Boivin et al., 2009). Mature indeterminate nodules consist of 5 different zones. At the distal end of the nodule is the apical meristem (Zone I), which gives rise to all tissues in the nodule. Cells in the meristem do not contain bacteria or infection threads. They are small and cytoplasm rich. Proximal to the meristem is the infection zone (zone II). Cells in this zone can contain infection threads. Cells in the infection zone are larger than the meristematic cells. In the distal part of the infection zone bacteria are released and can be found in the cell cytoplasm. The nitrogen-fixation zone (zone III) is proximal to the infection zone. In this zone, nodule cells contain a large, centrally placed vacuole, and have the cytoplasm filled with large, elongated bacteroids capable of fixing atmospheric nitrogen. Between the infection and nitrogen-fixation zones lies interzone II-III. Nodule cells in the interzone contain amyloplasts, and bacteria begin to differentiate into nitrogen-fixing bacteroids. Proximal to the nitrogen-fixation zone in older nodules is the senescent zone (zone IV). The internal contents of nodule cells and their bacteroids undergo degradation in the senescent zone (Monahan-Giovanelli et al., 2006). Overview of an indeterminate root nodule is given in Fig. 2. Cylindrical, indeterminate root nodules are well suited for the studies of nodule development and globular, determinate nodules for biochemical analysis (Xiao et al., 2014).

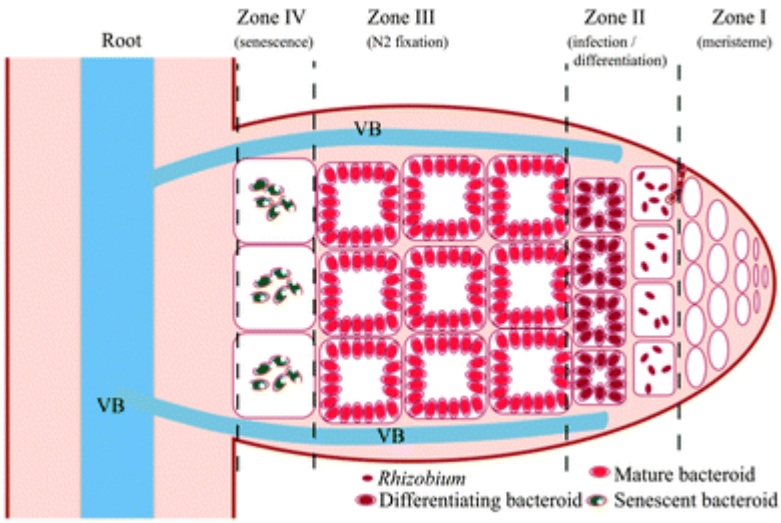

Figure 2. Overview of an indeterminate root nodule.
The elongated, mature root nodule is divided into 4 zones: Zone I (apical meristem), Zone II (infection zone), Zone III (nitrogen-fixation zone) and Zone IV (senescent zone) (taken from Rodríguez-Haas et al., 2013).

1.4 Multidrug (MDR) efflux systems

Bacteria have different survival strategies for interacting with antibacterial substances from the environment. In the past decade, the number of antibiotics, to which bacterial cells have developed resistance mechanisms, has dramatically increased (Piddock, 2006). One such mechanism is the inactivation of antibiotics through their modification or degradation, for example, with a mutation in the active site of the antibiotic, as in a β-lactamase-negative ampicillin-resistant bacterium *Haemophilus influenza*. Another, more common mechanism of resistance to cytotoxic agents in biological systems, is their elimination or efflux from the cell by membrane proteins (Borges-Walmsley et al., 2003). These proteins serve as pumps and reduce the intracellular concentration of toxic substances to sub-toxic levels (Borges-Walmsley et al., 2003).

In the late 1980s and early 1990s multidrug efflux systems were detected in bacteria (Paulsen, 2003). Such systems can excrete structurally different organic compounds from bacterial cells. This creates resistance to toxic substances and provides bacteria survival strategies in specific ecological niches (Paulsen, 2003; Piddock, 2006). Multidrug efflux systems confer resistance to a variety of toxic agents that are present in bacterial habitats (Wenzel et al., 2012). They are also involved in symbiotic interactions between rhizobia and legumes, and are required for the colonization of eukaryotic hosts (Ramos et al., 2005; Piddock, 2006).

Multidrug efflux systems are classified based on several factors: the number of components of an efflux pump and the transmembrane border regions of a transporter, as well as the power source and types of substrates that are exported by an efflux pump. The mechanism of energy generation is crucial (Piddock, 2006). Bacterial multidrug efflux systems are divided into five families: the ATP-binding cassette (ABC) superfamily, the Multidrug and toxic compounds efflux (MATE) family, the Small multidrug resistance (SMR) family, the Resistance/nodulation/cell division (RND) family, and the Major facilitator superfamily (MFS) (Fig. 3) (Saier et al., 1998).

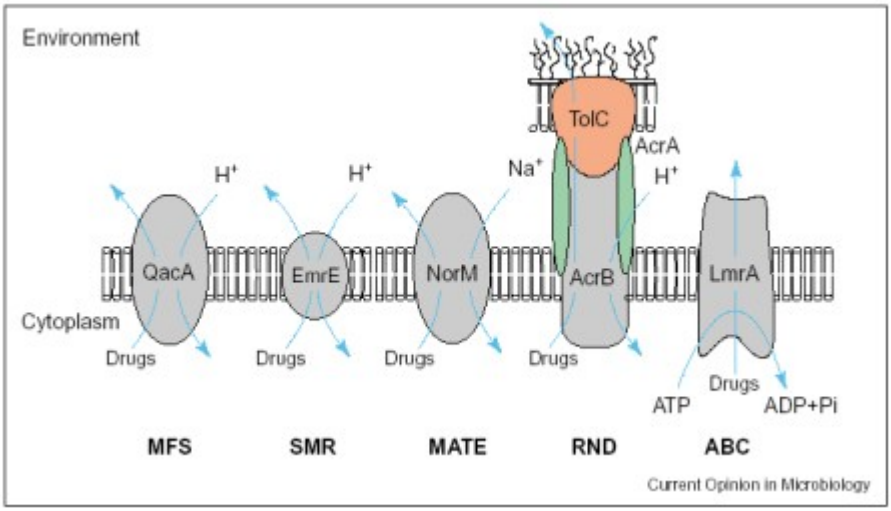

Figure 3. Overview of bacterial multidrug efflux systems.
In the picture an example from each family of multidrug efflux systems is shown. MFS: Major facilitator superfamily; SMR: small multidrug resistance; MATE: multidrug and toxic compounds efflux; RND: resistance nodulation division; ABC: ATP-binding cassette; E.g.: QacA from *Staphylococcus aureus*; E.g.: EmrE from *E. coli*; E.g.: NorM from *Vibrio parahaemolyticus*; E.g.: AcrAB-TolC from *E. coli*; E.g.: LmrA from *Lactococcus lactis* (taken from Paulsen, 2003).

In prokaryotes the efflux of substances is usually managed by proton coupled influx pumps (H^+), which are referred to as antiport (Borges-Walmsley et al., 2003). All multidrug efflux pumps consist of an inner membrane transporter, for example, MdfA efflux system from *E. coli* from the MFS family (Zgurskaya et al., 2009). However, multidrug efflux systems also include other components (Piddock, 2006; Eda et al., 2011). Multidrug efflux pumps in gram-negative bacteria typically consist of two additional components: a membrane fusion protein (MFP) and an outer membrane factor or channel (OMF). The function of the majority of known efflux systems from the RND, MFS and ABC families is dependent on these proteins (Zgurskaya et al., 2009).

During the transport of substances in gram-negative bacteria through the two membranes, the proteins in the outer membrane are also part of the system, for example TolC (Zgurskaya et al., 2009). TolC serves as a protein channel for various efflux pumps of the RND, MFS and ABC families. In *S. meliloti*, the TolC protein is required for successful symbiosis with *M. sativa* plants, primarily for protein secretion and oxidative stress response (Santos et al., 2014). The TolC protein is involved in interactions with membrane transporters. This cellular import and export are controlled by different compounds (Santos et al., 2010).

According to the analyses of genomic sequences of more than 100 organisms, the largest number of multidrug efflux systems has been found in soil and plant-

associated bacteria (Paulsen et al., 2003). A single organism can have various types of multidrug efflux systems (from one or more families) (Piddock, 2006). Studies have already shown the presence of one ABC, three MFS and ten RND type efflux systems, as well as three outer membrane proteins in nitrogen fixing *S. meliloti* (Eda et al., 2011; Rossbach et al., 2014).

The ABC transporters use ATP as an energy source. The efflux systems of the ABC family exist in prokaryotes and eukaryotes, and consist of a membrane protein with six transmembrane α helices and a protein for energy supply on the cytoplasmic side of the membrane, e.g. LmrA transporter from *Lactococcus lactis* (Saier et al., 1998; Borges-Walmsley et al., 2003). Transportation in the ABC family appears in two forms: a system with one component or a multi-component system (Eda et al., 2011).

Representatives of the SMR family consist of 100-110 amino acids which are organized into 4 helices. Transport proteins from this family are very hydrophobic. The EmrE efflux system from *E. coli* is one example of an SMR family transporter which confers ethidium bromide resistance (Saier et al., 1998; Borges-Walmsley et al., 2003). Structurally, SMR-pumps are composed of one component (Eda et al., 2011).

The transporters from the RND family typically consist of more than 1000 amino acids, and have a structure similar to MFS transporters. They have large periplasmic or extra-cytoplasmic domains of 1, 2, 7 and 8 helices. An example of RND transporters is AcrB from *E. coli* and *Erwinia amylovora* (Saier et al., 1998; Borges-Walmsley et al., 2003). Efflux systems of gram-negative bacteria from the RND family are organized in three-part systems. Such efflux pumps consist of a transporter protein, e.g. AcrB in the inner cell membrane, a membrane fusion protein, e.g. AcrA in the periplasmic space and an outer membrane protein, e.g. TolC (Piddock, 2006; Eda et al., 2011).

Transporters from the MATE family typically consist of 450 amino acids, which are organized in 12 helices. For example, the efflux system NorM from *Vibrio parahaemolyticus,* which confers aminoglycoside resistance, is part of the MATE family (Saier et al., 1998; Borges-Walmsley et al., 2003). MATE pumps are, similarly to SMR transporters, composed of one component (Eda et al., 2011).

1.5 Major facilitator superfamily (MFS) of multidrug efflux systems

The transporters from the MFS family typically consist of 400-600 amino acids, which are organized in 12-14 α-helical transmembrane domains with a large

cytoplasmic loop between the 6th and 7th helices (Borges-Walmsley et al., 2003). Two subfamilies are recognized, namely the 12 and 14 helices transmembrane transporters, e.g. TetA(B) and TetA(K), class B and K of the tetracycline transporter from *E. coli* and *Staphylococcus aureus* respectively (Saier et al., 1998; Borges-Walmsley et al., 2003). Pumps from the MFS family consist of one or several components (Eda et al., 2011). The MFS family is an evolutionarily very old family of bacterial transporters (Saier et al., 1998). This family includes transporters such as Bcr (*E. coli*), EmrAB (*E. coli*), EmrD (*E. coli*), NorA (*S. aureus*) QacA (*S. aureus*), BMR (*Bacillus subtilis*) and EmrAB (*S. meliloti*) (Piddock, 2006; Santos et al., 2014).

However, so far there have been only a few studies in which the regulation and role of MFS pumps was examined in rhizobia (Wenzel et al., 2012; Santos et al., 2014). For example, in *S. meliloti* strain 41, the *nolG* genes that encode an AcrB-like membrane protein of the RND family have been studied (Hernandez-Mendoza et al., 2007). Studies with a *smeAB* deletion strain have shown increased sensitivity of bacteria to toxic compounds and reduced nodulation. The *smeAB* gene is regulated by a TetR-type protein that is encoded within the same operon (Eda et al., 2011).

1.6 Transcriptional regulators of efflux systems

Survival in unstable environments is made possible in bacteria through rapid and adaptive responses that are provided by regulatory proteins. Such reactions respond to specific environmental signals that affect gene expression, in particular transcription or translation (Ramos et al., 2005).

The expression of multidrug efflux systems is regulated at the level of transcription by transcription regulators. Operons of multidrug efflux pumps are often connected to a regulatory gene (Eda et al., 2011). Many of these regulators are multidrug binding proteins which recognize and respond to the same substances that are exported by the transporters they regulate (Routh et al., 2009). They act as cytosolic chemical sensors and detect toxic compounds (Ramos et al. 2005; Routh et al., 2009).

In bacteria, the regulation of transcription is performed by one- or two-component regulatory systems (Routh et al., 2009). The majority of bacterial regulators are proteins that consist of two domains. One domain is used for signal recognition, while the second serves as a DNA-binding domain. Prokaryotic transcription factors can be divided into families based on sequence similarities, structure and function. Some of the most important families of transcription factors in prokaryotes include the LysR,

AraC/XylS, TetR, LuxR, LacI, ArsR, IclR, MerR, AsnR, MarR, NtrR, OmpR, DeoR, GntR and Crp families. Many of those are repressors, for example, the TetR, LacI, ArsR and DeoR families. DNA binding motifs can have a C- or N-terminal position. The most commonly found motif is helix-turn-helix (Ramos et al., 2005). The most important families of prokaryotic transcriptional regulators are summarized in Tab. 1.

Table 1. Overview of some transcriptional regulator families; DBD motif: DNA binding domain motif, HTH: helix-turn-helix (modified according to Ramos et al. 2005)

Family	Regulation type	Function of regulated genes	DBD motif	Position
AraC/XylS	Activator	Carbon metabolism, stress response, pathogenesis	HTH	C-terminal
LacI	Repressor	Use of carbon sources	HTH	N-terminal
LuxR	Activator	Quorum sensing, biosynthesis	HTH	C-terminal
LysR	Activator	Carbon and nitrogen metabolism	HTH	N-terminal
OmpR	Activator	Virulence and heavy metal resistance	Winged helix	C-terminal
TetR	Repressor	Antibiotic biosynthesis, efflux pumps, osmotic stress	HTH	N-terminal

1.7 TetR family of transcription factors

Many gram-negative bacteria have developed mechanisms of tetracycline resistance. Many efflux pumps coding genes are regulated by repressor proteins that belong to the TetR family of regulators, for example *emrAB* or *tetA* in *E. coli*. (Ramos et al., 2005; Rossbach et al., 2014). Typically, the mechanism consists of a membrane-bound protein, e.g. TetA from *E. coli*, which exports the antibiotic from the cell, and a regulator, e.g. TetR from *E. coli*. The gene *tetR* is divergently oriented and controls the expression of *tetA* as well as of *tetR*. The TetR binds to intergenic operator sequences and represses the transcription of both promoters (Ramos et al., 2005).

The members of the TetR family of transcriptional regulators are very common in bacteria and have a helix-turn-helix DNA-binding motif (Ramos et al., 2005). The majority of TetR regulators recognizes and binds to palindromic DNA sequences present in divergent orientations (Ramos et al., 2005; Eda et al., 2011). Another common feature of the TetR family is that binding activity of transcriptional regulators can be eliminated through interaction with a ligand (Eda et al., 2011).

In *S. meliloti* six multidrug efflux systems are associated with putative regulatory genes. For example, a transcription factor from the TetR family, SmeR negatively regulates the expression of *smeAB* genes (Eda et al., 2011). Other examples of transcription regulators, belonging to the TetR family, are the regulator of the *emrAB* efflux system from *E. coli* as well as the regulator of the *emrAB* (*smc03168-smc03167*) efflux system from *S. meliloti* (Ramos et al., 2005; Rossbach et al., 2014).

1.8 EmrAB efflux system of *S. meliloti* from the MFS family under the regulation of the transcription factor TetR

In *S. meliloti*, genes *emrB* (*smc03167*) and *emrA* (*smc03168*) encode an MFS efflux system and *emrR* (*smc03169*) encodes a repressor from the TetR family (Santos et al., 2014; Rossbach et al., 2014). The components of the system are adjacent and probably organized in an operon (Ramos et al., 2005). The *emrBAR* system is much conserved in the order *Rhizobiales* (Rossbach et al., 2014).

Genes, encoding the components for the transporter of multidrug efflux systems, are usually located in a tandem with genes encoding for the membrane fusion protein (Eda et al., 2011). In studies of transcriptomes of *S. meliloti* and *tolC* mutant strains, analyzes have shown that around 1,500 genes from *S. meliloti* (a symbiont of alfa-alfa) are differentially expressed in a *tolC* mutant strain (Santos et al., 2010). The absence of a functional TolC protein affects cell homeostasis and leads to the increase in the expression of genes which are responsible for cytoplasmic and extracytoplasmic stress response. *EmrA*, *emrB* and *emrR* genes appear to be significantly upregulated in the *tolC* mutant strain (Santos et al., 2010).

The deletion of the *emrAB* efflux system in *S. meliloti* exhibited no effect on the sensitivity to various toxic substances and on the symbiotic properties of the bacterium (Eda et al., 2011). However, it was shown that an *emrB* defective mutant had greater susceptibility to sulphonamide antibiotics, respiration inhibitors and 2-phenylphenol (Spini et al., 2014). The deletion of the regulator *emrR*, as well as the deletion of *emrR* together with *emrA* led to the formation of fewer nodules on plants infected with the mutant strain and the decrease in the expression of genes relevant for the formation of the Nod factor (Santos et al., 2014). The EmrR could be involved in the regulation of membrane and cell wall modifications for the colonization of plants (Santos et al., 2014).

1.9. Aims and objectives

The main focus of this study was the effect of the EmrBAR efflux system on the symbiosis between the legume *Medicago truncatula* and the rhizobium *Sinorhizobium meliloti*. The aim was to investigate the expression patterns of genes encoding both, the membrane fusion protein EmrA and the repressor EmrR. These two genes are involved in nodule development and there is currently no data available regarding their expression. For this purpose, two mutant strains would be constructed, which have a translational gene fusion. One strain would have an *emrA-uidA* (β-glucuronidase) fusion, while the other would have an *emrR-lacZ-uidA* fusion. To show the expression of both genes, β-glucuronidase staining assays would be performed. Furthermore, the effects of the EmrBAR efflux system on nodulation and the export of toxic substances would be investigated. A phenotypical analysis of nodulation of two *ΔemrBAR* mutant strains in symbiosis with *M. truncatula* would be conducted, in comparison to the wild type. Additionally, a bacterial growth assay of the two *ΔemrBAR* mutants and the wild type would be conducted. The results of this study would build upon and contribute to the work of Prof. Göttfert and his group at the Laboratory for Molecular Genetics, Institute of Genetics at the Department of Biology, Technische Universität Dresden.

2. MATERIALS AND METHODS

2.1 Materials

Equipment

Agarose gel electrophoresis power device
- CONSORT E831 Electrophoresis Power Supply; Consort

Agarose gel imaging
- AlphaImager®; Biozym Scientific GmbH

Centrifuges
- Sigma 1-14 Microfuge; SciQuip Ltd.
- Sigma 1-15K Refrigerated Microfuge; SciQuip Ltd.

Electroporation
- E. coli Pulser™ Transformation Apparatus; Bio-Rad Laboratories

Incubators
- Infors HT Multitron; Infors HT
- Heraeus® Function line B6 incubator; Kendro Laboratory Products

Microscopy
- Axio Observer.Z1 fluorescence microscope; Carl Zeiss AG
- Optical microscope; Thalheim Spezial Optik

PCR machine
- Biometra T1 Thermocycler; Biometra GmbH
- Biometra Tpersonal; Biometra GmbH

Photometer
- Helios Beta UV-Vis Spectrophotometer; Thermo Electron Corporation
- NanoDrop ™ 1000 Spectrophotometer; Peqlab Biotechnology

Plants chamber
- Sanyo MLR-350H; Sanyo Electric Biomedical Co.

Thermo shaker
- Thermomixer comfort with thermoblock 1.5 ml; Eppendorf

Vacuum infiltration
- Nucerite®; Nalge/Sybron Corporation

Sources of chemicals

All chemicals used for the experiments, unless otherwise indicated, were obtained from the companies Merck, Carl Roth, AppliChem, Kalys, Sigma-Aldrich and Difco in analytical quality.

Antibiotics

The antibiotics used and their concentrations are given in Tab. 2.

Table 2. Antibiotics and media additives

Substance	Solvent	Stock solution	Supplier
Kanamycin (Km)	ddH_2O	50 mg/mL	AppliChem
Nalidixic acid (Nx)	0.15 M NaOH	10 mg/mL	AppliChem
Streptomycin (Sm)	ddH_2O	100 mg/mL	AppliChem
Tetracyclin (Tc)	50% ethanol	50 mg/mL	AppliChem
AVG (ethylene synthesis inhibitor)	ddH_2O	2 mM	Sigma-Aldrich

Enzymes, standards, buffers, miscellaneous

The enzymes used are shown in Tab. 3.

Table 3. Enzymes with appropriate buffers and other components

Description	Supplier
Restriction endonucleases with 10x buffers	Thermo Scientific
Pfu DNA polymerase with buffer	Thermo Scientific
DreamTaq DNA polymerase buffer	Thermo Scientific
T4 DNA ligase with 10x buffer	Fermentas
Pronase E	Merck
RNase A	AppliChem
GeneRuler 1 kb DNA Ladder	Thermo Scientific
λ-DNA (0.3 µg/µL)	Thermo Scientific
X-Gluc	Thermo Scientific

Kits

The Zyppy™ Plasmid Miniprep Kit (Zymo Research) is used according to the manufacturer provided user manual unless specified otherwise.

Oligonucleotides

The oligonucleotides used are listed in Tab. 4.

Table 4. Oligonucleotides used in the study.

Primer	Sequence 5'→3'	Tm	Application
emrR_for	ATATTCTAGACCGACG TTATGCTCGTCACC	63 °C	Cloning an intergenic region between *emrA* and *emrR*
emrA_ATG	CATTACTTCGAAATCAT CGCCGACGG	65 °C	Cloning an intergenic region between *emrA* and *emrR*
SmnodDFor	GTGCGGCATCCATATC GCAG	59 °C	Detection of *S. meliloti* region between *nodD* and *nodA*
SmnodARev	CAGCATAGCTTCCACT GCAC	51 °C	Detection of *S. meliloti* region between *nodD* and *nodA*
pSUPPOLfor	ATAAACCAGCCAGCC GGAA	55 °C	Checking the integration of the homologus region into p_SUPPOL_uidA and the integration of the *emrA-uidA* fusion into *S. meliloti*
uidA_974_r	AGGGTAATGCGAGGT ACGGTAGG	61 °C	Checking the integration of the homologus region into p_SUPPOL_uidA and the integration of the *emrA-uidA* fusion into *S. meliloti*
lacZ_pSUP_rev	GGCCTCTTCGCTATT ACGC	52 °C	Checking the integration of the *emrR-lacZ-uidA* fusion into *S. meliloti*
Sm_P1F_N3F	CTCGGCCGAATGAG TATCC	56 °C	Checking the integration of the *emrR-lacZ-uidA* fusion into *S. meliloti*
03169R3	CATTCTGTCCAAAAT CACTAT	49 °C	Checking the integration of the *emrA-uidA* fusion into *S. meliloti*
DMseq01R	GTTTTCGCGATCCAG ACTGAATG	58 °C	Sequencing of pSMD013
Sm_RT03168	CCACCAATCATAGCC GTACC	56 °C	Checking the integration of the *emrA-uidA* fusion into *S. meliloti*
03169K_Int	GATCGTGCTTTCCGC TTCCATCTG	62 °C	Checking the integration of the *emrR-lacZ-uidA* fusion into *S. meliloti*

Plasmids

The plasmids used are listed in Tab. 5.

Table 5. Plasmids used in the study.

Plasmid	Characteristics	Reference
pSMD001	Derivative of pSUPlacZ481uidA with the *emrR-lacZ-uidA* fusion at the annotated start codon.	Master thesis of A. Welker, 2014
pSUPPOL2_uidA	Derivative of pSUPPOL2 containing the *uidA* gene.	A. Zdyb
pSMD013	Derived from pSUPPOL2 containing *uidA* for co-integration into the genome of *S. meliloti* for the purpose of creating a translational fusion of the *emrA* and *uidA* genes.	This work

Bacterial strains

Bacterial strains used are listed in Tab. 6.

Table 6. Bacterial strains used in the study.

Strain	Characteristics	Reference
E. coli		
DH10B	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80dlacZΔM15 ΔlacX74 *deoR recA1 endA1 araD139Δ* (*ara leu*)*7697 galU galK λ*$^-$ *rpsL nupG;* Sm^R	Invitrogen, Germany
S17-1	*hsdR pro thi* (RP4-2 *km*::Tn7 *tc*::Mu, integrated into the chromosome), used for the mobilization of plasmids; Sm^R Sp^R	Simon et al., 1983
S. meliloti		
S. meliloti 2011	Wild-type, Nx^R, Sm^R	Meade et al., 1982
SMD001	*emrR-lacZ* fusion at the annotated translation start integrated into the wild-type *S. meliloti* 2011; Sm^R, Tc^R	Master thesis of A. Welker, 2014
SMD011	Deletion of *emrBAR* in *S. meliloti* 2011, kanamycin resistance cassette in the direction of *smc03170*, Sm^R, Km^R	Master thesis of S. Krysenko, 2014
SMD012	Deletion of *emrBAR* in *S. meliloti* 2011, kanamycin resistance cassette in the direction of *smc03166*, Sm^R, Km^R	Master thesis of S. Krysenko, 2014
SMD013	A translational fusion of the *emrA* and *uidA* genes in *S. meliloti* 2011. (pSMD013 transconjugated into *S. meliloti*); Str^R, Tc^R, Nx^R	This work
SMD014	A fusion of the *emrR* and *lacZ* and *uidA* genes in *S. meliloti* 2011. (pSMD001 transconjugated into *S. meliloti*); Str^R, Tc^R, Nx^R	This work

2.2 Methods

2.2.1 Microbiological methods

Culture conditions and media for microorganisms

The culture of *E. coli* were incubated at 37 °C in Luria Bertani (LB) medium overnight and of *S. meliloti* at 28 °C on Tryptone Yeast (TY) medium for 2-3 days. Liquid cultures were incubated on a rotary shaker at 180 rpm. If necessary, the addition of antibiotics in the following concentrations was carried out during the incubation: nalidixic acid 10 µg/ml, streptomycin 200 µg/ml, tetracycline 10 µg/ml or kanamycin 100 µg/ml for *S. meliloti* or tetracycline 10 µg/ml for *E. coli*.

Luria-Bertani medium (LB) (Sambrook et al., 2001)	1% (w/v) tryptone (Carl Roth) 1% (w/v) NaCl (Carl Roth) 0.5% (w/v) yeast extract (Carl Roth)
Tryptone-Yeast medium (TY) (Beringer, 1974)	0.5% (w/v) Bacto tryptone (Difco) 0.3% (w/v) Bacto yeast extract (Difco) Addition of 10 mM $CaCl_2$ after autoclaving
Solid media	contained 1.5% agar (Carl Roth)

Preparation of glycerol stocks for long term storage of bacterial cultures

5 ml of medium with the appropriate antibiotic was inoculated with a single colony and incubated overnight (for *E. coli* strains DH10B and S-17) or for 2 days (for *S. meliloti* 2011 and derivatives). Two ml of the culture was spun down for 5 minutes at 3000 g and room temperature (RT), the pellet was resuspended in 500 µl of LB medium (for *E. coli* strains) or in TY medium (for *S. meliloti* 2011 and derivatives) with fresh antibiotic. The cell suspension was mixed with 500 µl of sterile glycerol by vortexing, incubated for 30 minutes at RT and stored at -80 °C.

Bacterial growth assay

5 ml of medium with the appropriate antibiotic was inoculated with a single colony of *S. meliloti* 2011, SMD011 or SMD012, and incubated overnight. A new culture was started, combining the overnight culture into new media with the appropriate antibiotic, to achieve an optical density of about 0.4 A at the wavelength of 600 nm (OD_{600}), to a total of 10 mL. The culture was then treated with different concentrations of 1M 2-phenylphenol (Sigma-Aldrich) dissolved in methanol. The culture was incubated for a few days and the OD_{600} was measured at different time points using the

spectrophotometer. For this, cuvettes were prepared containing 1 ml of the culture or 1 ml of the culture diluted with new media in a ratio of 1/10.

2.2.2 Genetic engineering methods

Plasmid DNA isolation, alkaline lysis method

Five mililiters of LB medium with the appropriate antibiotic was inoculated with a single colony and incubated overnight (for *E. coli* strains). 1.5 ml of the culture was spun down for 5 minutes at 14000 g and RT. The pellet was then resuspended in 0.1 ml ice-cold solution I and incubated for 5 minutes at RT. Afterwards, 0.2 ml of solution II were added and mixed carefully by inversion. After an incubation of 5 minutes on ice, 0.15 ml of ice-cold solution III were added and the mixture was mixed by inversion. After a further incubation for 5 minutes on ice, the cell debris was separated by centrifugation for 5 minutes at 17968 g and 4 °C. The supernatant was removed and mixed with 0.3 ml of phenol/chloroform/isoamyl alcohol, and then vortexed for 30 seconds and centrifuged for 3 minutes at 17968 g and RT. The upper phase was removed and mixed with 0.3 ml of methylene chloride and then again vortexed for 30 seconds and centrifuged for 3 minutes at 17968 g and RT. The upper phase was moved to a new tube and mixed with the 2.2 fold volume of absolute ethanol. The precipitated DNA was collected by centrifugation for 15 minutes at 17968 g and 4 °C. The supernatant was discarded and the DNA pellet was washed with 500 µL of ice-cold 70% ethanol. The pellet was briefly centrifuged, then dried in the air for about 10-15 minutes and resuspended in 25 µl of Tris-EDTA-RNAse (TE-RNAse) buffer (TE buffer content is on page 19). The DNA concentration determination and verification of the purity of the isolated DNA was carried out by gel electrophoresis or the nano-drop. The DNA was stored until use at -20 °C.

Solution I	50 mM glucose 20 mM Tris HCl, pH 8.0 10 mM EDTA, pH 8.0 4 mg/mL Lysozym Adjust pH to 8.0 with HCl
Solution II	1% (w/v) SDS 0.2M NaOH
Solution III	3M potassium acetate Adjust pH to 4.8 with acetic acid

Plasmid DNA isolation, Zyppy™ Plasmid Miniprep Kit

The kit was used according to the manufacturer provided user manual. Plasmid DNA was isolated from 3 mL of *E. coli* bacterial culture. The final resuspension of plasmid DNA was made in 40 µL of Zyppy Elution Buffer.

Genomic DNA isolation

For the isolation of genomic DNA from *S. meliloti* 10 ml of TY medium with the appropriate antibiotic was inoculated with a single colony and incubated for 2-3 days at 28 °C and 180 rpm. Two ml of the culture was spun down for 5 minutes at 6000 rpm and RT. The cell pellet was then resuspended in 1 ml of TE-buffer. The solution was again spun down for 5 minutes at 6000 rpm and RT. The cells were resuspended in 300 µl of TE buffer, followed by the addition of 100 µl of 5% SDS and 100 µl of Pronase-E with a subsequent incubation at 37 °C overnight. The DNA solution was shattered by passing through a needle (0.6 mm diameter) to a 2 mL syringe, which was repeated 5 - 10 times. After shearing, 300 µL of TE buffer was added. Afterwards, 300 µl of equilibrated phenol was added. The solution was then vortexed for 15 seconds and centrifuged for 3 minutes at 14000 rpm and RT. Without disturbing the whitish lower phase, the upper aqueous phase was moved to a new tube and the phenol extraction was repeated three more times. Subsequently, the upper phase was mixed with 300 µl of methylene chloride and centrifuged for 3 minutes at 14000 rpm and RT. After extraction, the upper phase was mixed with the 2.5 fold volume of absolute ethanol and mixed by inversion. The DNA was spun down for 10 minutes at 14000 rpm and RT. Finally, the genomic DNA was washed with 400 µl of 70% ice-cold ethanol, mixed by inversion to remove possible phenol leftovers from the Eppendorf tube wall and shortly centrifuged to remove the ethanol. The pellet was then air-dried, resuspended in 50 µl of TE-RNAse buffer and stored at -20 °C.

TE buffer	1 mM EDTA 10 mM Tris-Base Adjust pH to 8.0 with HCl
5% SDS	5% (w/v) SDS in ddH_2O
Pronase E	2.5 mg/mL in TE buffer

Determination of the DNA concentration

To determine the DNA concentration, the DNA was measured using either a NanoDrop ND-1000 spectrophotometer or compared with a length/amount standard on an electrophoretic gel. With a NanoDrop gauge the concentration was determined

photometrically by measuring the UV absorbance (OD_{260}). A zero value was determined using 1 µL of ddH_2O. The calculation of the concentration of DNA was carried out according to the following equation:

1 = OD_{260} = 50 µg of double-stranded DNA/mL

Agarose gel electrophoresis

For the preparation of the gel, agarose was added to 1xTris-acetate-EDTA (TAE) buffer and boiled. The liquid solution was poured into a gel tray and cooled at RT. The gel carrier was placed into an electrophoresis chamber and covered with 1x TAE buffer. For the separation of DNA fragments according to their size, the DNA samples were treated with 2 or 3 µL of loading buffer. Subsequently 5 µL were applied to the gel. To estimate the size and the amount of DNA, a DNA length standard was applied to the gel. Electrophoretic separation of the DNA fragments was carried out in a 1% (w/v) agarose gel at a varying voltage of 30 - 120 V for about 60 – 90 minutes. After electrophoresis the DNA was stained in an Ethidium bromide bath for 10 minutes. Afterwards the agarose gel was placed for 10 minutes in a water bath for decolorization of the background. The gel was evaluated by exciting the ethidium bromide with UV radiation using an AlphaImager device and then documented.

Agarose solution	1% (w/v) agarose in 1x TAE buffer
6x loading buffer	0.2% (w/v) Bromphenolblue 0.1 M EDTA 33% (v/v) glycerol
50x TAE	2 M Tris-base 50 mM EDTA (adjust pH to 8.0 with HCl) 1 M acetic acid
Ethidium bromide bath	10 µg/mL ethidium bromide in H_2O

2.2.3 Cloning of DNA fragments

Amplification of DNA by polymerase chain reaction (PCR)

For the amplification of DNA fragments PCR was carried out (Tab. 7). The *Pfu* polymerase was used for the amplification of DNA segments that would be used for cloning (Tab. 8). For the detection of DNA fragments the *Taq* polymerase was used (Tab. 9).

Table 7. PCR conditions

Step	Temperature	Time	Number of cycles
Initial denaturation	95 °C	5 min	1
Denaturation	95 °C	30 s	30-33
Annealing	Tm-5 °C	30 s	
Elongation	72 °C	*Pfu* DNA polymerase 2 min/kb *Taq* DNA polymerase 1 min/2 kb	
Final elongation	72 °C	10 min	1

Table 8. Components of the PCR for the amplification of DNA fragments

Template DNA	0.5-100 ng
Pfu DNA polymerase (5U/µL)	1 µL
Pfu buffer (with Mg SO_4)	5 µL
Forward primer (10 pmol/µL)	0.5 µL
Reverse primer (10 pmol/µL)	0.5 µL
10 mM dNTP mix	1 µL
ddH_2O	Fill up to 50 µL
Total volume	50 µL

Table 9. Components of the PCR for the detection of DNA fragments

Template DNA	10 µL
Taq DNA polymerase	0.5 µL
Taq DNA polymerase buffer (with $MgCl_2$)	2.5 µL
Forward primer (10 pmol/µL)	0.25 µL
Reverse primer (10 pmol/µL)	0.25 µL
10 mM dNTP mix	0.5 µL
ddH_2O	Fill up to 25 µL
Total volume	25 µL

Colony PCR

For quick verification of transformants colony PCR was performed. A single colony was added to 10 µL of ddH_2O. 10 µL of the suspension were used for the PCR as a template DNA.

Preparation of DNA length standard λ-DNA/*Pst*I

Alongside commercial DNA markers, also a manually produced length standard λ-DNA/*Pst*I was used for agarose gel electrophoretic analysis. For this purpose λ-DNA was digested with *Pst*I at 37 °C overnight. The endonuclease was then inactivated at 65 °C for 15 minutes. Subsequently, the DNA was mixed with 50 µL of 6x loading buffer and 50 µL of ddH_2O by vortexing and stored at -20 °C.

Restriction endonuclease digestion

For the specific enzymatic cleavage of the DNA restriction enzymes were used. The DNA was incubated at 37 °C for 3 h or overnight with a corresponding enzyme and buffer in a heating block. For each batch, the final volume was adjusted to 20 µL or 40 µL. The amount of DNA used was 100 ng - 1 µg per batch (Tab. 10). Finally, the restriction enzyme was inactivated for 15-20 minutes with a corresponding enzyme dependent temperature (65 °C or 80 °C) and the DNA mixture was purified by DNA precipitation. Some enzymes can function in the same buffer. In a double digestion, in which the restriction enzymes do not have full activity in the same buffer, the DNA was precipitated after the first digestion. After the digestive reaction, the DNA fragments were verified and documented by agarose gel electrophoresis.

Table 10. Components for restriction endonuclease digestion.

DNA	100 ng – 1 µg
Restriction endonuclease	1 – 2 µL
10x restriction enzyme buffer	2 – 4 µL
ddH_2O	fill up to 20 or 40 µL
Total volume	20 or 40 µL

Ligation

For the enzymatic linking of the DNA fragments, a ligation was performed. DNA fragments were used in a molar ratio of vector to insert 1:5 and mixed in a 10 µL reaction with T4 DNA ligase and an appropriate buffer (Tab. 11). Here, the previously digested vector and insert DNA were ligated by the activity of the T4 DNA ligase. For the ligation of DNA fragments the mixture was incubated at 16 °C overnight. The control was done by ligating the vector DNA and ddH_2O instead of insert DNA. Subsequently, the ligase was thermally inactivated for 20 minutes at 65 °C. Afterwards, a restriction digest was performed in order to eliminate religated plasmids. Here, the restriction enzyme (*Spe*I) was chosen so that it cuts in the vector sequence, which should be replaced with the desired DNA insert.

Table 11. Components for the ligation mixture.

Vector DNA	105 ng (3 µL)
Insert DNA (molar vector:insert ratio 1:5)	40 ng (4 µL)
T4 DNA ligase (1U)	1 µL
10x Tango buffer (for *Spe*I)	1 µL
ATP (5 mM)	1 µL
Total volume	10 µL

Sequencing of DNA fragments

The DNA sequencing was performed by the company GATC-Biotech (Light Run), Cologne, Germany. 5 µL of DNA at a concentration of 80-100 ng/µL (for plasmid DNA) or 20-80 ng/µL (for PCR-products) was mixed with 5 µL of the appropriate primer (each reaction used only one primer, either forward or reverse) with a concentration of 5 pmol/µL in a 1.5 mL Eppendorf tube. 10 µL of the mixture was prepared for each sample and sent for sequencing.

Preparation of electrocompetent cells

Electrocompetent cells were prepared for the electroporation. First, a 10 ml preculture with the appropriate antibiotic was incubated overnight (for *E. coli* strains DH10B and S-17) at 37 °C and 180 rpm. Afterwards, an 80 ml main culture was inoculated 1: 100 with the preculture. The bacterial culture was then incubated up to an OD_{600} of 0.5-0.8 and pre-cooled for 15-30 minutes on ice. The cells were spun down for 15 minutes at 4000 g and 4 °C and then resuspended in 80 ml of cold water. After another centrifugation for 10 minutes at 4000 g and 4 °C, the cell pellet was resuspended in 40 ml of cold water. The centrifugation was again repeated for 10 minutes at 4000 g and 4 °C. The pelleted cells were resuspended in 2 ml of 10% glycerol and again centrifuged for 10 minutes at 4000 g and 4°C. Subsequently, the cell pellet was resuspended in 800 µl of 10% (v/v) glycerol and divided into 40 µl aliquots. Finally, the bacterial cells were frozen in liquid nitrogen and stored at -80 °C.

Transformation by electroporation

For the transformation of *E. coli* with recombinant plasmids approximately 50 ng of DNA was used. For this, the DNA solution was dialyzed on a membrane filter with a pore size of 0.025 µM in ddH_2O for 45 minutes (MF-Millipore™ membrane filter, 0.025 µM pore size; Merck Millipore Ltd). The electroporation cuvettes (0.1 cm cuvette) were pre-cooled on ice. Aliquots (40 µL) of electrocompetent cells were also thawed on ice. Dialyzed plasmid DNA was mixed with 40 µL of electrocompetent cells

and transferred after 1 min of incubation on ice in electroporation cuvettes. Subsequently, an electric pulse (1.8 kV) was applied. Afterwards, the cells were mixed with 1 mL of warmed SOC medium and incubated for 1 hour at 37 °C in an incubator. After incubation, 50 µL, 100 µL and 200 µL of transformed cells were plated on selection plates with the appropriate antibiotic and incubated at 28 °C (*E. coli* S17-1) or 37 °C (*E. coli* DH10B) overnight.

SOC-medium	2% (w/v) tryptone
	0.5% (w/v) yeast extract
	10 mM NaCl
	2.5 mM KCl
	10 mM $MgCl_2$ x 6 H_2O
	10 mM $MgSO_4$ x 7 H_2O
	20 mM glucose in d H_2O

DNA transfer by bi-parental conjugation

Plasmid DNA was transferred into *S. meliloti* by bi-parental conjugation with the *E. coli* S17-1 strain carrying the desired plasmid. The plasmids were isolated and transformed by electroporation into the *E. coli* S17-1 strain which has the necessary transfer genes. This allowed the transfer and integration of the plasmids into the *S. meliloti* genome via homologous recombination. First, 10 mL of TY medium with the appropriate antibiotic were inoculated with a single colony of *S. meliloti* 2011, and incubated for 2-3 days at 28 °C and 180 rpm. In addition, 5 mL of LB medium with the appropriate antibiotic were inoculated with a single colony of *E. coli* S17-1 carrying the desired plasmid and incubated at 28 °C and 180 rpm overnight. Subsequently, 1 mL from each of the cultures was spun down for 5 minutes at 3000 rpm and RT, washed twice with 1 mL of physiological saline solution (0.9% (w/v) NaCl) and spun down again. After centrifugation, the cells of *S. meliloti* 2011 and *E. coli* S17-1 carrying the desired plasmid were resuspended in 100 µL of physiological saline solution. 20 µL of the resuspended cells of *E. coli* and 30 µL of the resuspended cells of *S. meliloti* were mixed in a reaction vessel. The mixed cells were suspended in a drop and carefully placed on top of a TY medium plate without antibiotic, dried and incubated for 2 days at 28 °C. Subsequently, the bacterial cell lawn was harvested with an inoculating loop and resuspended in 1 mL of physiological saline solution. From this, dilutions ranging from 10^{-1} to 10^{-7} were prepared and 100 µL of each dilution were plated. The undiluted solution, as well as dilutions of 10^{-1}, 10^{-2} and 10^{-3}, were plated on TY plates with

nalidixic acid and tetracycline. Dilutions of 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} were plated on TY plates with nalidixic acid and LB plates with tetracycline. The plates were incubated for 5 days at 28 °C and were checked in between if colonies are visible. Subsequently, after single colonies appeared on TY plates with nalidixic acid and tetracycline, four clones were replated on TY plates with nalidixic acid and tetracycline. Once more, after single colonies appeared on these plates, four clones were replated on TY plates with tetracycline. Correct clones were then further analyzed.

2.2.4 Plant nodulation tests

Sterilization of seeds

The seeds of *Medicago truncatula* were initially submerged in concentrated H_2SO_4 and incubated for 5–10 minutes with agitation. Seeds were then washed with big volumes of deionized water under an open tap and transferred to a 2 mL Eppendorf tube. Seeds were then sterilized with sodium hypochlorite (6%) diluted with sterile H_2O and a drop of Tween (2%). The solution was mixed by inversion and incubated for 45 seconds at RT. Afterwards the seeds were washed 6-10 times with sterile water. Subsequently, the seeds were placed on 0.8% sloped water agar plates and placed in a refrigerator at 4 °C for 3 days. After 3 days, seeds were transferred to a dark place for 24 hours at RT. If incubation longer than 24 h was required, seeds were transferred to a growth chamber with a light/dark cycle.

Media preparation

The B&D (Broughton and Dilworth, 1971) medium was prepared by mixing stock solutions A, B_1, B_2, C and D (Tab. 12). The pH of the medium was adjusted to 6.8 with stock solutions B_1 and B_2.

Table 12. Components of the B&D medium.

Stock solution	Component	Concentration of the stock solution	Final concentration in µM
Stock solution A	$CaCl_2 \bullet 2H_2O$	2 M	1000
Stock solution B_1	KH_2PO_4	1 M	500
Stock solution B_2	$K_2HPO_4 \bullet 3\ H_2O$	1 M	500
Stock solution C	Fe citrate	0.02 M	10
Stock solution D	$MgSO_4 \bullet 7H_2O$	0.5 M	250
	K_2SO_4	0.5 M	K: 1500; S:500
	$MnSO_4$	0.002 M	1
	H_3BO_3	0.004 M	2
	$ZnSO_4 \bullet 7H_2O$	0.001 M	0.5
	$CuSO_4 \bullet 5H_2O$	0.004 M	0.2
	$CoSO_4 \bullet 7H_2O$	0.002 M	0.1
	$Na_2MoO_4 \bullet 2H_2O$	0.002 M	0.1

Inoculation of *M. truncatula* with *S. meliloti*

S. meliloti strains were grown in 5 mL liquid TY cultures for 2 days at 28 °C and 180 rpm with the appropriate antibiotic. Two milliliters of the bacterial cultures were spun down for 5 minutes at 3000 rpm and RT. The cell pellet was resuspended in 1 mL of sterile water and additionally washed two more times with sterile water and finally resuspended in 1 mL of sterile water. From this cell suspension dilutions were made with sterile water to achieve the desired OD_{600} (0.05-0.1 A). The plants were then inoculated with 400 µL of diluted bacterial cell suspension per plant and 100 µL of the diluted cell suspension was plated on agar plates to check colony growth.

***M. truncatula* growth conditions in sloped agar tubes**

After germination on sloped water agar plates, seeds were transferred into B&D sloped agar tubes (1.5% of Merck or Kalys agar). The tubes were prepared by pouring 20 mL of B&D medium into the tubes and while the agar is still warm laying the tubes diagonally to get the slope and leaving them to solidify. It is known that ethylene accumulation inhibits plant growth. Therefore, when plant growth inhibition was observed, an ethylene synthesis inhibitor (AVG; Sigma Aldrich) was added to the medium at a concentration of 0.2 µM with great effect. AVG was added after sterilization when the medium was at 50 °C. The plants were grown for a varied number of days (3-35 days) in a plant chamber with a light/dark cycle of 16/8 hours at 23 °C during the light and 18 °C during the dark cycle and a light intensity of 200 µmol m^{-2} s^{-1}. In order to protect the roots from the light the tubes were wrapped in the root region with aluminum foil.

***M. truncatula* growth conditions in Magenta boxes**

Plants were grown in Magenta boxes (Magenta GA7 300 ml; Magenta) with a vermiculate/sand ratio of 1/1, and regularly watered with water B&D medium. The plants were grown for 21 or 28 days in a plant chamber with a light/dark cycle of 16/8 hours at 23 °C during the light and 18 °C during the dark cycle and a light intensity of 200 µmol m^{-2} s^{-1}.

Glucuronidase reaction

M. truncatula plants inoculated with different *S. meliloti* strains were grown for a varied number of days (3-21 days). After harvesting, shoots were discarded, while roots were cut into smaller pieces and placed into 1-2 mL of GUS buffer after which vacuum was used for 15 minutes to infiltrate the roots with the buffer (Tab. 13). Samples were then incubated for 6 hours or overnight at 37 °C. Nodules, collected from plants 21 days after inoculation, were incubated for 11 hours at 37 °C. After incubation, samples were washed 3 times with 50 mM HEPES for 10 minutes at 350 rpm and RT on a thermomixer. Finally, samples were fixed with 5% glutaraldehyde (GA) and 4% paraformaldehyde (PFA) for 10-60 minutes at 350 rpm and RT on a thermomixer and then stored in 4 °C. Samples were later put on microscope slides and examined. Some were counterstained with 0.05% or 0.1% Ruthenium red (incubation between 30 seconds and 15 minutes). Samples stained 21 days after inoculation were sent to the Biotechnology Center of the TU Dresden, Dresden for embedding in Technovit 7100 and slicing into 10 µm sections. Samples were also counterstained with 0.05% Ruthenium red for 15 minutes. Afterwards, the samples were mounted in Entellan (Merck) medium and covered with a cover slip. Sections were examined by light microscopy.

Table 13. Components of the GUS buffer.

Solution	Stock concentration	Final concentration
Sodium phosphate buffer pH 7.2	100 or 200 mM	50 or 100mM
Tween	2%	0.1%
EDTA	500 mM	1 mM
X-Gluc	100 mM	4 mM
ddH_2O		

2.2.5 Bioinformatic analysis and databases used

ApE (A plasmid Editor)
This program was used for the analysis of DNA sequences.
Chromas2LITE
This program was used to evaluate GATC sequencing chromatograms.
http://genome.microbedb.jp/rhizobase/
Genome database of rhizobia
http://blast.ncbi.nlm.nih.gov/Blast.cgi

3. RESULTS

3.1 Cloning strategy for creating the reporter gene fusion and characteristics of the vector

A translational reporter gene fusion of the *uidA* and *emrA* gene was constructed to perform expression analyzes. A plasmid containing a translational reporter gene fusion of the *lacZ*, *uidA* and *emrR* genes was received and used in this study. The gene *uidA* encodes the β-glucuronidase enzyme, which cleaves glucoronides. The two fusions represent a reporter gene construct, as the product of the enzymatic reaction can be measured.

The *uidA* gene was cloned in a previous experiment in a derivative of the cloning vector pSUPPOL2. The vector pSUPPOL2_uidA contains the following genes: *tetR* coding for tetracycline resistance, *cmR* coding for kanamycin resistance, *mob* (mobilization region) for the transfer of the plasmid during conjugation and *uidA* coding for β-glucuronidase. The size of the plasmid is 9847 bp. The plasmid pSMD001, which contains the *emrR-lacZ-uidA* fusion, was constructed in the previous work of A. Welker. The plasmid contains the following genes: *tetR* coding for tetracycline resistance, *mob* region for the transfer of the plasmid during conjugation, *lacZ* coding for β-galactosidase and *uidA* coding for β-glucuronidase. The size of the plasmid is 12468 bp.

Initially a vector based on the plasmid pSUPPOL2_uidA was constructed to transfer the *emrA-uidA* reporter gene fusion in the desired bacterial strain. The homologous region was obtained from the genomic DNA of *S. meliloti* by PCR with the oligonucleotides emrR_for and emrA_ATG. The homologous region includes the *emrA* start codon, the upstream intergenic region between *emrA* and *emrR* and a part of the *emrR* gene. The length of the homologous region was 703 base pairs. The primer emrR_for had a recognition sequence for the restriction enzyme *Xba*I and an additional sequence (ATAT) for the optimization of the restriction endonuclease digestion.

Then, the amplified homologous region was cleaved by restriction endonuclease digestion at the 5' end. The plasmid pSUPPOL2_uidA was cleaved by restriction endonuclease digestion at the *Xba*I and *Stu*I restriction sites. The homologous region was ligated into the plasmid so that the homologous region is in front of the *uidA* gene. The modified vector was transferred into the *E. coli* DH10B strain via transformation by

electroporation. The clones that had the recombinant plasmid were selected based on tetracycline resistance. There were 5 colonies tested by colony PCR with the primer pair pSUPPOLfor and uidA_974r, to verify the integration of the homologous region into the plasmid. The desired PCR amplified fragments with the expected length of 1973 bp were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. Smaller fragments (1230 bp) do not contain the insert. The expected band patterns were confirmed for 3 of the 5 selected clones (Fig. 4).

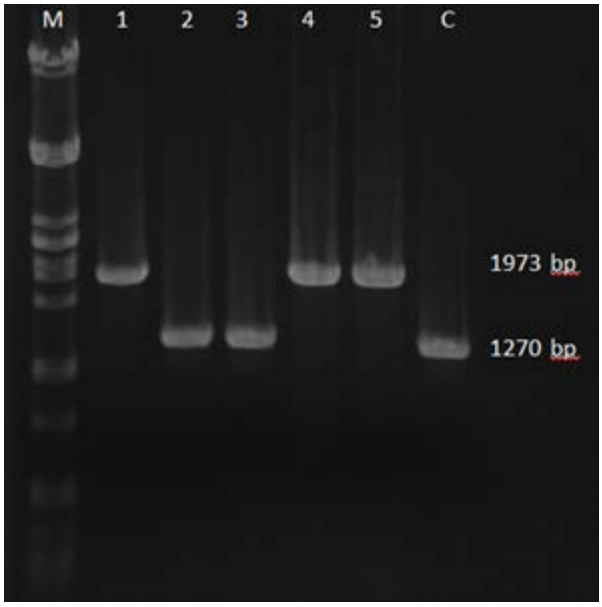

Figure 4. Colony PCR for verifying the recombinant plasmids.
The homologous region from the genome of *S. meliloti* was cloned into the plasmid vector pSUPPOL2_uidA. The recombinant plasmids were verified by colony PCR. The expected length of the fragment with the insert was 1973 bp (clones 1, 4 and 5). The length of the fragment without the insert was 1270 bp (clones 2 and 3). M: Lambda DNA/*Pst*I marker. C: insert-free vector pSUPPOL2_uidA.

The plasmid DNA (pSMD013) was isolated from clones 1, 4 and 5, and verified by restriction digestion with the *Pst*I enzyme, which cuts once in the plasmid and once in the inserted homologous region, and resolved by agarose gel electrophoresis. All three clones showed the two expected band patterns of 2165 and 8366 bp (Fig. 5). Then, the plasmid pSMD013 from clones 1, 4 and 5 was isolated and sent for sequencing to GATC, Cologne. All three clones had the correct sequence, so clone 5 was used in further work.

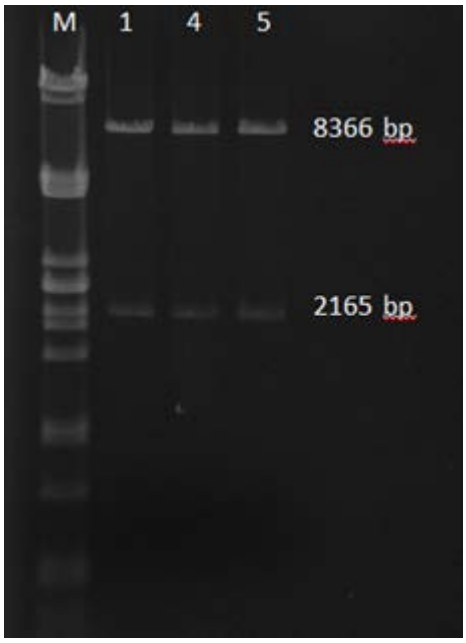

Figure 5. Restriction digestion of plasmid pSMD013 with *Pst*I.
The expected band patterns of 2165 and 8366 bp were shown in all three clones after restriction digestion with *Pst*I, which cuts twice in the pSMD013 plasmid. M: Lambda DNA/*Pst*I marker.

The plasmid pSMD013 was isolated and transformed by electroporation into the *E. coli* S17-1 strain. Desired clones were selected based on tetracycline resistance. The plasmid pSMD013 was transferred to *S. meliloti* via bi-parental conjugation. In the same way, the plasmid pSMD001 was transferred to *S. meliloti*. The plasmids were integrated into the genome via homologous recombination. The plasmid pSMD001 was integrated in front of the *emrR* gene, while pSMD013 was integrated in front of the *emrA* gene. The desired *S. meliloti* transformants were selected based on nalidixic acid and tetracycline resistance.

By integrating the plasmids into the *S. meliloti* genome, two new strains were created: *S. meliloti* SMD013 with the integrated plasmid pSMD013 and *S. meliloti* SMD014 with the integrated plasmid pSMD001. First, eight clones of SMD013 and eight clones of SMD014 were verified by colony PCR for specific *S. meliloti* genes with primers SmnodDfor and SmnodArev. These primers bind in the region between the nodulation genes *nodD* and *nodA*, and produce a fragment of 296 bp. All but one clone of SMD014 showed the expected PCR fragment (Fig. 6). This one clone was tested again and showed the expected PCR fragment (not shown).

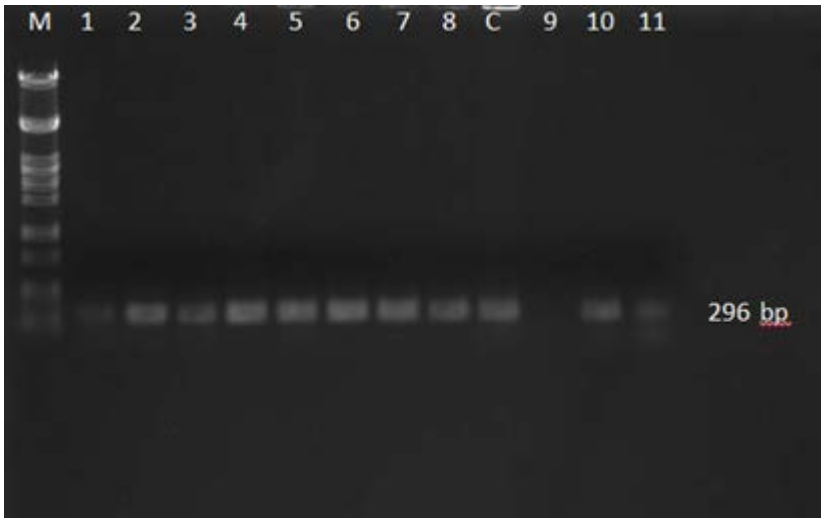

Figure 6. Verifying the clones for *S. meliloti* specific genes.
Clones of SMD013 and SMD014 were verified for *S. meliloti* specific nodulation genes. Expected fragment size is 296 bp. All but one SMD014 clone show the expected fragment. M: Lambda DNA/*Pst*I marker. 1-8: *S. meliloti* SMD013. C: *S. meliloti* 2011 control. 9-11: *S. meliloti* SMD014.

Clones were then verified for the integration of the plasmid. For eight clones of SMD013, primers 03169R3 and uidA_974r were used. These primers bind in the homologous region and the *uidA* gene and produce a fragment of 1230 bp (Fig. 7).

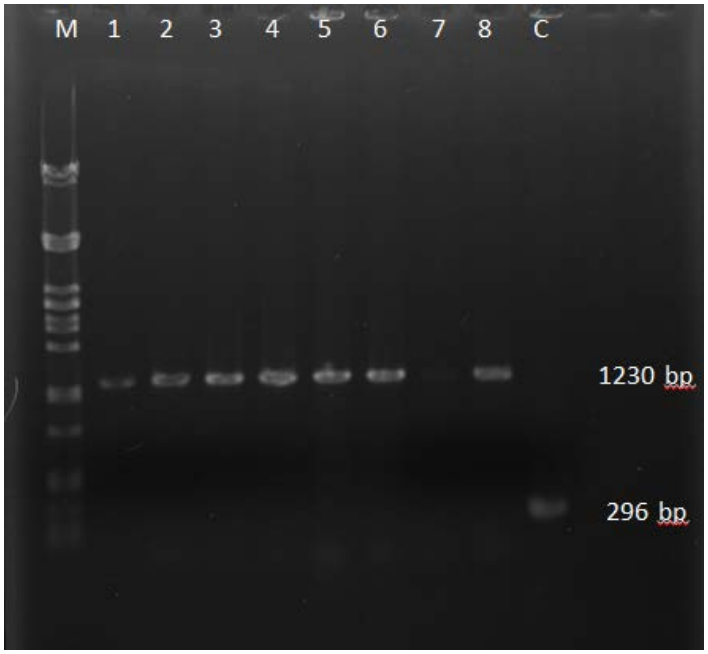

Figure 7. Verifying the integration of pSMD013 into the genome of *S. meliloti*.
Primers 03169R3 and uidA_974r bind in the homologous region and the *uidA* gene and produce a fragment of 1230 bp if the integration was successful. M: Lambda DNA/*Pst*I marker. 1-8: *S. meliloti* SMD013. C: *S. meliloti* 2011 control.

Furthermore, to verify the correct integration, primers Sm_RTO3168 and pSUPPOLfor were used. These primers bind in the *emrA* gene and the plasmid, both outside the

homologous region, and produce a fragment of 1139 bp (Fig. 8).

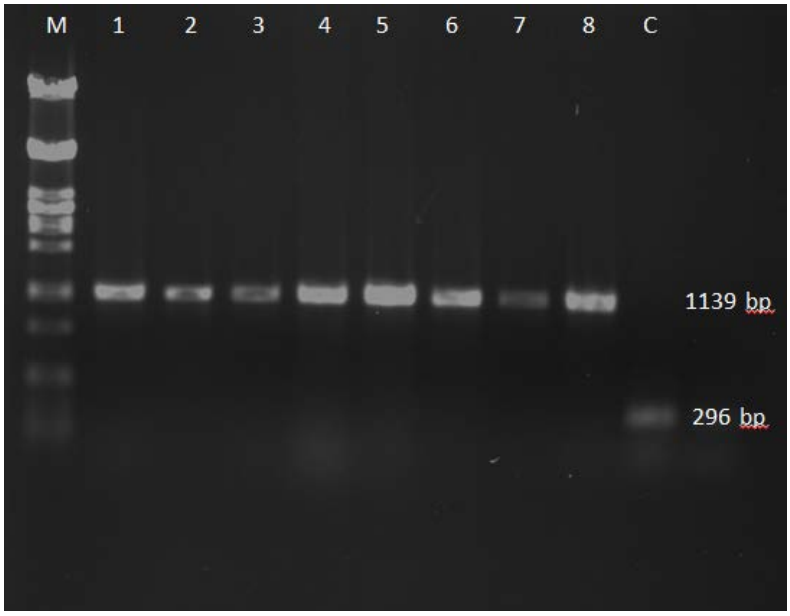

Figure 8. Verifying the correct integration of pSMD013 into the genome of *S. meliloti*.
Primers Sm_RTO3168 and pSUPPOLfor bind in the *emrA* gene and the plasmid, both outside the homologous region, and produce a fragment of 1139 bp if the integration was successful. M: Lambda DNA/*Pst*I marker. 1-8: *S. meliloti* SMD013. C: *S. meliloti* 2011 control.

In seven clones the integration of the plasmid was verified. The integration of the plasmid in clones of SMD014 was verified with the primers lacZ_pSUP_rev and Sm_P1F_N3F, which bind in the *lacZ* gene and the homologous region, and produce a fragment of 464 bp (Fig. 9).

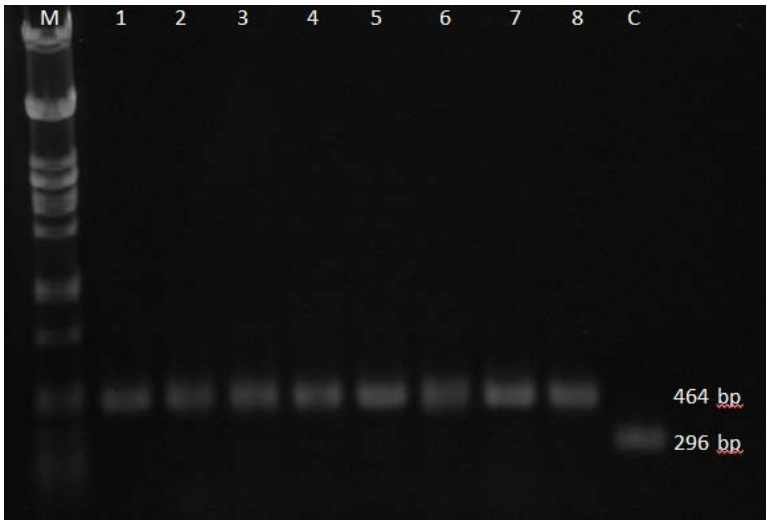

Figure 9. Verifying the integration of pSMD001 into the genome of *S. meliloti*.
Primers lacZ_pSUP_rev and Sm_P1F_N3F bind in the *lacZ* gene and the homologous region, and produce a fragment of 464 bp if the integration was successful. M: Lambda DNA/*Pst*I marker. 1-8: *S. meliloti* SMD014. C: *S. meliloti* 2011 control.

All eight clones showed the integration of the plasmid into the genome. Additionally, one clone of SMD014 was verified for the correct integration, using primers lacZ_pSUP_rev and 03169_KInt, which bind in the *lacZ* gene and the *emrA* gene outside the homologous region. These primers produce a fragment of 1109 bp (Fig. 10). The clone showed the correct integration of the plasmid. This confirmed the creation of two new *S. meliloti* strains, SMD013 and SMD014.

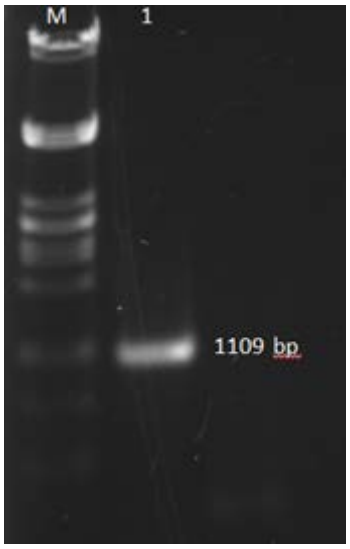

Figure 10. Verifying the correct integration of pSMD001 into the genome of *S. meliloti*.
Plasmids lacZ_pSUP_rev and 03169_KInt bind in the *lacZ* gene and the *emrA* gene outside the homologous region and produce a fragment of 1109 bp if the integration was successful. M: Lambda DNA/*Pst*I marker. 1: *S. meliloti* SMD014.

3.2 Plant growth medium optimization

Plants were first grown in sloped Merck agar tubes. However, two weeks after inoculation plants started to lose color and leaves. It is known that ethylene accumulation inhibits cell divison, DNA synthesis and growth in roots and shoots (Burg, 1973). Therefore, an ethylene synthesis inhibitor, aminoethoxyvinylglycine (AVG), was added to the media. AVG is an ethylene synthesis inhibitor that inhibits ACC synthase activity which converts S-adenosylmethionine to ACC, which is the immediate precursor of ethylene.

To test the beneficial effect of AVG on plant growth, *M. truncatula* plants were grown in three different growth conditions. Plants were inoculated with the *S. meliloti* 2011 strain and grown in sloped Merck agar tubes without AVG, in sloped Kalys agar tubes without AVG and in sloped Kalys agar tubes with 0.2 µM AVG. Plant growth was monitored for 35 days. Already, after 18 days the difference between the growth

media was apparent. Plants grown on AVG looked much greener and healthier than the ones grown without AVG, which started showing signs of stress and losing color. After 35 days the difference was even greater. Plants grown with AVG had grown bigger and looked much healthier than the ones without AVG, which also started losing leaves (Fig. 11).

Figure 11. Comparison of *M. truncatula* plants inoculated with *S. meliloti* 2011 and grown with or without AVG after 35 days
After 35 days plants grown without AVG on Merck agar (left) and Kalys agar (center) lost color and leaves and look much worse than ones grown with 0.2 µM AVG (right), which grew bigger and have a healthy, green color.

Although plants grown in sloped agar tubes did show pink nodules about two weeks after inoculation, none of them were elongated. For this reason, a new setup for the phenotypical nodulation analysis was made, in which plants were grown in Magenta boxes. Unfortunately, due to lack of time, the expression analysis of *emrA* and *emrR* was made only on plants grown in sloped agar tubes.

3.3 Expression analysis of *emrA* and *emrR*

After constructing the strains SMD013 and SMD014, expression patterns of *emrA* and *emrR* were determined, using a glucuronidase staining assay. Both strains have the *uidA* gene that codes for β-glucuronidase. When X-Gluc is added, the β-glucuronidase cleaves X-Gluc to produce colorless glucuronic acid and an intense blue precipitate of chloro-bromoindigo. This blue coloration indicates the activity of *emrA* and *emrR*. After inoculating *M. truncatula* plants with *S. meliloti* SMD013 and SMD014 strains, plants were grown in sloped Kalys agar tubes with 0.2 µM AVG.

Their roots were harvested at various time points and stained. First, the expression of *emrA* was studied in the early nodulation stage, which is the first week after infection when infection threads are formed. It was shown that the gene is expressed in infection threads (Fig. 12).

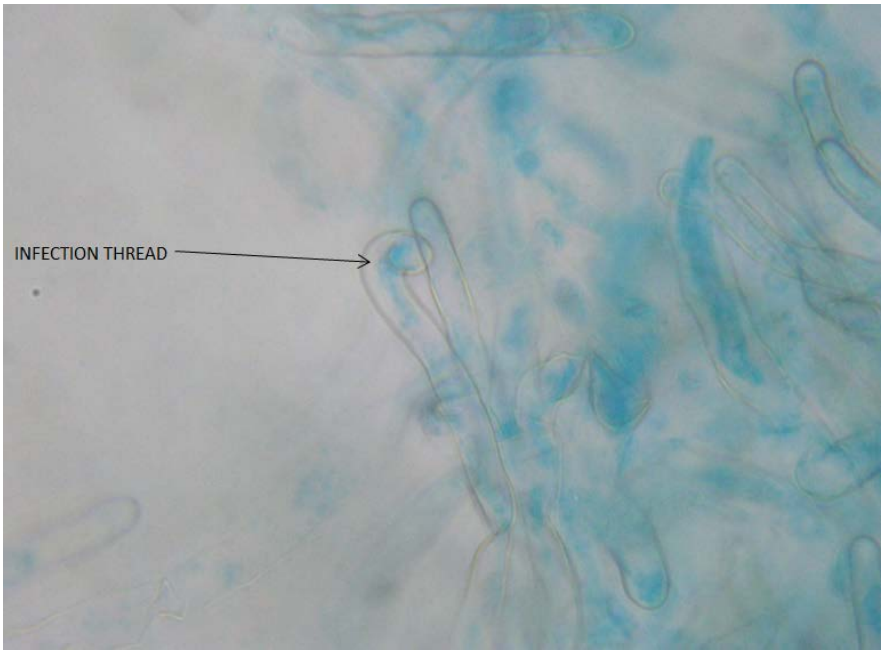

Figure 12. Expression of *emrA* in infection threads 7 days post inoculation (100x)
Infection threads formed in *M. truncatula* root hairs after infection with *S. meliloti* SMD013 and glucuronidase staining. Blue coloration indicates *emrA* expression.

Afterwards, the expression of the gene was observed in mature nodules. For this purpose, sections of developed nodules were made and stained 21 days post inoculation because then all nodule zones should be visible. It was shown that in the developed nodule, *emrA* was expressed in the infection zone and the nitrogen fixation zone (Fig. 13). Additionally, the gene was expressed in the infection threads formed inside the nodule (Fig. 14).

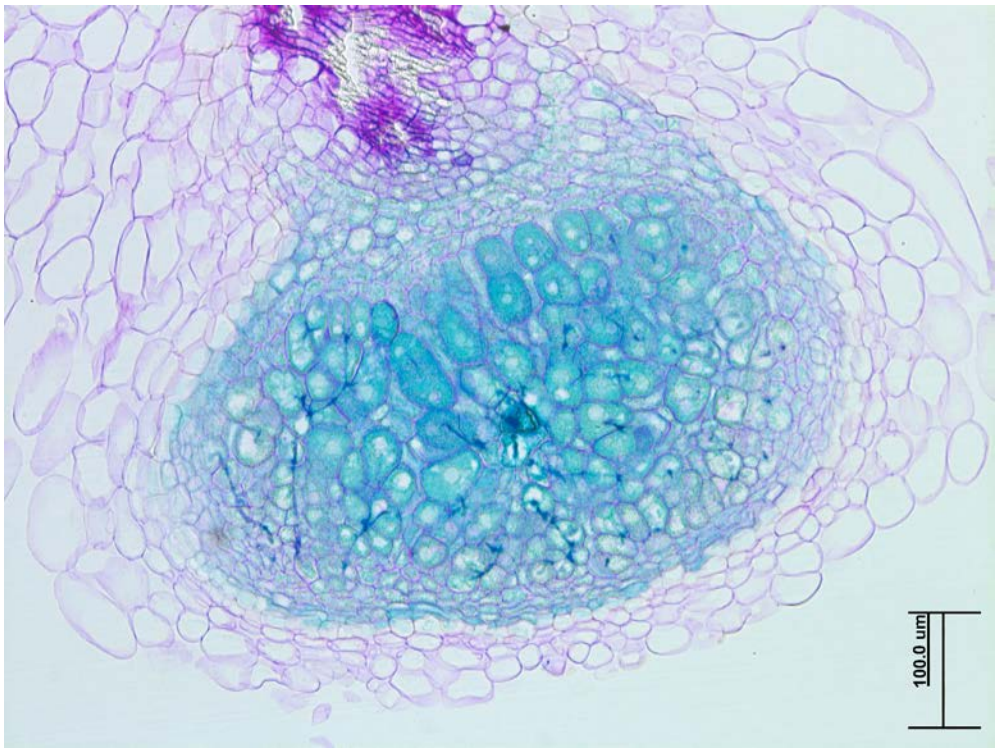

Figure 13. Expression of *emrA* in the nodule 21 days post inoculation
Nodules formed on M. truncatula roots after infection with S. meliloti SMD013 and glucuronidase staining. Blue coloration indicates *emrA* expression. Sample was counter-stained with 0.1% Ruthenium red.

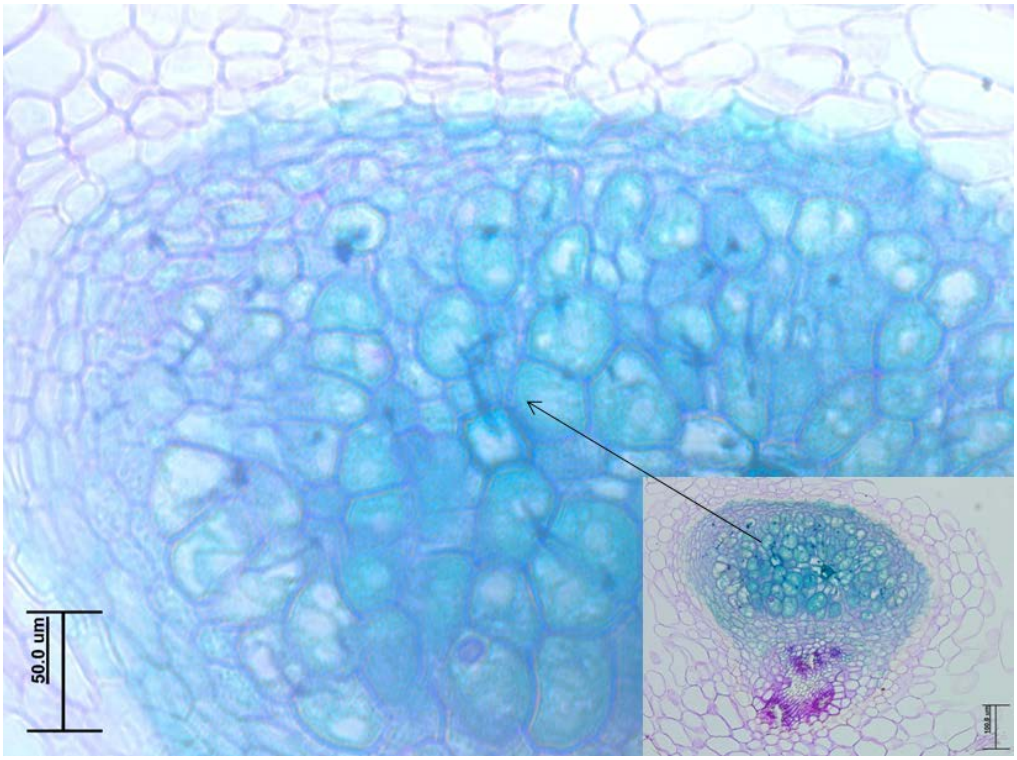

Figure 14. Expression of *emrA* in infection threads in the nodule 21 days post inoculation
Infection threads were formed in nodules on *M. truncatula* roots after infection with *S. meliloti* SMD013 and glucuronidase staining. Blue coloration indicates *emrA* expression. Sample was counter-stained with 0.1% Ruthenium red.

After examining roots of *M. truncatula* inoculated with *S. meliloti* SMD014, the expression of *emrR* was also shown in infection threads (Fig. 15).

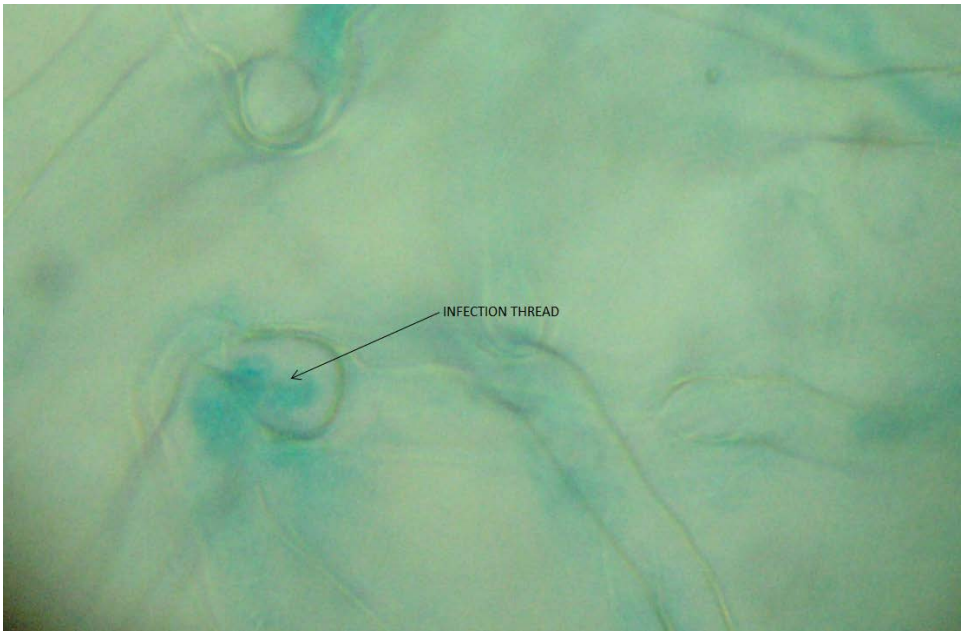

Figure 15. Expression of *emrR* in infection threads 4 days post inoculation (400x)
Infection thread formed in *M. truncatula* root hairs after infection with *S. meliloti* SMD014 and glucuronidase staining. Blue coloration indicates *emrR* expression.

The expression of *emrR* was also confirmed in the developed nodule 21 days post inoculation. The gene was expressed only in the infection zone (Fig. 16). Infection threads inside the nodule did not show the expression of the *emrR* gene.

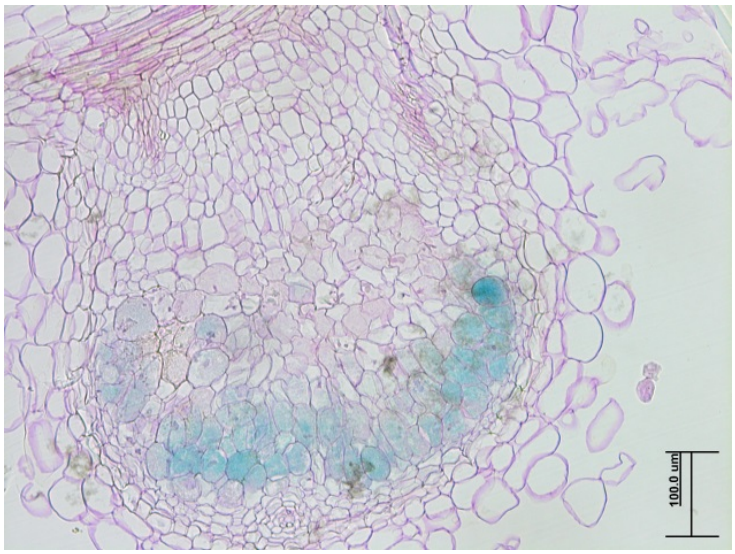

Figure 16. Expression of *emrR* in the nodule 21 days post inoculation
Nodules formed on *M. truncatula* roots after infection with *S. meliloti* SMD014 and glucuronidase staining. Blue coloration indicates *emrR* expression. Sample was counter-stained with 0.1% Ruthenium red.

3.4 Phenotypical nodulation analysis of *M. truncatula* plants inoculated with *S. meliloti* SMD011, SMD012 and the wild type

A phenotypical analysis of nodulation of two *S. meliloti emrBAR* deletion mutant strains and the wild type (*S. meliloti* 2011), in symbiosis with *M. truncatula,* was conducted. The two mutant strains have the *emrBAR* efflux genes deleted by introducing a kanamycin resistance cassette in the coding sequence. Two mutant strains were used because the kanamycin promoter is a strong promoter, so it might have an effect on neighbouring genes, depending on its orientation. The SMD011 mutant strain has the cassette oriented in the direction of the smc03166 gene, while the SMD012 mutant strain has the cassette oriented in the direction of smc03170 gene. In order to exclude the effect of the kanamycin promoter on one of the surrounding genes, both mutant strains should show an increase in nodule numbers in comparison with the wild type.

3.5 Phenotypical nodulation analysis in sloped agar tubes

M. truncatula plants were inoculated with *S. meliloti* and divided into 4 groups: 15 plants were inoculated with each of the three strains (2011, SMD011 and SMD012), plus 2 uninfected controls. Plants were grown in sloped Kalys agar tubes with 0.2 µM aminoethoxyvinylglycine (AVG) and monitored for 35 days. Plants grown with AVG looked green and healthy, and didn't show signs of stress (Fig. 11). One plant inoculated with the SMD011 strain showed stunted growth since the beginning and was excluded from the results.

Average nodule number in each group gradually increased after each time point (Fig. 17). After 7 days there was no significant difference in nodule numbers. After 14 days there was a significantly higher number of nodules in plants inoculated with the SMD012 strain in comparison with plants inoculated with the SMD011 strain ($p<0.05$) and the wild type ($p<0.001$). This trend also continued after 21, 28 and 35 days. In addition, after 28 and 35 days there was a significantly higher number of nodules in plants inoculated with the SMD011 strain in comparison with plants inoculated with the wild type ($p<0.05$). As expected, uninfected control plants didn't develop any nodules. After 35 days average dry shoot weight was measured, but there was no significant difference between the groups (Tab. 14, sloped agar tubes).

Table 14. Average dry shoot weight of *M. truncatula* plants inoculated with different *S. meliloti* strains measured 21, 28 or 35 days post inoculation.

Inoculated strain	***Dry shoot weight (sloped agar tubes, 35 dpi)/g***	***Dry weight (Magenta boxes, 21dpi)/g***	***Dry weight (Magenta boxes, 28dpi)/g***
S. meliloti 2011	0.009553	0.06968	0.08104
S. meliloti SMD011	0.009657	0.04286	0.07022
S. meliloti SMD012	0.009773	0.05503	0.04946

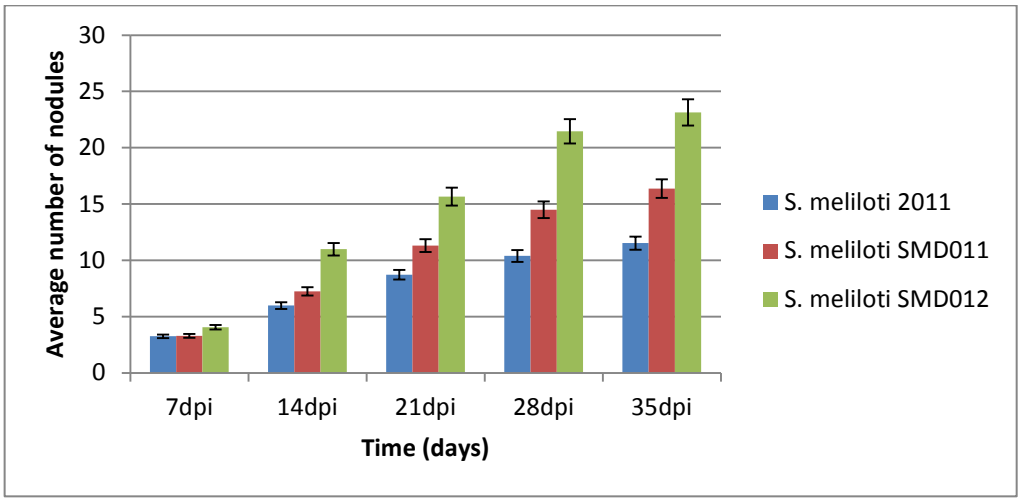

Figure 17. Comparison of average nodule numbers of *M. truncatula* plants inoculated with different *S. meliloti* strains (sloped agar tubes).
M. truncatula plants were inoculated with three different *S. meliloti* strains (2011, SMD011 and SMD012). Nodules were counted at 7, 14, 21, 28 and 35 days post inoculation (dpi). There is a significantly higher nodule number in the SMD012 group in comparison with the SMD011 ($p<0.05$) and 2011($p<0.001$) groups after 14, 21, 28 and 35 days. There is also a significantly higher nodule number in the SMD011 group in comparison with the 2011 group ($p<0.05$) after 28 and 35 days.

3.6 Phenotypical nodulation analysis in Magenta boxes

M. truncatula plants were inoculated with *S. meliloti* and divided into 4 groups: 20 plants were inoculated with each of the three strains (2011, SMD011 and SMD012), plus 4 uninfected controls. Plants were grown in Magenta boxes with a vermiculate/sand ratio of 1/1 and then dug out and examined after 21 and 28 days. At both time points, 10 plants per each strain were examined. Plants clearly showed elongated, pink nodules (Fig. 18).

Figure 18. *M. truncatula* plants inoculated with *S. meliloti* 2011 after 28 days.
Plants were grown in Magenta boxes, then dug out and examined after 28 days. Elongated, pink nodules are clearly visible.

Although, plants inoculated with the wild type showed a higher number of nodules than plants inoculated with the SMD011 and SMD012 strains after 21 days, there was no significant difference. Similarly, there was no significant difference in nodule numbers after 28 days (Fig. 19). However there was a significant difference in dry weight measured. Plants inoculated with the SMD011 strain and the wild type showed a much higher dry weight than plants inoculated with the SMD012 strain at both time points (Tab. 14, Magenta boxes). Uninfected control plants didn't develop any nodules.

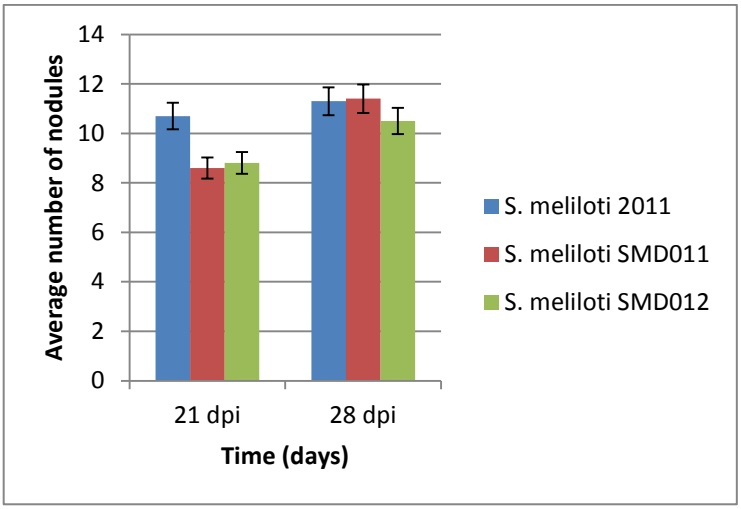

Figure 19. Comparison of average nodule numbers between different *S. meliloti* strains (experiment 3).
M. truncatula plants were inoculated with three different *S. meliloti* strains (2011, SMD011 and SMD012). Nodules were counted at 21 and 28 days post inoculation (dpi). There is no significant difference in nodule numbers between the groups.

3.7 Bacterial growth assay of *S. meliloti* SMD011, SMD012 and the wild type

To examine the influence of the EmrBAR efflux system on bacterial growth, a bacterial growth assay was conducted. Liquid bacterial cultures of *S. meliloti* 2011, SMD011 and SMD012 were grown with the addition of 2-phenylphenol. The growth of the bacteria was represented by their optical density at the wavelength of 600 nm (OD_{600}). It was shown that adding 0.2 mM 2-phenylphenol to the media inhibits growth of all three strains. However, the growth of the wild type is only moderately inhibited, while the growth of the two deletion mutants is almost completely inhibited (Fig. 20).

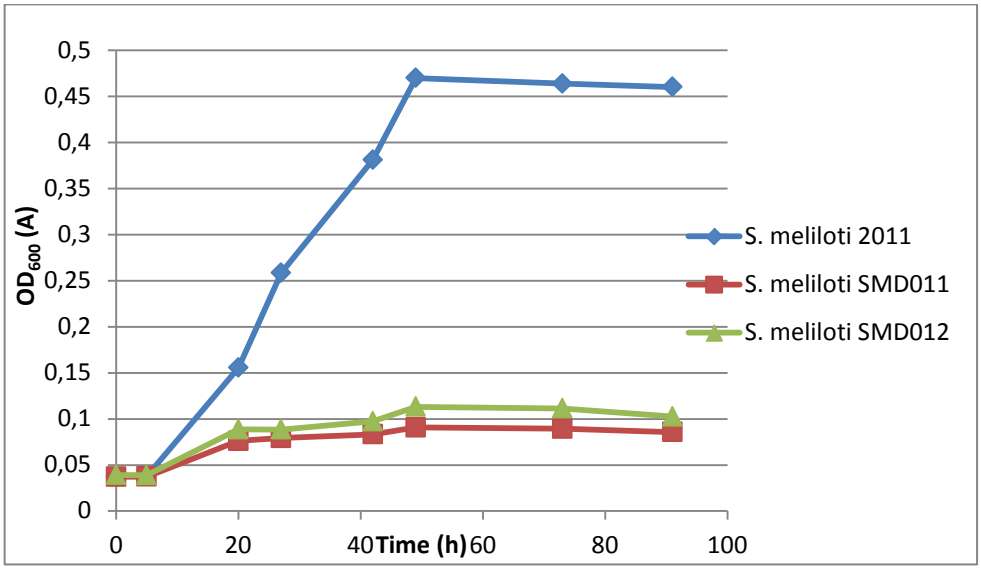

Figure 20. Growth of different *S. meliloti* strains treated with 0.2 mM 2-phenylphenol
Liquid cultures of *S. meliloti* 2011, SMD011 and SMD012 were treated with 0.2 mM 2-phenylphenol. Optical density at the wavelength of 600 nm was measured (OD_{600}). Growth of two deletion mutants (SMD011 and SMD012) is strongly inhibited.

4. DISCUSSION

The expression of the membrane fusion protein EmrA and the transcriptional regulator EmrR was analyzed by translational *emrA-uidA* and *emrR-lacZ-uidA* reporter gene fusions. The product of a reporter gene can be visualized much easier in most cases, than a product of the target gene (Goldenkova et al., 2003). Among the most widespread and widely used regulator gene systems in prokaryotes are β-galactosidase (LacZ) from *E. coli* and β-glucuronidase (GUS), also from *E. coli* (Jefferson et al., 1986).

The LacZ-system is commonly used in bacteria due to its efficiency and sensitivity (Piruzian et al., 2002). Unfortunately, in *S. meliloti* the background activity of β-galactosidase has already been reported (MacLean et al., 2006). The background activity of β-glucuronidase was not detected in *S. meliloti.* The GUS reporter gene system is a good tool for the analysis of gene expression in *S. meliloti* due to the absence of background activity and the availability of numerous commercial substrates (Goldenkova et al., 2003). Therefore, to detect expression patterns of *emrA* and *emrR*, two new *S. meliloti* strains, which have a GUS reporter gene fusion, were constructed. The strain SMD013 has an *emrA-uidA* translational gene fusion, while SMD014 has an *emrR-lacZ-uidA* translational gene fusion.

The expression patterns of *emrA* and *emrR* were determined in a symbiosis between *S. meliloti* and *M. truncatula* plants grown in sloped agar tubes. The expression of the genes was first followed in the early stages of symbiotic interaction. During these first few days after infection, root hairs are colonized by bacterial cells and infection threads started to form. It was previously shown that the *emrA* gene is highly inducible by flavonoids and that an *emrA* mutant does not affect symbiosis with *M. sativa* (Eda et al., 2011; Rossbach et al., 2014). The expression of the *emrA* gene, which was expected due to the naturally secreted plant flavonoids in the first stages of symbiotic interaction, was observed (Fig. 12). On the other hand, it was shown that the inoculation of *M. sativa* with an *S. meliloti emrR* mutant results in small, nitrogen starved plants, which would suggest the importance of *emrR* in symbiotic interactions. The expression of the *emrR* gene was also observed (Fig. 15) and it correlates with previous findings that the EmrR regulator is expressed during the colonization of *M. sativa* root nodules (Santos et al., 2014).

Afterwards, an expression analysis of *emrA* and *emrR* in the mature nodule was

made 21 days post inoculation. At this stage, elongated, fully formed and active nodules that fixate nitrogen should be visible (Fig. 2). Although the nodules were not elongated, they still showed the different nodule zones.

The *emrA* gene was expressed in the infection zone and the nitrogen-fixation zone (Fig. 13). In addition, the infection threads in the nodule also showed *emrA* expression (Fig. 14). In contrast, the *emrR* gene was expressed only in the infection zone, and the infection threads in the nodule did not show *emrR* expression (Fig. 16). This reported expression of both genes in the infection zone, would suggest that a careful balance in their expression is critical in not yet fully differentiated bacteria. Meanwhile, only *emrA* is expressed in the nitrogen-fixation zone and in the infection threads inside the nodule. This would indicate that the EmrAB efflux system is highly active in mature, nitrogen fixing bacteroids, as well as in the actively dividing bacteria inside the infection threads. In contrast, it seems that the EmrR repressor is not active in the bacteroids and infection threads.

To investigate the effects of the EmrBAR efflux system on symbiotic interactions, a phenotypical analysis of nodulation was conducted. It was previously shown that the deletion of *emrA* had no effect on symbiosis (Eda et al., 2011; Rossbach et al., 2014). The deletion of *emrB* also showed no effect on symbiosis (Santos et al., 2014). However, the deletion of *emrR*, as well as the deletion of *emrR* together with *emrA*, showed a negative effect on nodule number and plant growth (Rossbach et al., 2014; Santos et al., 2014). For these reasons, the effect of the deletion of all three genes on symbiosis was tested. *M. truncatula* plants were inoculated with *S. meliloti* SMD011, SMD012 and the wild type. The SMD011 and SMD012 strains have an inserted kanamycin resistance cassette, which results in the deletion of the *emrBAR* genes.

Plants were grown in sloped agar tubes. There was a significantly higher number of nodules in plants inoculated with the SMD012 strain in comparison with plants inoculated with the wild type and the SMD011 strain after 14, 21, 28 and 35 days (Fig. 17). Furthermore, there was a significantly higher number of nodules in plants inoculated with the SMD011 strain in comparison with plants inoculated with the wild type after 28 and 35 days. However, a large number of nodules on plants inoculated with the two deletion strains were small and white. Also, interestingly, there was no significant difference between the plants in dry shoot weight (Tab. 14, sloped agar tubes). It would suggest that the increase in nodule numbers did not provide better plant growth. This

would indicate that the increased nodule numbers are due to most nodules being ineffective in fixing nitrogen, so the plant responds by making new nodules. In addition, while pink nodules were visible, there were no typical, elongated nodules present on plants grown in sloped agar tubes. Therefore, a new setup was made with plants grown in Magenta boxes.

Plants were grown in Magenta boxes, which should eliminate any effects of ethylene accumulation on plant growth that might have affected the plants in sloped agar tubes. All plants grown in Magenta boxes showed pink, elongated nodules. Although, plants inoculated with the wild type showed a slightly higher number of nodules than plants inoculated with the SMD011 and SMD012 strains after 21 days, there was no significant difference. Similarly, there was no significant difference in nodule numbers after 28 days (Fig. 19). However there was a significant difference in dry weight measured after both 21 and 28 days. Dry weight of plants inoculated with the the wild type was much higher in comparison with plants inoculated with the SMD011 and SMD012 strain (Tab. 14, Magenta boxes). These results suggest that, while the *emrBAR* deletion doesn't affect nodule numbers, it might affect overall plant growth and fitness by decreasing nodule efficiency.

The influence of the EmrBAR efflux system on bacterial growth was examined by a bacterial growth assay. It was previously shown that an *emrB* defective mutant has a higher susceptibility to 2-phenylphenol than the wild type (Spini et al., 2014). It is therefore expected that the *emrBAR* deletion mutants would have impaired growth in comparison with the wild type. The growth of the bacteria was represented by their optical density at the wavelength of 600 nm (OD_{600}). It was shown that adding 0.2 mM 2-phenylphenol to the media inhibits growth of both mutant strains and the wild type. However, the growth of the wild type is only moderately inhibited, while the growth of the two deletion mutants is almost completely inhibited (Fig. 20). This could suggest the importance of the EmrBAR efflux system in the extrusion of toxic substances, specifically 2-phenylphenol and its derivatives.

5. CONCLUSIONS

From this research it can be concluded that both, the *emrA* and *emrR* genes are expressed in early symbiotic interactions, during the formation of infection threads. The *emrA* gene is also expressed in the infection and nitrogen-fixation zones, as well as in the infection threads inside the nodule. The *emrR* gene is expressed only in the infection zone of the nodule and is not expressed in the infection threads inside the nodule.

Phenotypical nodulation analysis of the *emrBAR* deletion mutants and the wild type in sloped agar tubes showed a higher nodule number in plants inoculated with the two *emrBAR* deletion strains in comparison with the wild type. However, a large number of these nodules were white and inefficient at fixing nitrogen. In addition, there was no difference in dry shoot weight between the plants. It would suggest that the increase in nodule numbers did not provide better plant growth.

Phenotypical nodulation analysis of the *emrBAR* deletion mutants and the wild type in Magenta boxes showed no significant difference in nodule numbers between plants inoculated with the wild type and the two deletion strains. However, dry weight of plants inoculated with the wild type was higher in comparison with plants inoculated with the deletion mutants. This suggests that, while the *emrBAR* deletion doesn't affect nodule numbers, it might affect overall plant growth and fitness by decreasing nodule efficiency.

A bacterial growth assay showed that 2-phenylphenol inhibits the growth of the two *emrBAR* deletion mutant strains in comparison with the wild type. This seems to indicate the importance of the EmrBAR efflux system in the extrusion of toxic substances. Further studies should investigate the effect of other toxic substances on the growth of the *emrBAR* deletion mutants.

The results of this study will contribute to the work of Prof. Göttfert and his group at the Laboratory for Molecular Genetics, Institute of Genetics at the Department of Biology, Technische Universität Dresden.

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7. CURRICULUM VITAE

PERSONAL INFORMATION

Damjan Mitić

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Sex Male | Date of birth 31/10/1991 | Nationality Croatian

WORK EXPERIENCE

October 2015 – today	Splitska banka (office in Zagreb) – delivery jobs
September 2015	Student Center Zagreb – jobs on the arranging of student dormitories
2011 – today	Occasional proofreading and translation of texts in English
2011	HRT (Croatian Radio-Television) – Work through an author's contract

EDUCATION AND TRAINING

2015 (March 1st –September 1st)	Technische Universität Dresden, Institute of Genetics at the Department of Biology, Laboratory for Molecular Genetics (Erasmus exchange – work on the Master thesis))
2013 – today	Faculty of Science, University of Zagreb, Department of Biology, University graduate study in molecular biology
2010 – 2013	Faculty of Science, University of Zagreb, Department of Biology; title: Bachelor's degree in molecular biology
2006 – 2010	V. Gymnasium, Zagreb
1999 – 2006	Foreign Language School: Butković strain jezici, BSJ (English - 8 degrees; German – 4 degrees)
1998 – 2006	Elementary school Stenjevec, Zagreb

PERSONAL SKILLS

Mother tongue(s): Croatian

Other language(s):

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	C2	C2	C2	C2	C2
German	A2	A2	A2	A2	A2

Communication skills

- Excellent communication skills gained by participating in many scientific projects in both Croatia and abroad.
- Studious researcher, self-reliant and reliable team worker

Computer skills

- Good command of Microsoft Office™ tools.
- Basic understanding of computer programming and coding.

Other skills

- Laboratory skills (molecular methods techniques – DNA isolation and cloning, PCR, conjugation and concomitant integration of constructs into genome, expression analyses in planta and ex planta, nodulation assays etc.)
- Translations and proofreading of manuscripts on English
- Tennis player (Active member of the Tennis club "Ponikve")

Driving licence	▪ B

ADDITIONAL INFORMATION

2015	Receiver of the ERASMUS scholarship (Dresden, Germany)
2014	Co-author of a poster presentation on the congress of the Croatian Ecological Society, Zagreb 2014
2010-2014	Participant in the event “Night of Biology” (University of Zagreb, Faculty of Science, Department of Biology)
2008-2012	Receiver of the scholarship of the city of Zagreb
2010	Co-author of a poster presentation on the congress of NEOBIOTA, Copenhagen 2010
2009	Third place in the international competition „International Conference of Young Scientists“, Psczyna, Poland
2009	Second place at the State competition and festival in biology, Crikvenica
2008-2009	Active participation (field research and participation in writing of congress abstracts) on the project „Countdown towards 2010 in Zagreb: community involvement in biodiversity assessment”
2008	First place at the State competition and festival in biology, Podgora
2008	Co-author of a poster presentation on the congress of NEOBIOTA, Prag 2008
2005-2009	Repeatedly participated in county competitions in biology, geography and English language