

CHARACTERIZATION OF THE *SINORHIZOBIUM MELILOTI* EXOR PROTEIN

By

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ABSTRACTCHARACTERIZATION OF THE *SINORHIZOBIUM MELILOTI* EXOR PROTEIN

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The soil bacterium *Sinorhizobium meliloti* is capable of establishing a symbiotic relationship with its leguminous plant host alfalfa by forming nitrogen-fixing root nodules through a series of signal exchanges and structural changes. The presence of a potential bacterial signal molecule, succinoglycan, is required for the invasion step of this symbiosis. The production of succinoglycan, which is inversely coupled with the production of flagella, is tightly regulated by the *S. meliloti* ExoR protein and the ExoS/ChvI two-component regulatory system. To better understand the regulatory function of ExoR and its relationship with the ExoS/ChvI system, I have carried out extensive genetic and biochemical analyses of ExoR and ExoS proteins. I found that ExoR is a periplasmic protein and it functions only in the periplasm. Interestingly, the C-terminal 20 amino acids appear not to be essential for the regulatory function of ExoR. Most importantly, the ExoR protein is digested in the periplasm, which appears to be the molecular mechanism regulating the amount of functional ExoR protein in the periplasm. The genetic analysis of my collection of *exoR*, *exoS*, and *chvI* mutants suggests that ExoR functions upstream of the ExoS/ChvI two-component signal transduction pathway. This conclusion was further supported by the analysis of the *exoR* expression in different

genetic backgrounds, which suggests the ExoR-ExoS-ChvI pathway is feedback regulated by ExoR. The combination of biochemical and genetic analyses suggest that ExoR functions in the periplasm through interaction with the ExoS sensing domain to keep ExoS in the off state. The proteolysis of ExoR would reduce the amount of functional ExoR and lead to the activation of ExoS and the expression of the genes regulated by the pathway. With the continuous discovery of ExoR/ExoS/ChvI homologous systems in a wide range of bacteria, my findings will contribute to a better understanding of the *S. meliloti*-alfalfa symbiosis and the pathogenicity of some bacterial plant and animal pathogens.

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1 CHAPTER I INTRODUCTION

1.1 The *Sinorhizobium meliloti*-alfalfa symbiosis

The Gram-negative soil bacterium *Sinorhizobium meliloti* is capable of establishing a symbiotic relationship with alfalfa plants (*Medicago sativa*) (112). This symbiosis is a complex development process and requires the exchanges of a series of signals between the plant and bacterium (13, 51, 67, 112). Under conditions of poor nitrogen availability, alfalfa plant secretes flavonoids (2-phenyl-1,4-benzopyrone derivatives) from roots to the soil, which attract *S. meliloti* cells in the rhizosphere to move toward the roots and attach themselves to the surface of the root hairs (94). These bacterial cells then begin to produce Nod factor, a lipochito-oligosaccharide signal molecule (62, 95). Nod factor induces the formation of curled root hairs and root cell divisions in the inner root cortex, which results in the formation of nodule primordium (68). *S. meliloti* cells trapped inside the curled root hairs elicit the formation of infection threads inside root hairs (113). The infection threads penetrate several layers of cells, enter root cortex, and deliver bacterial cells to the plant cells in the newly formed nodule primordium, where they are released and differentiate into nitrogen-fixing bacteroids (88, 114).

1.2 The *S. meliloti* surface polysaccharides required in establishing symbiosis

The establishment of the *S. meliloti*-alfalfa symbiosis is a complex developmental process. The results of extensive research have demonstrated that the presence of bacterial polysaccharides is essential for successful invasions of root nodules but the underlying mechanisms are still not clear (34, 43, 110). In the *S. meliloti*-alfalfa

symbiosis, at least one of the three bacterial polysaccharides: succinoglycan (exopolysaccharide I or EPS I), galactoglucan (exopolysaccharide II or EPS II), and capsular polysaccharides (KPSs) must be present for a successful nodulation (20, 60, 86, 92).

Succinoglycan is one of the most studied and best understood of all the symbiotically active exopolysaccharides in rhizobia-legume symbiosis, and it is more effective than the other two polysaccharides (92). Succinoglycan is a polymer of octasaccharide subunits containing one galactose and seven glucose residues joined by β -1 $\rightarrow$ 3, β -1 $\rightarrow$ 4 and β -1 $\rightarrow$ 6 glycosidic bonds, with acetyl, succinyl, and pyruvyl modifications (61). It is synthesized by more than 20 *exo* and *exs* gene products, most of which are clustered in a 27 Kb region on megaplasmid pSymB of its genome (40, 101). They are responsible for the synthesis of precursors and the repeating unit, decoration, polymerization, export and degradation of succinoglycan. The protein products of the *exoC* (also named *pgm*), *exoB* and *exoN* genes catalyze production of precursors, such as UDP-glucose and UDP-galactose (15, 111). The *exoY* gene encodes a glycosyl transferase which catalyses the first step of succinoglycan biosynthesis by adding the first sugar, galactose, to the lipid carrier on the cytoplasmic side of bacterial inner membrane (98, 101). *exoF* gene encodes a protein that is also needed for addition of the galactose to the lipid carrier (58, 83). All *exoA*, *exoL*, *exoM*, *exoO*, *exoU* and *exoW* encode glucosyl transferase and are involved in the subsequent addition of glucose residues (8, 101). ExoZ, ExoH, and ExoV function in the addition of acetyl, succinyl, pyruvyl groups to the sugar residues, respectively (40, 59). The completed octasaccharide repeating unit is then flipped to the periplasmic side of the inner membrane, where ExoP, ExoQ, and ExoT

proteins are involved in the polymerization of the subunits or secretion of the completed polymer (9, 40).

Succinoglycan can be grouped into high molecular weight (HMW) form with hundreds of the octasaccharide repeating unit, and low molecular weight (LMW) form composed of monomers, dimers and trimers of the repeating unit (121, 122). The ratio of HMW to LMW succinoglycan can be affected by two extracellular endo-glycanases, encoded by *exoK* and *exsH* genes (121, 122). The LMW succinoglycan was reported to be symbiotically active (41).

Exopolysaccharide II (EPS II) is a linear polysaccharide consists of repeating disaccharide unit of glucose and galactose joined by α -1 $\rightarrow$ 3, β -1 $\rightarrow$ 3, and the repeating unit can be decorated by acetyl- and pyruvyl groups (46, 63). It can suppress the symbiotic defects of EPS I-deficient strains on alfalfa, but not in invasion of other plant hosts (39). The *exp* genes cluster on megaplasmid pSymB of genome is involved in EPSII biosynthesis and export (10, 39). *S. meliloti* strain Rm1021, a streptomycin-resistant derivative of the wild-type SU 47, produces succinoglycan (EPS I) but does not produce galactoglucan (EPS II) at detectable levels due to an insertion into the *expR* gene (93). Both HMW and LMW EPS II were identified, but only LMW was shown to be symbiotically active (41).

S. meliloti capsular polysaccharides, named K-antigens, are also symbiotically important polysaccharides (34). Their importance was first discovered in *S. meliloti* strain AK631 (an *exoB* mutant of Rm41), which cannot produce neither EPS I nor EPS II but is able to elicit nitrogen-fixing nodules on alfalfa (102). Different from exopolysaccharides, K-antigens, containing a dimeric repeating unit of one hexose and a 3-deoxy-D-manno-

oct-2-ulopyranosonic acid (Kdo) or a related sugar, are analogous to group II KPSs found in *Escherichia coli* (102). These KPSs adhere tightly to *S. meliloti* cell surface and are not excreted into the culture medium (102). Their production involves at least fifteen genes and has been shown to be modulated by host plant-derived compounds, such as apigenin, and also environmental conditions like temperature and pH (54). The nature and the size of KPS are variable within the genus (17). It has been shown that the presence of HMW KPS, not the LMW form, is biological efficient (7). *S. meliloti* strain 1021 is able to synthesize only a unique LMW KPS which is not symbiotically active (35, 106).

While a lot is known about the structure and biosynthesis of these symbiotically important polysaccharides, their precise roles in mediating nodule invasion remain unknown. They could play important roles in the attachment of *S. meliloti* cells to the plant root, the suppression of host defense responses, and the activation of enzymes involved in infection thread growth or root hair curling. It is also possible that they might act as signals to induce plant responses leading to the formation of infection threads.

1.3 The regulation of succinoglycan biosynthesis

Succinoglycan synthesis by *S. meliloti* Rm1021 is regulated both in the free-living state and during symbiosis. In the free-living state, succinoglycan production is affected by the concentration of ammonia, phosphate, and sulfate, with ammonia being the main regulatory factor (27, 60, 77). During symbiosis, it is believed succinoglycan biosynthesis is turned off after the bacterial cells are released into plant cells, since the expression of *exo* structural genes decreases during bacteroid maturation (101).

The regulation of succinoglycan production at the molecular level is very complicated. Several genes have been reported to regulate succinoglycan production, including *exoR*, *exoS*, *exoX*, *exoD*, *exsB*, *mucR*, *syrM* and *syrA*, most of which are located in the chromosome (107). An *exoX* mutant overproduced succinoglycan (124). The inner membrane-attached ExoX protein was suggested to regulate the succinoglycan production by inhibiting ExoY, through protein-protein interactions (98). *exoD* mutations caused reduced amount of succinoglycan, and the reason is unknown (58, 98). The ExsB protein, encoded by the *exsB* gene, also negatively influences the amount of succinoglycan production, probably at a posttranscriptional or posttranslational level (9). MucR is homologous to *Agrobacterium tumefaciens* Ros protein, which regulates *vir* genes and succinoglycan biosynthesis (23). MucR is a zinc finger protein which binds to promoter regions of the genes it regulates, including the *exoH*, *exoX* and *exoY* genes (83). It was shown that MucR has a weak activation on *exoY* and mutations in *mucR* results in a reduction of EPS I production (12, 53). It also negatively regulates *exp* genes for EPS II biosynthesis (3, 53, 65). The *syrM* gene is involved in regulation of *nod* genes expression, but its overexpression resulted in overproduction of succinoglycan (5). The *syrA* gene is located very close to *syrM*, and is activated by SyrM (6, 84). SyrA mediates transcriptional regulation of *lpsS* and *exo* genes through the ExoS/ChvI (52).

1.4 The *S. meliloti* ExoR protein and the ExoS/ChvI two-component regulatory system are the key regulators of succinoglycan production

The *S. meliloti* ExoR protein and the ExoS/ChvI two-component regulatory system (2CRS) are the key regulators of succinoglycan production in free-living *S.*

meliloti cells. The involvement of *exoR* and *exoS* genes in regulating succinoglycan was discovered in a screen for transposon Tn5 insertion mutants that formed mucoid colonies (27). Transposon insertion mutations of *exoR95::Tn5* and *exoS96::Tn5* dramatically increase the levels of succinoglycan production (27).

Increased expression of *exoP*, *exoA*, *exoF*, and *exoQ* genes was detected in both *exoR95* and *exoS96* mutant backgrounds (100). Consistent with this finding, high levels of mRNA for *exo* genes were detected in the *exoR95* mutant, suggesting that the ExoR protein negatively regulates the expression of the *exo* genes (98). An ExoR homolog has been found in *Rhizobium leguminosarum*, which shares 71% identity at amino acid level with *S. meliloti* ExoR (99). A transposon insertion mutation in *R. leguminosarum* *exoR* also resulted in EPS I overproduction, which indicates a negative regulation of EPS I biosynthesis by this gene (99).

Many bacterial two-component regulatory systems are involved in sensing and responding to the changes of external environmental conditions. In the ExoS/ChvI 2CRS, ExoS is the membrane-bound sensor histidine kinase, and ChvI serves as the response regulator (21, 90). ExoS may form homodimers in the inner membrane. Its sensor domain located in cell periplasm can be activated by unknown stimuli and then passes the signal through the membrane and activates the cytoplasmic kinase by trans-autophosphorylation (118). The active ExoS phosphorylates ChvI, the regulator, which then activates the transcription of the *exo* genes (21). The *exoS* allele, *exoS96::Tn5*, encodes an N-terminal truncated derivative of ExoS, ExoS96, which was thought to be a constitutively active version of the sensor, causing the upregulation of the *exo* genes and the overproduction of succinoglycan (21).

The *exoR* gene product, ExoR, appears to mediate the ammonia regulation of succinoglycan (27). The wild type strain synthesizes only a small amount of succinoglycan in the presence of ammonia, but synthesizes much more in the absence of ammonia. In contrast, the *exoR95* mutant overproduces succinoglycan regardless of the presence of ammonia in the medium, suggesting that the *exoR* gene might be involved in the ammonia sensing pathway that regulates succinoglycan production (27). But in another study, *exoR* was reported not required for the regulation of succinoglycan biosynthesis by ammonia (91). The *exoS96::Tn5* mutant still responds to ammonia as the wild type, while it produces a higher level of succinoglycan compared to the wild type (27). In addition, the *exoR95* mutant exhibits severe nodulation deficiency, while *exoRS96* mutant shows no significant nodulation defect (21, 27, 91, 120).

1.5 The *S. meliloti* ExoR protein and the ExoS/ChvI two-component regulatory system regulate flagellum production

S. meliloti is a rod shaped bacterium with several peritrichous flagella. The motility of the bacterium is important for survival in the soil under adverse environmental conditions and also could be important for the establishment of symbiosis with its host plant, a process which starts from bacterial chemotaxis toward plant root exudates (16). The motility and chemotaxis genes are located in one contiguous chromosomal region named flagellar regulon (37). These genes are expressed in a hierarchical order (109). Class IA is composed of the *visN* and *visR* genes, and class IB is composed of recently discovered *rem*, which is under the control of *visNR* (109). Class I genes control class II genes, which comprise class IIA, flagella assembly genes and class IIB, motor genes (*mot*)

(105). Class IIA genes control class III genes which comprise flagellin genes (*fla*) and chemotaxis genes (*che*) (109). Any disruption of gene function in the hierarchy could result in the loss of cellular motility (11, 16, 103).

In addition to regulating succinoglycan production, the ExoR and ExoS proteins also regulate bacterial flagellum production (117, 120). The *exoR95* and *exoS96* mutant cells do not produce flagella in free-living conditions while the wild type cell does (120). Therefore, the *exoR95* and *exoS96* mutants lose motility and only form small size colonies on 0.3% soft agar (117, 120). The *exoR95* and *exoS96* mutants also overproduce succinoglycan, but it has been shown that succinoglycan overproduction is not responsible for blocking flagellum biosynthesis (120). The expression levels of the *fla* genes were decreased in both *exoR95* and *exoS96* mutants compared to that in the wild-type strain (120).

The regulation of EPS II production has been shown to be coupled with regulation of motility by the ExpR/Sin quorum-sensing system, through the regulators VisNR and Rem (47). A recent study showed that MucR and ExpR inhibit *ram* and *visN* at transcriptional level, respectively (3). Thus a model of regulatory network correlating EPS production and motility through MucR and ExpR was proposed (3).

In fact, the closely coupled regulation between succinoglycan production and flagellar motility is also seen in other bacteria, especially during bacterial biofilm formation. For example, in *Pseudomonas aeruginosa*, SadC and BifA proteins coregulate bacterial flagellum and exopolysaccharide production as the bacterium transitions from a planktonic to a surface-associated lifestyle (56, 78). In *Vibrio cholerae*, the extracellular protein secretion pathway genes (*eps*) affect the biosynthesis of flagellum and

exopolysaccharide during biofilm formation (2, 115). Likewise, this coordination by ExoR and ExoS/ChvI in *S. meliloti* might be important for bacterial biofilm formation in the soil or invasion of plant host.

1.6 The *S. meliloti* ExoR protein functions upstream of the ExoS/ChvI two-component regulatory system

The 268 aa ExoR protein has been demonstrated to be a periplasmic protein in a recent publication (117), as well as my own findings. In a previous study, an *exoR95exoS** strain was isolated from a pink nitrogen-fixing nodule on alfalfa elicited by the *exoR95* mutant (91). The spontaneous *exoS** mutation in *exoR95* background reduced succinoglycan production to an almost normal level. Complementary assay demonstrated that the suppressor *exoS** was mapped to the *exoS* locus (91). In another study, a suppressor mutation of *exoR95* was found in *chvI* gene (117). These findings suggest that ExoR functions upstream of the ExoS/ChvI system.

Bioinformatic analysis of ExoR amino acid sequence showed that it has four Sel1 protein-protein interaction domains (81). The Sel1 family of proteins was named after the *Caenorhabditis elegans* Sel1 protein which regulates cell development through protein-protein interactions (42). Sel1 proteins typically contain multiple copies of α -helix (Sel1 domain) that mediate protein-protein interactions that are required for many different cellular functions (81). In addition to eukaryotic organisms, Sel1 family proteins have also been found in bacteria including *Helicobacter pylori* HcpA (81), *Legionella pneumophila* EnhC (24) and LpnE (85), *Pseudomonas aeruginosa* AlgK (50), *Caulobacter crescentus* PodJ (25), *Vibrio parahaemolyticus* MotX (87), and

Pseudomonas strain K-62 MerG (55). The *P. aeruginosa* AlgK in particular, was thought to rely on its Sell domains to form protein complexes which facilitate the export of polysaccharide alginate and prevent alginate degradation by AlgL (alginate lyase) in the periplasmic domain (50). These findings further suggest that ExoR might rely on protein-protein interactions with ExoS or other intermediate proteins to regulate gene expression indirectly. And recently, the ExoR protein was shown to interact directly with the ExoS protein by immunoprecipitation and point mutations in the Sell domain of ExoR interrupted binding to ExoS (19).

S. meliloti ExoS/ChvI 2CRS is homologous to the *A. tumefaciens* C58 ChvG/ChvI 2CRS. Microarray study has revealed that acid conditions induce the expression of ChvG/ChvI, which is involved in the regulation of acid-inducible genes (18). The *S. meliloti* ExoS/ChvI 2CRS is also homologous to the BvrR/BvrS 2CRS in *Brucella abortus* (108). Mutations in *bvrR* and *bvrS* genes affect the expression of a few outer membrane proteins, causing altered cell permeability, higher sensitivity to microbicidal peptides (44, 57, 69, 74, 108). It has been proposed that BvrR/BvrS controls cell envelope changes, which are necessary to the transit between extracellular and intracellular environments (44). Both *A. tumefaciens* and *B. abortus* have ExoR proteins, which sequences have highly similarity to the *S. meliloti* ExoR protein, although it is not clear how closely they are functionally related.

2 CHAPTER II THE CELLULAR LOCATION OF THE *S. MELILOTI* EXOR PROTEIN

2.1 INTRODUCTION

The *S. meliloti* ExoR protein negatively regulates the expression of succinoglycan biosynthesis genes and positively regulates the expression of flagellum biosynthesis genes (120). However, this small 268 amino acid protein does not resemble any known transcription factors (98). By using bio-software prediction, a highly conserved N-terminal signal peptide was found in ExoR, which suggests that it could be located in the periplasm. *Escherichia coli* alkaline phosphatase gene (*phoA*) has been used widely to determine protein cellular location in topology studies (48, 72), because PhoA is enzymatically active only when it is translocated to the periplasm (79, 80). Translational fusion between a target gene and a truncated *phoA* gene which lacks a signal peptide sequence will result in a hybrid protein. If the target gene encodes a periplasm protein, the hybrid fusion protein will exhibit PhoA activity (72, 73). If the hybrid fusion is retained inside cytoplasm, the PhoA protein cannot form disulfide bonds and it will not show any enzymatic activity (4, 26). To understand how ExoR functions, three different ExoR-PhoA fusion proteins were constructed and PhoA activities were determined to examine the cellular location of ExoR.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this project are listed in Table 1-1. *E. coli* strains used in this study were grown in Luria-Bertani (LB) medium at 37°C (104). *S. meliloti* was grown in LB medium supplemented with 2.5 mM MgSO₄ and 2.5 mM CaCl₂ (LB/MC) at 30°C (60). Agar (1.5%) was used to solidify media. When required, IPTG (isopropyl-β-d-thiogalactopyranoside) was added to induce gene expression. The following antibiotics were used at the concentrations indicated: ampicillin (Ap), 100 µg/mL; chloramphenicol (Cm), 10 µg/mL; gentamycin, 50 µg/mL; kanamycin (Km), 25 µg/mL; neomycin (Nm), 200 µg/mL; spectinomycin (Sp), 100 µg/mL; streptomycin (Sm), 500 µg/mL; and tetracycline (Tc), 10 µg/mL.

2.2.2 Computer aided topology models

The TMAP program on Biology Workbench (<http://workbench.sdsc.edu>) was used to predict protein transmembrane segments.

2.2.3 Molecular cloning methods

General molecular cloning techniques were done as described before (104). DNA primers were ordered from Invitrogen (Carlsbad, CA). DNA polymerase *pfu* was purchased from Stratagenes (Santa Clara, CA). Dextroribose triphosphate (dNTPs), DNA ligase, and all restriction enzymes were purchased from NEB Biolabs (Ipswich, MA) and used according to the guidelines provided by the manufacturer. Genomic DNA was isolated using Promega genomic DNA isolation kit (Promega, Madison, WI). DNA was

purified by QIAquick® PCR purification kit (Qiagen, Valencia, CA). All plasmid constructs were sequenced to confirm their accuracy at either Biotechnology Resource Center of Cornell University or DNA Sequencing Facilities at Albert Einstein College of Medicine.

2.2.4 Construction of the *exoR-phoA* fusions

To construct a translational fusion of *exoR* and *phoA*, an *XhoI/XbaI* DNA fragment containing complete *exoR* open reading frame (ORF) except the stop codon was obtained in PCR using *S. meliloti* Rm1021 genomic DNA as the template and two PCR primers: *exoRf-20x* and *exoRr895x*. An *XbaI/KpnI* DNA fragment containing a truncated *E. coli phoA* gene (lacking its original signal sequence coding region) was obtained in PCR using the genomic DNA of *S. meliloti* *exoF265::TnphoA* mutant strain as the template and two primers: *phoAf79x* and *phoAr1416k*. The *XhoI/XbaI* *exoR* DNA fragment and the *XbaI/KpnI* *phoA* DNA fragment were first treated with restriction enzymes *XhoI/XbaI* and *XbaI/KpnI*, respectively, and then ligated in one single reaction into *XhoI* and *KpnI* treated vector pSW213 to generate plasmid pHC528, on which a full length ExoR-PhoA protein will be expressed from an IPTG inducible *lac* promoter.

To construct a translational fusion between an *exoR* without its signal peptide coding region (start codon with the 91st - 807th bp of the *exoR* ORF) and *phoA*, an *XhoI/KpnI* DNA fragment containing the fusion was obtained in PCR using plasmid pHC528, the template, and two primers, *exoRfATG91x* and *phoAr1416k*. This DNA fragment was treated with restriction enzymes *XhoI* and *KpnI* and cloned into similarly

treated vector pSW213 to generate plasmid pHC555, on which a signal peptide lacking-ExoR-PhoA, ExoR Δ SP-PhoA, will be expressed from an IPTG inducible *lac* promoter.

To construct a translational fusion of the *exoR* signal peptide and the *phoA* gene, the *exoR* signal peptide coding region and a truncated *phoA* ORF (without *phoA* signal peptide coding region) were obtained individually from two PCR reactions. The primers for amplifying the *exoR* signal peptide coding region were *exoRf-20x* and *exoR91-phoAr*, and the primers for amplifying the *phoA* gene were *exoR91-phoAf* and *phoAr1416k*. After purification, the two fragments were ligated together via overlapping PCR using primers *exoRf-20x* and *phoAr1416k*. This PCR product was then purified and treated with restriction enzymes *XhoI* and *KpnI*, and ligated with similarly treated vector pSW213 to generate plasmid pHC533, on which a PhoA fused with an N-terminal ExoR signal peptide, SP_{ExoR}-PhoA, will be expressed from an IPTG inducible *lac* promoter.

2.2.5 Construction of *exoR*-expressing plasmids

To express the *exoR* gene under its own promoter, a *HindIII/XhoI* DNA fragment containing the *exoR* promoter region and its entire ORF was obtained in a PCR reaction using *S. meliloti* Rm1021 genomic DNA as template and two PCR primers: *exoRf-662h* and *exoRr807x*. The PCR product was first treated with restriction enzymes *HindIII* and *XhoI*, and then ligated with similarly treated vector pMB393 to generate plasmid pHC510.

To express a mutant version of *exoR* encoding ExoR without its signal peptide under its own promoter, the *exoR* promoter and part of *exoR* ORF (without the 4th – 90th

bp) were obtained individually from two PCR reactions, using primers *exoRf-662h* and *exoRrATGw91* for amplifying the *exoR* promoter part, and *exoRfATGw91* and *exoRr807x* for the *exoR* ORF part. After purification, the two fragments were ligated together via overlapping PCR using primers *exoRf-662h* and *exoRr807x*. This PCR product was then purified and treated with restriction enzymes *HindIII* and *XhoI*, and ligated with similarly treated vector pMB393 to generate plasmid pHC557.

2.2.6 Transformation of *S. meliloti*

Triparental mating was used to introduce plasmids into *S. meliloti* cells as reported previously, with *E. coli* MT616(pRK600) providing transfer functions (32).

2.2.7 Detection of alkaline phosphatase activity

For detecting alkaline phosphatase activity, 5-bromo-4-chloro-3-indolyl phosphate (XP) was added at 40 µg/mL to solid medium. The presence of enzymatically active PhoA will hydrolyze XP and turn bacterial colonies blue.

2.2.8 Exopolysaccharide detection

To examine succinoglycan production on solid medium, calcofluor white M2R (CF, Fluorescent Brightener 28, Sigma) was added to a final concentration of 0.02% in LB/MC agar, which was buffered to pH 7.4 with 10 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) (60). To quantify succinoglycan production, bacterial cells were first grown in LB/MC medium to mid-log phase, washed with Z-MGS medium (70), and resuspended to approximately OD₆₀₀ of 0.1 in Z-MGS

medium. The cultures were then incubated at 30°C with shaking for 72 hours. To collect succinoglycan, the cultures were centrifuged at 13,000 g for 15 minutes at 4°C. Culture supernatants were then used to measure the amount succinoglycan with anthrone-H₂SO₄ method (66). The amount of succinoglycan was normalized to cell density of each culture.

2.2.9 Cell motility

Cell motility was examined using swarming plates as described previously (120). Briefly, fresh cell cultures were prepared and diluted to OD₆₀₀ of 0.1. Then 2 µL of each diluted culture was transferred into LB/MC soft agar (0.3%) plate and incubated for 3 days.

2.2.10 Alfalfa nodulation assays

Alfalfa nodulation on plates were carried out as previously described (60). Briefly, alfalfa seeds were surface-sterilized in 50% freshly diluted bleach for 10 minutes. The seeds were washed at least 4 times using sterile distilled water, and then spread on 0.8% agar in Petri dishes which were then wrapped in aluminum foil and placed in plant growth chamber (26°C) for 40 hours for germination. The germinated seedlings (about 1 inch long) were placed on Jensen's nitrogen-free agar medium in square Petri dishes; 8 seedlings per dish.

S. meliloti cells from overnight cultures in LB/MC medium were collected, washed, and diluted with sterile distilled water to an OD₆₀₀ of 0.03. Then 1 mL of the cell suspension was spread evenly on the seedlings in each square Petri dish. The Petri dishes

were left stand for a few hours to absorb the liquid and then wrapped with aluminum foil on three sides to cover roots. The Petri dishes with alfalfa plants were placed in plant growth chamber for four weeks. The number of nodules and the nodule color were examined to determine the overall symbiosis efficiency.

2.3 RESULTS

2.3.1 ExoR is a periplasmic protein

The ExoR protein is predicated to have a signal peptide at its N-terminus, with a conserved cleavage site between residues 30 (Alanine) and 31 (Phenylalanine). To determine the cellular location of the ExoR protein, *E. coli* PhoA protein was used as a reporter. Three different ExoR-PhoA translational fusions were constructed (Figure 2-1A) and expressed in three *S. meliloti phoA*⁻ mutant backgrounds, Rm8002 (*phoA*⁻), SmHC22 (*phoA*⁻, *exoR95*), and SmHC23 (*phoA*⁻, *exoS96*).

As shown in Figure 2-1B, upon induction by IPTG, the presence of the SP_{ExoR}-PhoA fusion protein turned all three strains blue on medium containing XP. This suggests that expression of gene fusions between the coding region of *exoR* signal peptide and *phoA* resulted in a correctly folded PhoA with enzymatic activities, and that the presence of ExoR signal peptide was sufficient for the fusion protein to be exported to the periplasm.

Fusion of PhoA with full length ExoR protein also turned colony blue in all strain backgrounds, which suggests that this fusion protein was exported to bacterial periplasm. In comparison, the expression of ExoRΔSP-PhoA fusion protein did not turn any of the

three strains into blue color, suggesting that this fusion protein remained inside the cytoplasm.

Altogether, these findings suggest that ExoR is located in the periplasm and that the presence of ExoR N-terminal signal peptide is essential and sufficient for the export of ExoR protein into periplasm.

2.3.2 ExoR functions only in *S. meliloti* periplasm

2.3.2.1 The signal peptide is required for the function of ExoR in regulating succinoglycan production

The *S. meliloti* ExoR protein is involved in regulating the expression of both *exo* and *fla* genes. To determine whether the signal peptide is required for the function of ExoR, plasmid pHC510 and plasmid pHC557, which carry genes encoding full length ExoR protein and an ExoR without its signal peptide (ExoR Δ SP), respectively, were introduced into wild type strain Rm1021 and the *exoR95* mutant individually.

The levels of succinoglycan production by strains carrying different constructs were examined on medium containing succinoglycan specific fluorescence dye, calcofluor. As shown in Figure 2-2, since the wild type strain Rm1021 produced succinoglycan at low levels and the *exoR95* mutant produced succinoglycan at high levels, Rm1021 showed low levels of fluorescence and the *exoR95* mutant showed high levels of fluorescence under ultraviolet (UV) light. The *exoR95* mutant carrying plasmid pHC510 showed reduced fluorescence levels compared to that of the wild type strain, but the *exoR95* mutant carrying plasmid pHC557 still presented the same high levels of fluorescence as the *exoR95* mutant. These results demonstrated that only the full length

ExoR protein can complement the *exoR95* mutation in repressing succinoglycan production, and the mutated ExoR without signal peptide can not.

2.3.2.2 The signal peptide is required for the function of ExoR in regulating flagellum production

To determine whether the signal peptide of ExoR is required for its ability to regulate flagellum production, the swarming ability of a set of strains carrying either plasmid pHC510 or pHC557 were examined on soft agar (0.3%) medium. As shown in Figure 2-3, the wild type strain Rm1021 produced flagella and formed big size colony on soft agar. The *exoR95* mutant formed a very small colony on soft agar, because the expression of flagella genes in this mutant was greatly repressed (120). The wild type strain Rm1021 carrying either plasmid pHC510 or pHC557 formed similar size colony to Rm1021. The *exoR95*(pHC510) strain formed big colonies, but *exoR95*(pHC557) still formed the same small size colony as the *exoR95* mutant. These results demonstrated that only the full length ExoR protein can complement the *exoR95* mutation in flagellum production, and the mutated ExoR without signal peptide cannot.

2.3.2.3 The signal peptide is required for the function of ExoR in symbiosis with alfalfa plants

The ExoR protein was shown to be important in *S. meliloti*-alfalfa symbiosis. To determine whether the signal peptide of ExoR is required for bacterium to establish a successful symbiosis, alfalfa plants were inoculated with a set of strains carrying either plasmid pHC510 or pHC557 and examined after 4 weeks. As shown in Figure 2-4,

without *S. meliloti*, no nodules were formed on plant roots and plants died of lacking of fixed nitrogen. The control strain, the *exoY210* mutant, which does not produce succinoglycan, only elicited non-nitrogen-fixing white nodules on alfalfa roots and plants died as well. The wild type Rm1021 mainly elicited pink nitrogen-fixing nodules on alfalfa roots and the plants were healthy. When ExoR Δ SP was expressed from plasmid pHC557 in Rm1021, plants were still healthy. When extra ExoR was expressed from plasmid pHC510 in Rm1021, a few white nodules on plant roots were observed and plants were less healthy than those which had Rm1021 alone. The *exoR95* mutant mainly elicited non-nitrogen-fixing white nodules on alfalfa roots which resulted in poor plant growth. Expressing ExoR Δ SP in the *exoR95* mutant did not rescue the poor symbiosis. But expressing ExoR in the *exoR95* mutant background elicited more pink nitrogen-fixing nodules on alfalfa roots which made the plants grow better.

Taken together, the presence of the altered ExoR lacking signal peptide (ExoR Δ SP) was not able to suppress the mutant phenotypes of the *exoR95* mutant, including succinoglycan production, the swarming ability, and nodulation efficiency. These results suggest that without the predicted N-terminal signal peptide, the ExoR protein is not functional. Therefore, ExoR functions only in *S. meliloti* periplasm.

2.3.3 ExoR-PhoA fusion is a bifunctional protein but with limited function

The full length ExoR-PhoA fusion protein was shown to reserve the PhoA enzymatic activity. To examine whether this ExoR-PhoA fusion protein also reserves the regulatory function of ExoR, bacterial succinoglycan production and swarming ability were tested. As shown in Figure 2-5 and Figure 2-6, Rm8002 behaved like the wild type

strain Rm1021 and produced small amount of succinoglycan and formed big size swarming colony. SmHC22 behaved like the *exoR95* mutant, producing large amount of succinoglycan and forming small size colony on soft agar. Upon induction by IPTG, ExoR-PhoA fusion protein expressed from plasmid pHC528 in SmHC22 was able to partially restore the swarming ability by forming a medium size colony on soft agar (Figure 2-6), but it was unable to repress succinoglycan production in SmHC22 (indicated by strong calcofluor-fluorescence) on LB/MC/CF plate (Figure 2-5). As a control, the ExoR Δ SP-PhoA fusion protein expressed from plasmid pHC555 in SmHC12 did not suppress any phenotypes of the *exoR95* mutation.

To further verify these results, anthrone-H₂SO₄ assay was used to quantitatively measure the succinoglycan production (pg/cell) by different strains grown in liquid medium. As shown in Table 2-3, Rm8002 produced low amount of succinoglycan, 1.64 ± 0.34 and 1.48 ± 0.36 , with and without IPTG induction, respectively. SmHC22, carrying the *exoR95* mutation, produced large amount of succinoglycan, 23.55 ± 2.22 and 26.93 ± 1.63 , with and without IPTG induction, respectively. In comparison, without IPTG induction, SmHC22(pHC528) still produced large amount of succinoglycan, 9.27 ± 1.00 ; upon IPTG induction, SmHC22(pHC528) only produced a low amount of succinoglycan, 2.21 ± 0.15 , which was similar to Rm8002. This suggests that the fusion of full length ExoR-PhoA protein repressed succinoglycan production in the *exoR95* mutant background in liquid medium to almost the wild type levels. Therefore, the ExoR-PhoA fusion is bi-functional (exhibiting both PhoA and ExoR functions) only in liquid medium. The reason that SmHC22(pHC528) produced less succinoglycan than SmHC22 in the

absence of IPTG induction could be due to the low leakage of the *lac* promoter on the plasmid.

2.4 DISCUSSION

Determining the cellular location of ExoR is important for understanding the signal transduction mechanism of ExoR protein. If ExoR is a cellular protein, it would be able to regulate gene expression directly; if ExoR is located in the periplasmic space, it would have to rely on other proteins to regulate gene expression indirectly. The possibility of ExoR being in the periplasmic space was raised by the predicted N-terminal signal peptide. Earlier, this predication was based on the hydrophobicity of the peptide sequence. Later it became clear that it is highly likely a functional signal peptide as the sequence analysis and prediction software become more and more sophisticated.

To determine the ExoR cellular location, different ExoR-PhoA fusions were constructed and tested for phosphatase activities. As our results showed, the presence of the ExoR signal peptide alone was sufficient for PhoA to be exported to the periplasmic space. The fusion of full length ExoR with PhoA was also clearly exported to the periplasmic space. In contrast, ExoR Δ SP-PhoA, the fusion of PhoA with ExoR missing its signal peptide stayed inside the cell. Because there is no other transmembrane sequence being predicted in the ExoR protein, these findings suggest that ExoR is most likely located in the periplasmic space.

The ExoR-PhoA fusion was shown to exhibit PhoA enzymatic activity. But to our surprise, the ExoR-PhoA fusion was able to repress succinoglycan production in liquid media, but not in solid media. But in semi-solid media (0.3% soft agar), the fusion

partially restored the swarming ability. It is possible that under osmotic pressure, the presence of the PhoA protein in the ExoR-PhoA fusion interferes with the function of ExoR due to three dimensional conformation changes. Therefore, the correct folding of the ExoR or its interactions with other proteins in periplasm may be delayed or blocked by the PhoA part. Because the ExoR-PhoA fusion is at least bifunctional in liquid media, anti-PhoA antibody can be used to study the fusion protein biochemically, which will be further described in Chapter 6.

S. meliloti ExoS protein is the membrane sensor of the ExoS/ChvI two-component regulatory system. Similarly to ExoR, it also regulates bacterial succinoglycan and flagellum biosynthesis genes (120). Before the start of my research, it was not clear whether these two proteins represent two different signal transduction pathways or part of the same signal transduction pathway. My data have shown that the predicted signal peptide is absolutely required for ExoR to regulate succinoglycan and flagellum production, and also to establish successful symbiosis with host plant. The fact that ExoR functions only in periplasm have raised the possibility that ExoR and ExoS may interact and belong to the same signal transduction pathway.

ExoR is one of the better known polysaccharide regulatory genes. While some of the ExoR homologues, such as *Rhizobium leguminosarum* ExoR (99), have been characterized individually, most others have not been characterized in detail. Most of the ExoR homologues do share two common features, the N-terminal signal peptide and Sell domains, which suggest that they could all function in the periplasm in regulating essential cellular functions by interacting with other proteins or by changing multimeric state. Further studies of the signaling mechanism of *S. meliloti* ExoR, such as the possible

protein-protein interactions through Sel1 domains should provide more insights into the regulatory mechanism of ExoR and contribute to the general understanding of protein-protein interactions in cellular signaling.

Table 2-1. Strains, plasmids and phage.

Strain or plasmid	Relevant properties	References
<i>E. coli</i>		
DH5 α	General purposed strain	(45)
MT616	MT607, pRK600, Cm ^R	(32)
<i>S. meliloti</i>		
Rm1021	SU47, Sm ^R	(60)
Rm7095	Rm1021 <i>exoR</i> 95::Tn5, Nm ^R	(27)
Rm7096	Rm1021 <i>exoS</i> 96::Tn5, Nm ^R	(27)
Rm7210	Rm1021 <i>exoY</i> 210::Tn5, Nm ^R	(60)
Rm8002	Rm1021 <i>phoA</i> ⁻	(38)
Rm8265	Rm8002 <i>exoF</i> 265::Tn <i>phoA</i>	(100)
SmHC22	Rm8002 <i>exoR</i> 95::Tn5, Nm ^R	This work
SmHC23	Rm8002 <i>exoS</i> 96::Tn5, Nm ^R	This work
Plasmids		
pMB393	Cloning vector, Sp ^R	(36)
pHC510	pMB393, expressing <i>exoR</i> under the control of its native promoter, Sp ^R	This work
pHC557	pMB393, expressing a signal peptide lacking <i>exoR</i> under the control of its native promoter, Sp ^R	This work
pSW213	Cloning vector, <i>lacI</i> ^Q , P _{lac} <i>lacZ</i> , Tc ^R	(75)
pHC528	pSW213, expressing <i>exoR-phoA</i> fusion under an inducible promoter, Tc ^R	This work
pHC555	pSW213, expressing a signal peptide lacking <i>exoR-phoA</i> fusion under an inducible promoter, Tc ^R	This work
pHC533	pSW213, expressing <i>phoA</i> with <i>exoR</i> signal peptide under an inducible promoter, Tc ^R	This work
pRK600	Helper plasmid, Cm ^R	(32)
Phage		
ϕ M12	Generalized transducing phage for <i>S. meliloti</i>	(31)

Table 2-2. Oligo DNA primers used for plasmid constructions.

Plasmid	Primer	Primer sequence
pHC510	<i>exoRf-662h</i>	CCCAAGCTTCATCGGCCACAAGTTCAACG
	<i>exoRr807x</i>	CCGCTCGAGTCATCAATCGTCGTCGTTCTGC
pHC557	<i>exoRf-662h</i>	CCCAAGCTTCATCGGCCACAAGTTCAACG
	<i>exoRrATGw91</i>	CGGCTCCGGGATCGAACATAACAATCAGTTTCTTTCGTT
	<i>exoRfATGw91</i>	GAAAGAAACTGATTGTTATGTTTCGATCCCGGAGCCG
	<i>exoRr807x</i>	CCGCTCGAGTCATCAATCGTCGTCGTTCTGC
pHC528	<i>exoRf-20x</i>	CCGCTCGAGTAACGAAAGAACTGATTGTTATG
	<i>exoRr895x</i>	GCTCTAGAATCGTCGTCGTTCTGCA
	<i>phoAf79x</i>	GCTCTAGACCTGTTCTGGAAAACCGGG
	<i>phoAr1416k</i>	GGGGTACCTTATTTTCAGCCCCAGAGCGGC
PHC555	<i>exoRfATG91x</i>	CCGCTCGAGTAACGAAAGAACTGATTGTTATGTTCGA TCCCGGAGCCGGCGTGA
	<i>phoAr1416k</i>	GGGGTACCTTATTTTCAGCCCCAGAGCGGC
pHC533	<i>exoRf-20x</i>	CCGCTCGAGTAACGAAAGAACTGATTGTTATG
	<i>exoR91-phoAr</i>	CCGGTTTTCCAGAACAGGAGCCCGTGCTGGCCCGGC
	<i>exoR91-phoAf</i>	GCCGGGCCAGCACGGGCTCCTGTTCTGGAAAACCGG
	<i>phoAr1416k</i>	GGGGTACCTTATTTTCAGCCCCAGAGCGGC

Table 2-3. Succinoglycan production by different *S. meliloti* strains*.

Strain	Succinoglycan production (pg/cell)					
	0 IPTG			0.8mM IPTG		
Rm8002	1.64	±	0.34	1.48	±	0.36
SmHC22	23.55	±	2.22	26.93	±	1.63
SmHC22(pHC528)	9.27	±	1.00	2.21	±	0.15

* *S. meliloti* strains were cultured in Z-MGS medium for 72 hours. The results are the average value of two independent experiments and the corresponding standard deviations are indicated.

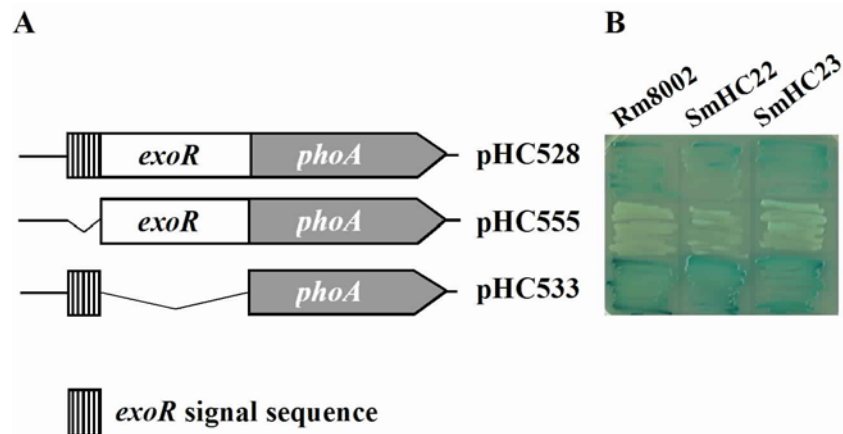


Figure 2-1. The cellular location of the ExoR protein. A composite figure showing A, a schematic representation of three ExoR-PhoA fusions; B, Alkaline phosphatase activities of Rm8002(*phoA*⁻), SmHC22(*phoA*⁻, *exoR*95), and SmHC23(*phoA*⁻, *exoS*96) strains expressing the three different ExoR-PhoA fusions. Only strains carrying pHC528 or pHC533 were turned blue on LB/MC plate with XP.

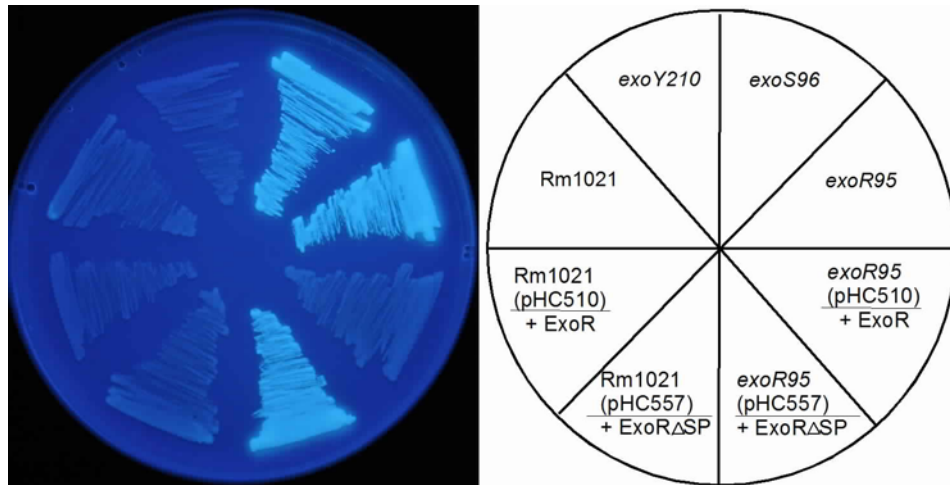


Figure 2-2. Calcofluor fluorescence image showing the levels of succinoglycan produced by different *S. meliloti* strains. Bacterial cells were grown on LB/MC solid medium with calcofluor.

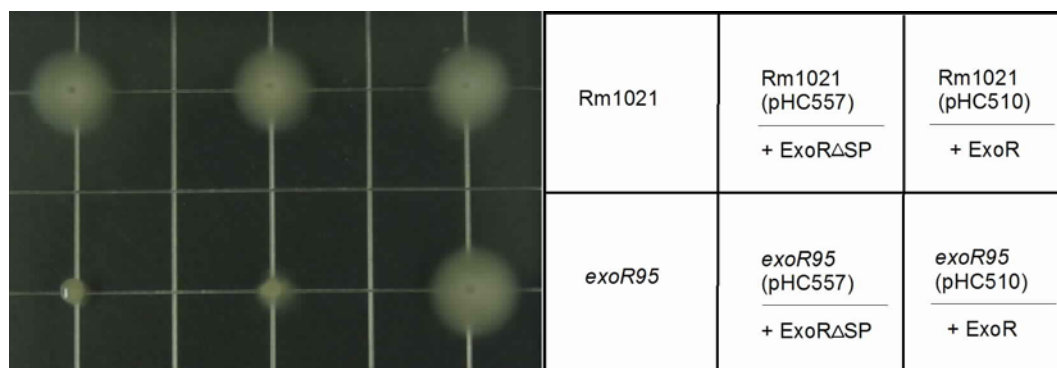


Figure 2-3. *S. meliloti* colonies on LB/MC plate with 0.3% agar. Cell cultures were loaded into the medium and grown for 3 days. Different swarming abilities of bacterial strains were observed.

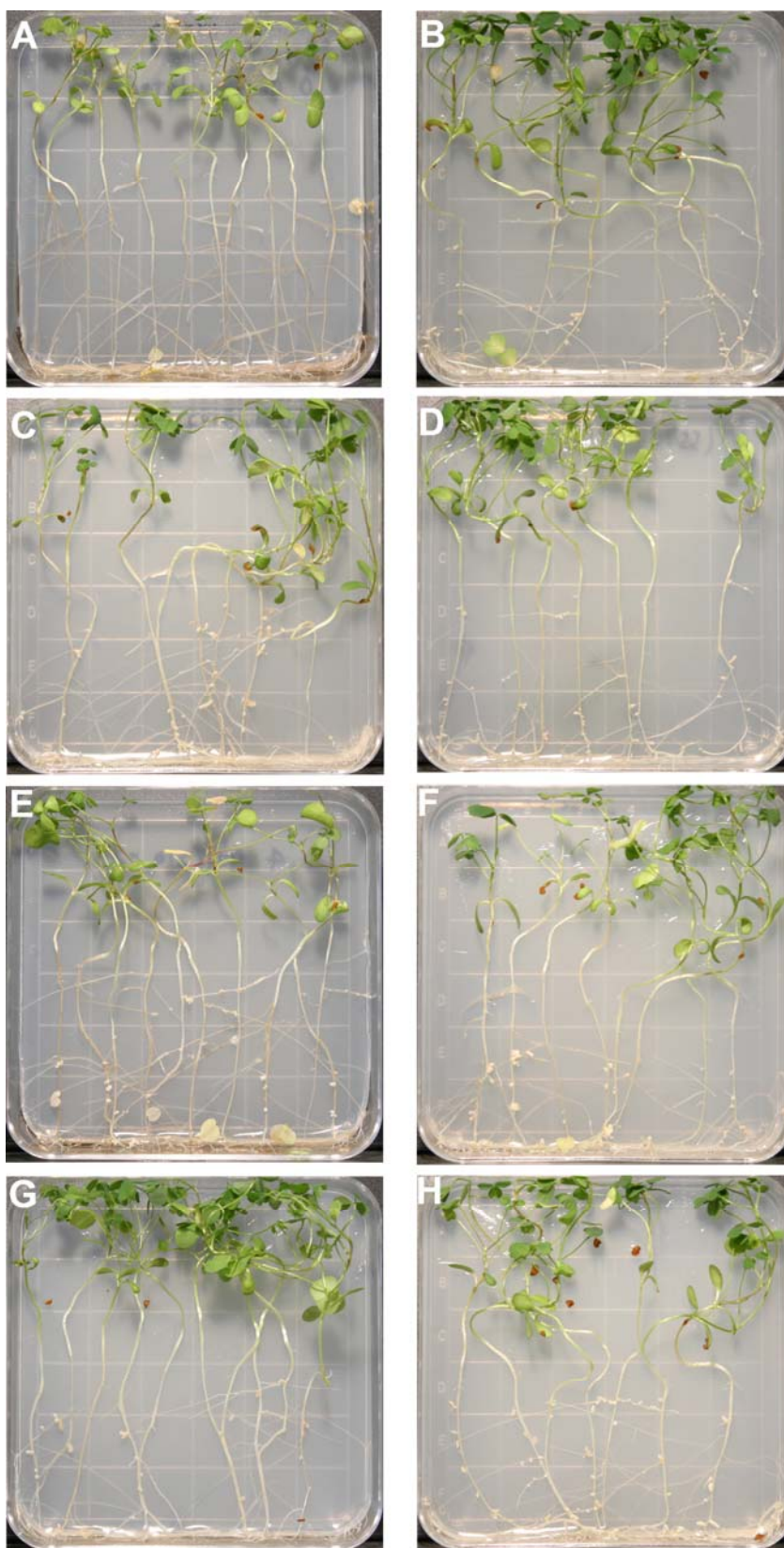


Figure 2-4. Alfalfa plants inoculate with different *S. meliloti* strains. A, No bacteria; B, Rm1021; C, Rm1021 (pHC510); D, Rm1021 (pHC557); E, *exoY210*; F, Rm7095, the *exoR95* mutant; G, *exoR95* (pHC510); H, *exoR95* (pHC557). Plants were grown for 4 weeks.

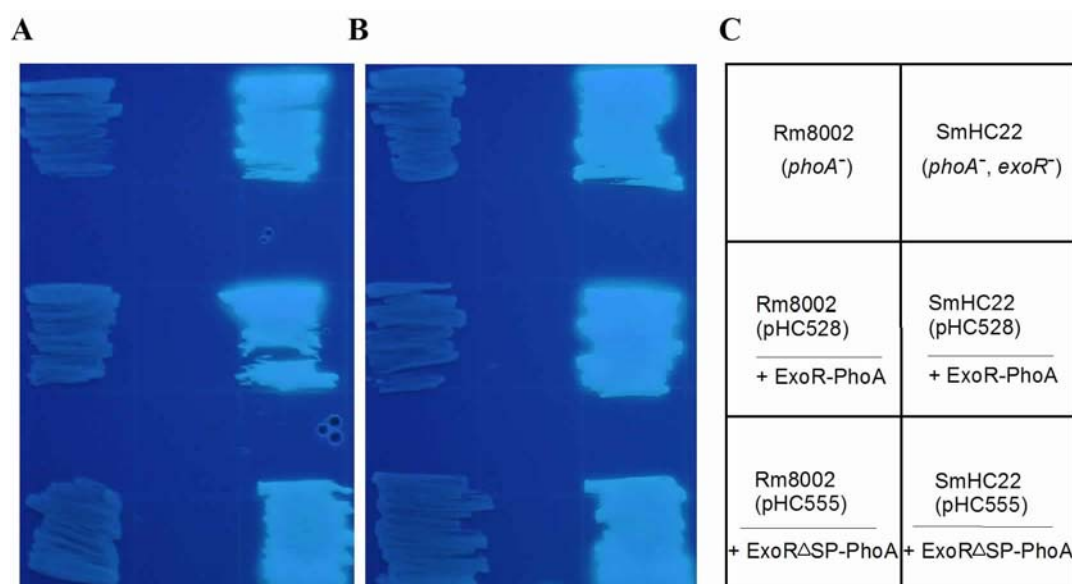


Figure 2-5. Calcofluor fluorescence image showing the levels of succinoglycan produced by different *S. meliloti* strains. Panel A, without IPTG induction; Panel B, with IPTG induction; Panel C, corresponding bacterial strains used in Panel A and B. Cells were grown on LB/MC solid medium with calcofluor.

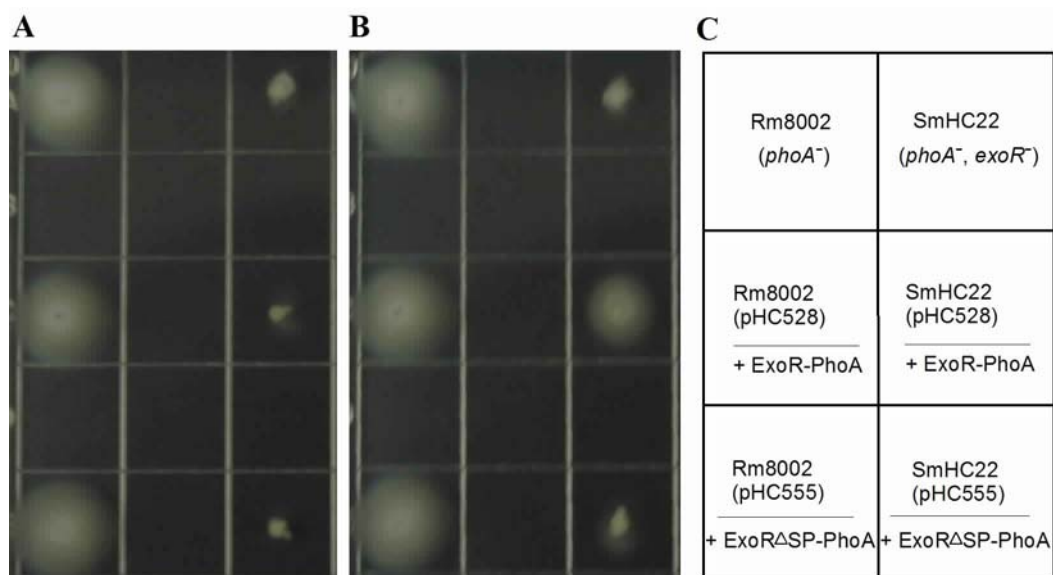


Figure 2-6. *S. meliloti* colonies on LB/MC plate with 0.3% agar. Panel A, without IPTG induction; Panel B, with IPTG induction; Panel C, corresponding bacterial strains used in Panel A and B. Cell cultures were loaded into the medium and grown for 3 days.

3 CHAPTER III THE *S. MELILOTI* EXOR REGULATORY PATHWAY

3.1 INTRODUCTION

The *S. meliloti* ExoR protein and the ExoS/ChvI two-component regulatory system are key regulators of succinoglycan production in free-living *S. meliloti* cells. The *exoR* and *exoS* genes were first discovered in two succinoglycan overproducing mutants, *exoR95::Tn5* and *exoS96::Tn5*, respectively, where higher levels of mRNA of the *exo* genes were observed (27, 98). Later, both mutants were found to be defective in flagellum biosynthesis, indicating both genes are also involved in regulating flagellum gene expression (120).

The ExoR protein does not resemble any known transcriptional regulators. The transposon Tn5 insertion in the *exoR95* mutant was believed to be a loss-of-function mutation (98). ExoS is the membrane-bound sensor of the ExoS/ChvI 2CRS, and ChvI serves as the response regulator in cytoplasm (21). The *exoS96::Tn5* allele encodes an N-terminal truncated derivative of ExoS which was thought to be a constitutively active version of the sensor (21). The *exoS96* mutant is sensitive to ammonia in regulating succinoglycan production and does not show significant nodulation defect (27). On the contrary, the *exoR95* mutant overproduces succinoglycan with or without ammonia, and exhibits great nodulation deficiency by inducing mainly non-nitrogen-fixing white nodules on alfalfa roots (27).

In a previous study, an *exoR95exoS** strain was isolated from a pink nitrogen-fixing nodule on alfalfa elicited by the *exoR95* mutant (91). The spontaneous *exoS** mutation in *exoR95* background reduced succinoglycan production to an almost normal

level (91). Complementary assay demonstrated that the suppressor *exoS** was targeted to the *exoS* locus (91).

To understand how ExoR exerts its functions in regulating succinoglycan and flagellum biosynthesis, I have further characterized the previously isolated *exoR95exoS** mutant. In addition, two more suppressor mutations of *exoR95* were isolated and studied in this work.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this project are listed in Table 3-1. *E. coli* strains used in this study were grown in Luria-Bertani (LB) medium at 37°C (104). *S. meliloti* was grown in LB medium supplemented with 2.5 mM MgSO₄ and 2.5 mM CaCl₂ (LB/MC) at 30°C (60). Agar (1.5%) was used to solidify media. To examine succinoglycan production, calcofluor white M2R (Fluorescent Brightener 28, Sigma) was added to a final concentration of 0.02% in LB/MC agar, which was buffered to pH 7.4 with 10 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) (60). The following antibiotics were used at the concentrations indicated: ampicillin (Ap), 100 µg/mL; chloramphenicol (Cm), 10 µg/mL; gentamycin, 50 µg/mL; kanamycin (Km), 25 µg/mL; neomycin (Nm), 200 µg/mL; spectinomycin (Sp), 100 µg/mL; streptomycin (Sm), 500 µg/mL; and tetracycline (Tc), 10 µg/mL.

3.2.2 Construction of *exoR*-expressing plasmids

Refer to Chapter 2.

3.2.3 Construction of *exoS*-expressing plasmid

To express the *exoS* gene from its own promoter on a plasmid, an *NdeI/XbaI* DNA fragment containing the *exoS* promoter region and ORF was obtained in PCR using *S. meliloti* Rm1021 genomic DNA as the template and two PCR primers: *exoSfNdeI* and *exoSrXbaI* (Table 3-2). The PCR product was treated with restriction enzymes *NdeI* and *XbaI*, and ligated with similarly treated pHC93 to generate plasmid pHC517. The *exoS** allele was cloned into pHC93 in the same way except that *exoR95exoS** genomic DNA was used as the template. The resulting plasmid was named pHC521. Similarly, the *exoS_{supA}* allele was cloned into pHC93 by using *exoR95supA* genomic DNA as the template. The resulting plasmid was named pHC534.

3.2.4 Generation of plasmid insertion mutation in the *exoS* gene

To interrupt the *exoS* gene with a plasmid insertion, a 0.3 Kb DNA fragment containing the middle part (758-1096) of the *exoS* gene was obtained in PCR using *S. meliloti* Rm1021 genomic DNA as template and two PCR primers: *exoSdf* and *exoSdr* (Table 3-2). The PCR product was treated with restriction enzymes *HindIII* and *BsrGI*, and ligated with similarly treated suicide vector pK19mob2ΩHMB to generate plasmid pHC507. The resulting plasmid was conjugated into wild type *S. meliloti* Rm1021 using helper MT616 in a triparental mating (Figure 3-1A). One of the few single colonies arose from the selective medium containing streptomycin and neomycin was purified. The *exoS* gene with plasmid insertion was transduced into Rm1021 background to ensure that the mutant strain carried a single mutation. Genomic DNA was then isolated from the

bacterial cells using a Wizard genomic DNA purification kit (Promega) and used as a template in a PCR reaction with primer *pK19Mp* which annealed to the suicide plasmid and primer *exoSf* which annealed to the promoter region of the *exoS* gene (Table 3-2). A DNA fragment of the predicted size was generated by PCR and confirmed that the plasmid insertion occurred in the *exoS* gene (Figure 3-1B). The genomic DNA of this mutant was isolated, and the *exoS* gene region was sequenced and confirmed the plasmid insertion mutation. The mutant was name *exoS107*.

3.2.5 Construction of the *exoR395exoS107* mutant

To facilitate the construction of *exoR* and *exoS* double mutant, *S. meliloti* strain Rm8395 which carries the *exoR395::Tn5-233* mutation was used. It is the same as the *exoR95::Tn5* mutant with the exception of the antibiotic markers. Double mutations were constructed by using phage Φ M12-mediated transduction as previous described (31).

3.2.6 Sequencing the *chvI* gene in different genetic backgrounds

The genomic DNA of *S. meliloti* wild type strain Rm1021, and mutant strains *exoS96*, *exoR95exoS**, *exoR95supA*, *exoS107*, and *exoR395exoS107*, were isolated and used individually as the template in PCR reactions with two primers, *chvIf-27* and *chvIr759* (Table 3-2) to generate DNA fragments covering the *chvI* ORF. The DNA products were sequenced at the Albert Einstein College of medicine DNA facility.

3.2.7 Recovery of bacterial cells from root nodules

Bacterial cells were recovered from alfalfa root nodules as previously described. Briefly, nodules were removed from roots, surface-sterilized with 2-minute incubation in 50% Clorox bleach, washed 6 times in sterile distilled water, and crushed inside the wells of 96-well plates in 100 µl LB/MC liquid medium plus 5.4% glucose. The suspensions were then diluted 1:100 in the same medium and plated on LB/MC agar plates with antibiotics for *S. meliloti* single colonies.

3.2.8 Physiological stress assays

Bacterial cultures were streaked on LB/MC medium containing different antibiotics or detergents indifferent concentrations. Bacterial growth was checked after 2-day incubation at 30°C. Each sample had two duplications. The detergent sodium dodecyl sulfate (SDS) was used at 0.1 g/L and 0.2 g/L. Sodium deoxycholate (DOC) was used at 2 g/L and 4 g/L. Sarkosyl was used at 2 g/L and 4 g/L. Tetracycline was used at 0.2 mg/L and 0.4 mg/L. Novobiocin was used at 1 mg/L and 2 mg/L. Carbenicillin was used at 2 mg/L and 4 mg/L. Bacterial growth at pH 5.5 was determined on LB/MC agar buffered with 50 mM MES (4-Morpholineethanesulfonic acid), pH 5.5.

3.3 RESULTS

3.3.1 Characterization of a previously isolated *exoR95* suppressor mutation *exoS**

The *exoR95exoS** mutant was previously isolated from a pink nodule elicited by the *exoR95* mutant (91). The presence of the spontaneous *exoS** mutation reduces succinoglycan production by the *exoR95exoS** mutant to the wild type Rm1021 levels

(91) (Figure 3-2). Cosmid pM13, carrying 20 Kb *S. meliloti* Rm1021 genomic DNA covering the *exoS* and *chvI* genes region, was isolated which complemented the non-mucoid phenotype of *exoR95exoS**. Random mutagenesis of pM13 and restriction mapping revealed that the *exoS** mutation belonged to the *exoS* locus (91).

To further characterize this *exoR95exoS** mutant, I tested its swarming ability and also nodulation efficiency. Like the wild type strain, the mutant formed big swarming colonies on soft agar, and showed normal nodulation efficiency (Table 3-3). To confirm the original mapping result and to ensure that the phenotypes of the *exoS** mutant is the result of a single spontaneous mutation, the wild type *exoS* gene was cloned with its native promoter on plasmid pHC517 and expressed in the *exoR95exoS** mutant. Expressing ExoS in the *exoR95exoS** mutant increased its succinoglycan production to the levels of the *exoR95* mutant. However, Expressing ExoS in the *exoR95exoS** mutant did not change the swarming ability of the mutant, because the *exoR95exoS*(pHC517)* strain still formed large colonies on soft agar (Figure 3-2). These data suggest that the spontaneous *exoS** mutation can be partially complemented by the wild type *exoS* gene.

To determine the site and the nature of the *exoS** suppressor mutation, the entire *exoS* ORF in *exoR95exoS** was sequenced. The results showed that nucleotide at position 448 of the *exoS* ORF (1788 bp) has been changed from A (ACG) to G (GCG) in the *exoR95exoS** mutant, which resulted in the change of the amino acid at position 150 from threonine to alanine (Figure 3-3). The amino acid threonine is located in the periplasmic sensing domain of the ExoS protein, which suggests that change in the ExoS sensing domain can suppress the succinoglycan overproduction by the *exoR95* mutant.

3.3.2 Isolation of two other *exoR95* suppressor mutations, *supA* and *supB*

A new suppressor screen was carried out to isolate additional *exoR95* suppressor mutations independently. A total of 9 pink alfalfa root nodules were isolated from 12 plants inoculated with the *exoR95* mutant. *S. meliloti* cells were recovered from the root nodules and examined on LB/MC/CF medium to determine the levels of their succinoglycan production. Only two isolates, *exoR95supA* and *exoR95supB*, produced less succinoglycan and restored cell swarming ability by forming big colonies on soft agar (Figure 3-2 and data not shown). Both mutants showed normal nodulation efficiency as the wild type strain Rm1021 (Table 3-3 and data not shown). These data suggest the spontaneous *supA* and *supB* mutations are suppressor mutations of the *exoR95* mutation.

To map the sites of *supA* and *supB* mutations, cosmid pM13 was conjugated into the *exoR95supA* and *exoR95supB* mutants. This cosmid was originally used in identifying the *exoS** mutation in the *exoR95exoS** mutant (91), and it is possible that it can complement the unknown *supA* and *supB* mutations as well. My results showed that the presence of pM13 complemented the mutations and brought the succinoglycan production by the two mutants up to the *exoR95* mutant levels (Figure 3-4), suggesting that both *supA* and *supB* mutations are located within the 20 Kb region that includes both *exoS* and *chvI* genes. To further test whether these two mutations are located in the *exoS* gene, the plasmid pHC517 was conjugated into both *exoR95supA* and *exoR95supB* mutants. The expression of ExoS in the two mutants did increase their succinoglycan production to the levels of the *exoR95* mutant. Again, the expression of ExoS did not change the swarming ability of the two mutants. The data suggest that both *supA* and *supB* mutations could be located in the *exoS* gene, similar to the *exoS** mutation.

The DNA sequence of the *exoS* gene was determined for *exoR95supA* and *exoR95supB* mutants. The *supA* mutation resulted in the C to T change at nucleotide 227 of the *exoS* ORF, which would result in the change of alanine to valine at position 76 of the ExoS protein, which is also in the periplasmic sensing domain of ExoS (Figure 3-3). Interestingly, the *supB* mutation resulted in the A to G base substitution at nucleotide 448 of the *exoS* ORF, which is the exactly the same as the *exoS** mutation. Therefore, the *exoR95supB* mutant was not continually examined. The finding of the two independently isolated mutants provides more evidence that the ExoR functions upstream of ExoS and mutations in the sensing domain of ExoS will suppress mutation in ExoR.

3.3.3 Characterization of the *exoS** and *exoS_{supA}* mutations

To directly examine the phenotypes of the *exoS** and *exoS_{supA}* mutations, *exoR* was expressed with its own promoter on pHC510 in the *exoR95exoS** and *exoR95supA* mutants to complement the *exoR95* mutation. As shown in Figure 3-2, expressing the wild type ExoR in the two mutants did not significantly change their succinoglycan production levels or swarming abilities, because they still produced less succinoglycan and formed big swarming colony. As a control, the *exoS96* mutant produced large amount of succinoglycan and formed small swarming colony. That their phenotypes were different from the *exoS96* mutant clearly indicates that the *exoS** and *exoS_{supA}* mutations are not the same as the *exoS96* mutation, which results in a constitutively active form of ExoS.

Also shown in Figure 3-2, expressing more ExoS* from pHC521 in the *exoR95exoS** mutant did not change its succinoglycan production levels or the swarming

abilities. Similarly, expressing more ExoS_{supA} from pHC534 in the *exoR95supA* mutant did not change any of its phenotypes either. These data suggest that the functional defects of ExoS* or ExoS_{supA} cannot be compensated by increased amount of proteins.

Taken together, these data suggest both the *exoS** and *exoS_{supA}* mutations may have induced a structural change in the periplasmic sensing domains of the ExoS protein which keeps the protein inactive, thereby preventing it from phosphorylating ChvI, the response regulator of the ExoS/ChvI 2CRS.

3.3.4 Construction and characterization of a mutant strain containing a plasmid insertion mutation in the *exoS* gene

The only existing *exoS* mutation is the *exoS96* which is a consistently active mutation (21). A loss of function *exoS* mutant is needed to directly test whether ExoS functions downstream of the ExoR protein. So an *exoS* insertion mutation, *exoS107*, was constructed by inserting a suicide plasmid pHC507 in the *exoS* ORF in Rm1021 genome. Only few conjugants were obtained after several attempts. The insertion of the suicide plasmid in the *exoS107* mutant was verified by PCR amplification of the junction of suicide plasmid and *S. meliloti* chromosome (Figure 3-1). The interrupted *exoS* gene in the *exoS107* mutant was transduced into wild type strain Rm1021 to ensure that it is a single plasmid insertion mutation in the genome. To further rule out the possibilities of suppressor mutation, the *chvI* gene, located downstream of *exoS* at chromosome was sequenced. Surprisingly, a C to A base substitution at nucleotide 319 in *chvI* ORF was identified, resulting in the amino acid change from leucine to isoleucine at position 107 in the ChvI protein, so the mutant was renamed *exoS107chvI107*. Both leucine and

isoleucine are hydrophobic amino acid, but leucine at position 107 seems to be quite conserved in other rhizobia species, such as *Rhizobium leguminosarum* and *Sinorhizobium medicae*, and also in *Agrobacterium tumefaciens* and *Brucella abortus*. The amino acid is in the receiver domain of the protein which interacts with the sensor partner in the two-component system, so it seems to be difficult to predict the functional effect of the *chvI107* mutation.

When characterizing this *exoS107chvI107* double mutant, I found it produced similar levels of succinoglycan to the wild type strain and it formed a slightly smaller swarming colony on soft agar than the wild type (Figure 3-5). This mutant was able to establish a successful symbiosis relationship with alfalfa plants (Table 3-3).

The *S. meliloti* *exoS* and *chvI* are homologous to the *chvG* and *chvI* genes, respectively, in *A. tumefaciens* (90). The *A. tumefaciens* ChvG regulates acid-inducible genes and the *chvG* mutant is highly sensitive to acidic pH, detergents and antibiotics (18, 64). To test whether the *exoS107chvI107* exhibits an altered growth phenotype as *A. tumefaciens chvG* mutant, different detergents and antibiotics were added into the growth media. As shown in Table 3-4, the *exoS107chvI107* double mutant showed sensitivities to detergents SDS (0.2 g/L) and sarkosyl (2 g/L), and to antibiotics tetracycline (0.2 mg/L) and carbenicillin (2 mg/L). It did not grow well at 2 g/L novobiocin, but showed a same normal growth as the wild type in 4 g/L DOC. As controls, both the *exoR95* and *exoS96* mutants exhibited the same resistance as the wild type strain to the detergents and antibiotics tested. But neither the wild type nor the *exoS107chvI107* grew well in acidic condition, which is quite different from *A. tumefaciens*.

This set of physiological stress testing suggests that like *A. tumefaciens chvG* mutant, the *exoS107chvI107* double mutant may exhibit some kind of membrane defect which renders the cell to be more sensitive to detergents and antibiotics. However, ExoS may have different functions from ChvG in *A. tumefaciens*, which is involved in regulation of acid-inducible genes and can be easily deleted (18, 64).

3.3.5 The *exoR395* mutation can be suppressed by combined mutations in the ExoS/ChvI 2CRS

To determine whether the *exoS107chvI107* mutation affects the phenotypes of the *exoR395* mutant (similar to *exoR95* except the antibiotic marker), it is necessary to construct the *exoR395exoS107chvI107* mutant. My results showed that this *exoR395exoS107chvI107* mutant strain produced succinoglycan at levels similar to the wild type strain, and also formed big colonies on swarming plate (Figure 3-5). This suggests that mutations in ExoS and ChvI together have suppressed the mutation in the *exoR* gene, which is consistent with the model that ExoR functions upstream of the ExoS protein. When pM13, which carries both of *exoS* and *chvI* genes, was introduced into the *exoR395exoS107chvI107* mutant, it brought up the levels of succinoglycan production by this mutant to that by the *exoR395* strain. However, cosmid pM13 did not completely complement the *exoS107chvI107* mutation either. The *exoR395* mutant formed small swarming colony on soft agar, but *exoR395exoS107chvI107*(pM13) still formed large colonies as *exoR395exoS107chvI107* did.

3.4 DISCUSSION

The ExoR protein has been demonstrated to be a periplasmic protein and it would have to interact with proteins in its signal transduction pathway in order to regulate the expression of *exo* and *fla* genes. The ExoS protein is also involved in regulating succinoglycan production and swarming ability (120). To help establish ExoR signal transduction pathway and the relationship between the ExoR and ExoS proteins, I focused on identifying proteins that function downstream of ExoR. Mutations in the proteins function downstream of ExoR should suppress mutations in the ExoR protein. Therefore, isolating suppressor mutations of the *exoR95* mutation could be an effective approach.

Characterization of a previously isolated *exoR95exoS** mutant from a pink nodule induced by the *exoR95* mutant revealed that the *exoR95* mutation can be suppressed by a point mutation in the ExoS periplasmic sensing domain. In the effort of finding more *exoR95* suppressors, I adopted this method and found two mutations located in the *exoS* gene, *supA* and *supB*. The finding of these two spontaneous suppressor mutations not only provided more evidence that the ExoR functions upstream of ExoS, but also demonstrated that it is an effective approach to identify suppressors of the *exoR95* mutation.

Since point mutations in ExoS suppressed the phenotypes of the *exoR95* mutant, the loss of ExoS should be able to suppress the *exoR95* mutation as well. However, making a completely loss-of-function *S. meliloti* *exoS* mutation seems to be very difficult, as been reported before (21, 90) and also in this study. The plasmid insertion-generated *exoS107* mutant harbors a mutation in *chvI*, the regulator of the ExoS/ChvI 2CRS,

suggesting the *exoS* gene is essential and cannot be fully deleted. Indeed, this mutant strain was found to be more sensitive to detergents and antibiotics, which suggests it has some kinds of defects. It is very possible that ExoS/ChvI must be maintained at a certain level in *S. meliloti*. Nevertheless, the combined mutations, *exoS107chvI107*, were able to suppress the *exoR395* mutation, which demonstrated again that ExoS/ChvI mediates the gene expression regulation by ExoR.

Analyses of all data support a model that ExoR functions as a repressor of the sensor ExoS of the ExoS/ChvI 2CRS and indirectly regulates the expression of *exo* and *fla* genes. This model relies on the hypothesis that ExoR interacts with ExoS in the bacterial periplasm. The *exoR95* mutation changes the C-terminus of ExoR (Chapter 5) so that the ExoR95 loses the ability to interact with and suppress the ExoS protein. ExoS becomes active and phosphorylates ChvI which then upregulates the *exo* gene expression and downregulates *fla* gene expression. The unique structure of ExoS96 periplasmic sensing domain may have disabled the repression by ExoR by preventing its interaction with ExoR so that ExoS96 remains active. The *exoS** and *exoS_{supA}* mutations may have changed the structure of ExoS periplasmic sensing domain and blocked the necessary conformational changes required for the ExoS to be active, therefore, ChvI cannot be phosphorylated, and no upregulation of the *exo* genes or downregulation of the *fla* gene is expected.

An interesting question raised from this study is how ExoS/ChvI 2CRS regulates succinoglycan and flagellum synthesis at the same time. Noticeable, the expression of extra ExoS from plasmid pHC517 in both *exoR95exoS** and *exoR95supA* mutants complemented the succinoglycan production, but not the swarming ability, since only

succinoglycan production was upregulated while swarming ability was not repressed.

Meanwhile, the expression of extra ExoS and ChvI proteins from cosmid pM13 in *exoR395exoS107chvI107* mutant did not complement the *exoS107chvI* mutation in swarming ability either. A possible explanation is that activation of the *exo* genes and repression of *fla* genes may require different phosphorylation levels of ChvI. The ExoS protein functions mostly likely in dimeric manner, so formation of ExoS*-ExoS, ExoS_{supA}-ExoS and ExoS107-ExoS heterodimers could interfere with the histidine kinase activity of ExoS and results in lower phosphorylation levels of ChvI. It is possible that only strong activation of ExoS/ChvI can repress the flagellum production, while a low activation of ExoS/ChvI is sufficient to upregulate the *exo* gene expression. Further investigation of the ExoS/ChvI should reveal more interesting findings.

Taken together, the ExoR protein in *S. meliloti* may function as a negative regulator that controls the activities of the ExoS, the sensor of ExoS/ChvI 2CRS. The interactions between ExoR and ExoS might be regulated, through proteases in the periplasm which disassociate ExoR from ExoS, or through regulating the amount of ExoR being expressed. The two possibilities are discussed in detail in other chapters of the dissertation.

Table 3-1. Strains plasmids and phage.

Strain or plasmid	Relevant properties	References
<i>E. coli</i>		
DH5 α	General purposed strain	(45)
MT616	MT607, pRK600, Cm ^R	(32)
<i>S. meliloti</i>		
Rm1021	SU47, Sm ^R	(60)
Rm7095	Rm1021 <i>exoR95::Tn5</i> , Nm ^R	(27)
Rm7096	Rm1021 <i>exoS96::Tn5</i> , Nm ^R	(27)
Rm7210	Rm1021 <i>exoY210::Tn5</i> , Nm ^R	(60)
Rm8395	Rm1021 <i>exoR395::Tn5-233</i> , Sp ^R	(120)
SmHC20	<i>exoR95exoS*</i> ; Rm7095 with A76V mutation in ExoS, Nm ^R	(91)
SmHC21	<i>exoR95supA</i> ; Rm7095 with T150A mutation in ExoS, Nm ^R	This work
SmHC17	<i>exoS107chvI107</i> , Nm ^R	This work
SmHC24	<i>exoR395exoS107chvI107</i> , Nm ^R , Sp ^R	This work
Plasmids		
pHC93	Cloning vector, Tc ^R	Lab stock
pMB393	Cloning vector, Sp ^R	(36)
pK19mob2 Ω HMB	A suicide plasmid, Km ^R	(70)
pHC510	pMB393, expressing <i>exoR</i> under <i>exoR</i> promoter, Sp ^R	This work
pHC517	pHC93, expressing <i>exoS</i> under <i>exoS</i> promoter, Tc ^R	This work
pHC521	pHC93, expressing <i>exoS*</i> under <i>exoS</i> promoter, Tc ^R	This work
pHC534	pHC93, expressing <i>supA</i> under <i>exoS</i> promoter, Tc ^R	This work
pHC507	pK19mob2 Ω HMB, <i>exoS</i> suicide plasmid, Nm ^R	This work
pRK600	Helper plasmid, Cm ^R	(32)
Phage		
ϕ M12	Generalized transducing phage for <i>S. meliloti</i>	(31)

Table 3-2. Oligo DNA primers used for gene constructions and sequencing.

Primer	Primer sequence
<i>exoRf-20x</i>	CCGCTCGAGTAACGAAAGAACTGATTGTTATG
<i>exoRr807k</i>	GGGGTACCTATCAATCGTCGTCGTTCTGC
<i>exoRf-662h</i>	CCCAAGCTTCATCGGCCACAAGTTCAACG
<i>exoRr807x</i>	CCGCTCGAGTCATCAATCGTCGTCGTTCTGC
<i>exoSdf</i>	GCCAAGCTTCCGTGCGGTCCT
<i>exoSdr</i>	GGCTGTACATCGGCGGCGAAATT
<i>exoSfNdeI</i>	AGCGGGACATATGAAGTTCAAGATGGTCGACAACGAC
<i>exoSrXbaI</i>	GCTCTAGACCCGTGGACATTGACGA
<i>exoSf-61</i>	ACGGGCGAATGCGACAGG
<i>exoSr1918</i>	CCCCGGTGCCTCACTTCC
<i>exoSr407</i>	CGCTCCTGAATGATCGGGAAT
<i>exoSr829</i>	CGGCATGGACGATCTTGTCG
<i>exoSf653</i>	CGATCTATCCGGAGGTGATGAAC
<i>exoSf1098</i>	GAGCCACGAACTCAAGAACCC
<i>exoSr1912</i>	TGCCTCACTTCCGAAAGACAG
<i>chvIf-27</i>	CCGGGCCTCGAAACGGAGTA
<i>chvIr759</i>	GGCCACGAGTGATCCGCAAT
<i>chvIf293</i>	TCATCACCAAGCCCTTCTCACAG
<i>chvIr419</i>	GGGAAGGCGTGTCGGCATT

Table 3-3. Nodulation efficiency of different *S. meliloti* strains.*

Strain	Red nodule percentage (%)		Nodule number/plant	
Rm1021	94.15	± 5.58	4.79	± 0.38
<i>exoY210</i>	0.00	± 0.00	5.43	± 0.65
<i>exoR95</i>	15.74	± 4.03	7.00	± 0.65
<i>exoS96</i>	79.33	± 6.51	5.93	± 1.17
<i>exoR95exoS*</i>	95.96	± 7.00	5.43	± 1.08
<i>exoR95supA</i>	95.63	± 5.12	4.43	± 0.76
<i>exoS107chvI107</i>	70.67	± 1.15	6.07	± 1.15

* The red nodule percentage and average nodule numbers per plant were determined from 7 four-week old alfalfa plants inoculated with 1 mL OD₆₀₀ of 0.003 bacterial cells. The results represent three independent experiments.

Table 3-4. Growth of *S. meliloti* strains in the presence of detergents and antibiotics and under condition of acidic pH. *

Strain	Growth in the presence of						Acidic pH (pH5.5)
	DOC (4g/L)	SDS (0.2g/L)	Sarkosyl (2g/L)	Tetracycline (0.2mg/L)	Novobiocin (2g/L)	Carbenicillin (2mg/L)	
Rm1021	+	+	+	+	+	+	±
<i>exoR95</i>	+	+	+	+	+	+	NA
<i>exoS96</i>	+	+	+	+	+	+	NA
<i>exoS107chvI107</i>	+	-	-	-	±	-	±

* Growth was determined by the ability to form colonies after 2 days at 30°C. +, growth present; ±, limited growth present; -, no growth; ND, not determined. The assay was repeated twice with similar results.

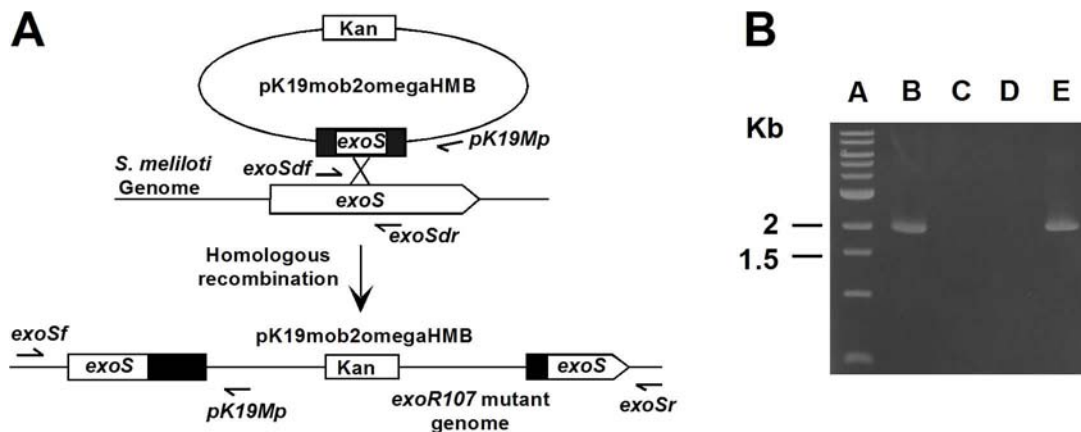


Figure 3-1. Generation of plasmid insertion mutation in the *exoS* gene and conformation of the insertion in *exoS*. A, Schematic representation of the strategies for plasmid insertion into the *exoS* gene in the genome. The locations of primers used to amplify the middle part of *exoS* and the junction between *exoS* and the suicide plasmid are indicated. B, Image of an agarose gel with a DNA ladder and DNA fragments amplified with different sets of primers from the wild type strain Rm1021 or *exoS107* mutant. Lane A, detection of the entire *exoS* ORF from Rm1021 using *exoSf* and *exoSr*; lane B, no PCR product was obtained from the *exoS107* genomic DNA using *exoSf* and *exoSr*; lane C, no PCR product was obtained from Rm1021 using *exoSf* and *pK19Mp*; lane D, detection of the junction of the *exoS* gene and the inserted plasmid from the *exoS107* genomic DNA using *exoSf* and *pK19Mp*.

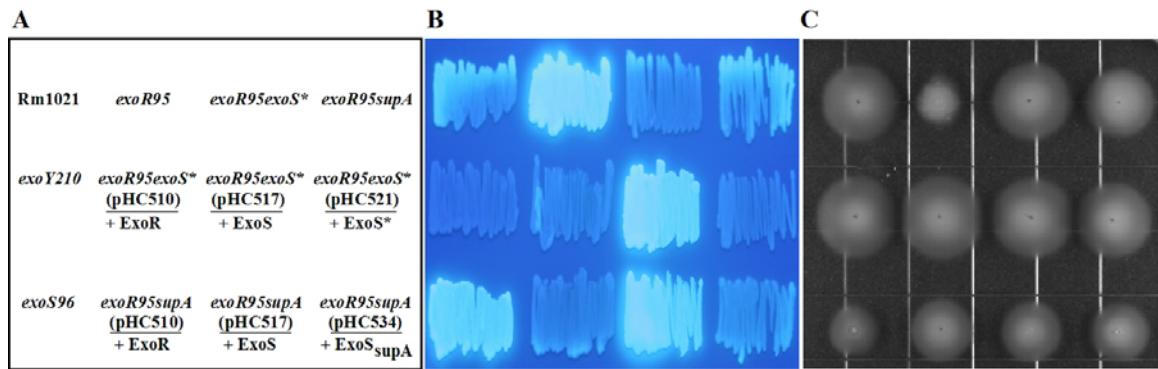


Figure 3-2. The effects of the mutations in the *exoR* and *exoS* genes on bacterial succinoglycan production and swarming ability. *S. meliloti* strains (panel A) were examined on LB/MC/CF plate (panel B) and LB/MC with 0.3% agar (panel C). Plasmid pHC510 carries the wild type *exoR* gene; pHC517 carries the wild type *exoS* gene; pHC521 carries the *exoS** allele, and pHC534 carries the *exoS_{supA}* allele.

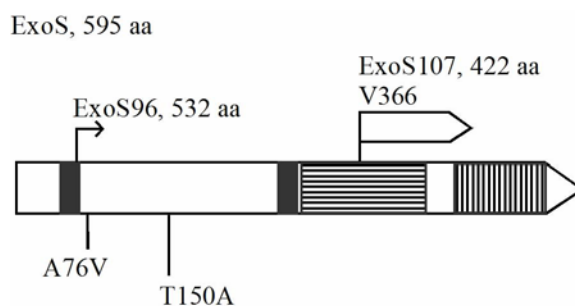


Figure 3-3. Diagram of the ExoS protein. Schematic diagram of the ExoS protein showing two putative transmembrane domains (black box), a putative histidine kinase domain (horizontal lines) consisting of a dimerisation and phosphoacceptor region, and a putative ATP-binding region (vertical lines). Two point mutations A76V and T150A were found in the periplasmic domain of ExoS in the *exoR95supA* and *exoR95exoS** mutant backgrounds, respectively. The ExoS96 (532 aa) is an N-terminal truncated ExoS, missing the first 63 amino acids. The ExoS107 ORF (422 aa) is predicted to have the N-terminal 366 aa from ExoS and C-terminal 56 aa from the suicide plasmid, resulted from the plasmid insertion.

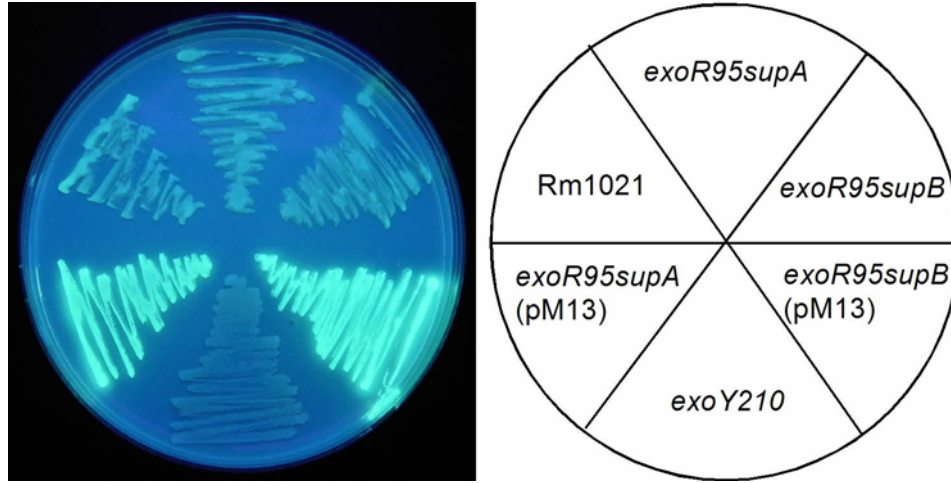


Figure 3-4. A picture of calcofluor fluorescence of *S. meliloti* strains on LB/MC/CF plate. The *exoR95supA* and *exoR95supB* mutants produced succinoglycan at similar levels to the wild type, which were less than the *exoR95* mutant. Cosmid pM13 complemented the *supA* mutation in the *exoR95supA* mutant and also the *supB* mutation in the *exoR95supB* mutant, causing the strains to produce more succinoglycan.

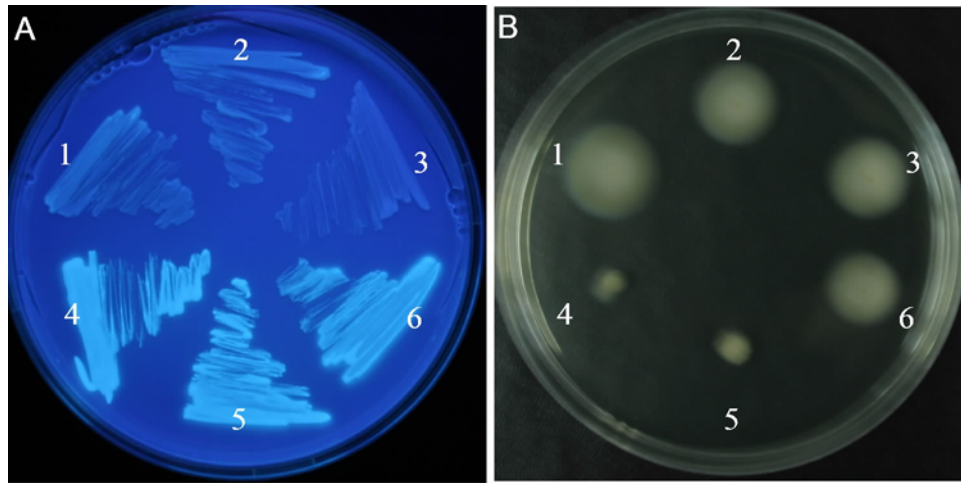


Figure 3-5. Effects of plasmid insertion in the *exoS* gene on succinoglycan production and swarming ability. Panel A, LB/MC/CF plate fluorescence picture; Panel B, Swarming colonies on 0.3% soft agar. Bacterial strains used were: 1, Rm1021; 2, *exoS107chvI107*; 3, *exoR395exoS107chvI107*; 4, *exoR395*; 5, *exoS96*; and 6, *exoR395exoS107chvI107*(pM13).

4 CHAPTER IV THE *EXOR* GENE EXPRESSION IN *S. MELILOTI*

4.1 INTRODUCTION

The *S. meliloti* wild type strain Rm1021 produces a small amount of succinoglycan and forms large size colonies on swarming plates (0.3% soft agar) (27). Both *exoR95* and *exoS96* mutations cause overproduction of succinoglycan by upregulating the expression of *exo* genes, including the *exoY* gene, which carries out the first step of succinoglycan biosynthesis (22, 27, 98). Both mutations also cause the downregulation of flagellum biosynthesis (120). In addition, the *exoR95* mutation causes a dramatic reduction in the efficiency of establishing symbiosis with alfalfa when compared to the wild type Rm1021 (27). To better understand how ExoR protein functions, I characterized the regulation of the *exoR* gene expression in this work using the green fluorescent protein (GFP) gene as a reporter. A set of plasmids containing *exoR* promoter-*gfp* fusions was constructed and the *exoR* promoter activities in different *S. meliloti* strains were examined by measuring the specific GFP expression.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains and growth media

The bacterial strains and plasmids used in this project are listed in Table 4-1. *E. coli* strains used in this study were grown in Luria-Bertani (LB) medium at 37°C (104). *S. meliloti* was grown in LB medium supplemented with 2.5 mM MgSO₄ and 2.5 mM CaCl₂ (LB/MC) at 30°C (60). Agar (1.5%) was used to solidify media. When required, IPTG (isopropyl-β-d-thiogalactopyranoside) was added to induce gene expression at the final

concentration of 0.8 mM. The following antibiotics were used at the concentrations indicated: chloramphenicol (Cm), 10 µg/mL; neomycin (Nm), 200 µg/mL; spectinomycin (Sp), 100 µg/mL; streptomycin (Sm), 500 µg/mL; tetracycline (Tc), 10 µg/mL; and ampicillin (Ap), 100 µg/mL.

4.2.2 The computer analysis of *exoR* promoter sequence

To locate the *exoR* promoter, total of 662 bp *exoR* gene upstream region was analyzed with a prokaryote promoter prediction program on Berkeley Drosophila Genome Project website (http://www.fruitfly.org/seq_tools/promoter.html).

4.2.3 Construction of *exoR* promoter-*gfp* fusions

DNA fragments covering different *exoR* gene upstream regions were amplified from genomic DNA of *S. meliloti* Rm1021. The DNA fragment containing the *gfp* ORF was amplified from plasmid pJK19-1. Primers used are listed in Table 4-2. The *exoR* promoter-*gfp* fusions (P_{exoR} -*gfp*) were constructed by either joining the *exoR* promoter fragment with the *gfp* fragment at a common *NheI* site or by recombinant PCR (Figure 4-1). Each of the P_{exoR} -*gfp* fusions was treated with restriction enzymes *HindIII* and *XhoI*, and ligated with similarly treated vector pMB393. This set of P_{exoR} -*gfp* fusions covering the *exoR* gene upstream regions of -20 to -1, -662 to -1, -325 to -1, and -20 to -1 fused with -662 to -353 were expressed from plasmids pHC505, pHC501, pHC514 and pHC548, respectively. All plasmids were moved into *S. meliloti* strains through conjugation using MT616 as the helper.

4.2.4 Construction of ExoR-expressing plasmids

An *XhoI/KpnI* DNA fragment containing complete *exoR* ORF was obtained in PCR using *S. meliloti* Rm1021 genomic DNA as template and two PCR primers: *exoRf-20x* and *exoRr807k*. The PCR product was treated with restriction enzymes *XhoI* and *KpnI*, and ligated with similarly treated vector pSW213 to generate plasmid pHC530, on which the wild type *exoR* gene will be expressed from an IPTG inducible *lac* promoter. Similarly, an *XhoI/KpnI* DNA fragment containing part of *exoR* ORF without the signal peptide coding region was obtained in PCR using *S. meliloti* Rm1021 genomic DNA as template and two PCR primers: *exoRfATG91x* and *exoRr807k*. This mutated *exoR*, *exoRΔsp*, was cloned into vector pSW213, resulting in plasmid pHC556, on which *exoRΔsp* is expressed under the control of the same IPTG inducible *lac* promoter.

The *exoR* gene along with its entire promoter was cloned into vector pMB393 to generate plasmid pHC510 (Refer to Chapter 2).

4.2.5 Construction of an ExoS-expressing plasmid

A *BamHI/KpnI* DNA fragment containing complete *exoS* ORF (1788 bp) was obtained in PCR using *S. meliloti* Rm1021 genomic DNA as template and two PCR primers: *exoSf1-atgb* and *exoSr1818k*. The forward primer *exoSf1-atgb* introduced ATG as the *exoS* gene start codon, replacing the original TTG (predicted) in the genome. The PCR product was treated with restriction enzymes *BamHI* and *KpnI*, and ligated with similarly treated vector pSW213 to generate plasmid pHC560, on which the *exoS* gene will be expressed from the IPTG inducible *lac* promoter on the vector.

4.2.6 Measurement of *exoR* and *exoY* promoter activities

The GFP fluorescence intensity was used to measure the *exoR* promoter activities in *S. meliloti* cells carrying the P_{exoR} -*gfp* constructs. Briefly, bacterial cultures were collected, washed and resuspended in 0.85% sterile NaCl solution to about OD₆₀₀ 0.1. The diluted culture was transferred into wells (100 μ L/well) in a black 96-well microplate and a transparent 96-well microplate. The black microplate was put into the fluorescence microplate reader (Spectra Max Gemini XS, Molecular Device, Sunnyvale, CA) to determine the GFP fluorescence intensities of the cell suspensions. The transparent microplate was put into the absorbance microplate reader (Spectra Max 340PC, Molecular Device, Sunnyvale, CA) to measure the cell density (OD₆₀₀). GFP fluorescence intensity of individual cell suspension was normalized to its corresponding cell density using the computer program Softmax Pro by Molecular Device. The normalized GFP fluorescence intensities were used to represent the *exoR* promoter activities.

The *exoY* promoter activities in *S. meliloti* cells were similarly determined using plasmid pH77 expressing a fusion of *exoY-gfp* from native *exoY* promoter (22).

4.2.7 Alfalfa nodulation assays

Refer to Chapter 2.

4.2.8 Exopolysaccharide detection

Refer to Chapter 2.

4.3 RESULTS

4.3.1 Analysis of the *exoR* promoter region

Total 662 bp upstream region of the *S. meliloti* *exoR* gene was analyzed with a prokaryote promoter prediction program (http://www.fruitfly.org/seq_tools/promoter.html). Three putative *exoR* promoters were identified at -302 bp, -262 bp, and -161 bp. A putative ribosome binding site was predicated at -17 bp (98). A transcriptional terminator of the *exoR* upstream *fad* gene is found at -352 bp, which should prevent any reading through from the *fad* promoter. To confirm these predictions, a set of plasmids which carry different *exoR* upstream regions fused with the *gfp* gene was constructed (Figure 4-1).

4.3.2 Identify the *exoR* gene promoter region

To identify the location of *exoR* promoter region, the set of plasmids carrying P_{exoR} -*gfp* fusion constructs was introduced into the wild type strain Rm1021. GFP fluorescence intensities of strains were determined to represent the activities of *exoR* promoters (Figure 4-2).

As shown in Figure 4-2, the wild type Rm1021 carrying the vector pMB393 produced basal levels of GFP fluorescence, 1.99 ± 0.17 . The levels of GFP fluorescence from Rm1021 carrying pHC505 were 2.81 ± 1.38 , which was similar to the values detected from Rm1021(pMB393), indicating that *gfp* cannot be expressed by any promoters on the vector itself. The levels of GFP fluorescence from Rm1021(pHC501) and Rm1021(pHC514) were about the same, which were 26.95 ± 0.41 and 26.35 ± 1.33 , respectively. In comparison, only basal levels of GFP fluorescence (similar to the

controls), 2.94 ± 0.76 , were detected from Rm1021(pHC548). Altogether, these data suggest that all of *exoR* promoters are located within the intergenic region between *exoR* and its upstream gene (-325 to -1), which is included in the plasmid pHC514. There are no additional *exoR* promoters (in the *fad* gene) far upstream the intergenic region.

4.3.3 The *exoR* expression is upregulated in the *exoR95* and *exoS96* mutant backgrounds

The same set of plasmids carrying P_{exoR} -*gfp* fusion constructs was also introduced into the *exoR95* and *exoS96* mutants to analyze the expression of the *exoR* gene in both backgrounds (Figure 4-2). As expected, the levels of GFP fluorescence from the control plasmid pHC505 in the two mutants were just as low as the levels detected from the vector control strains, 2.91 ± 0.42 and 3.19 ± 1.20 for *exoR95*(pMB393) and *exoR95*(pHC505), respectively, and 3.16 ± 0.74 and 3.47 ± 2.19 for *exoS96*(pMB393) and *exoS96*(pHC505), respectively. Basal levels of GFP fluorescence were also detected from both *exoR95*(pHC548) and *exoS96*(pHC548), 3.03 ± 0.90 and 2.64 ± 0.56 , respectively. Noticeably, in the *exoR95* mutant background, the specific GFP fluorescence was 88.32 ± 4.92 for *exoR95* (pHC501) and 87.56 ± 4.40 for *exoR95* (pHC514). In the *exoS96* mutant background, the specific GFP fluorescence was 74.38 ± 7.32 for *exoS96* (pHC501) and 74.07 ± 3.88 for *exoS96* (pHC514). These values in both mutants were significantly higher than that in the wild type background, suggesting that the expression of the *exoR* gene is upregulated in the *exoR95* and *exoS96* mutants. And these data also confirmed that the -325 to -1 region contains all the elements required for the upregulation of the *exoR* gene by *exoR95* and *exoS96* mutants. Therefore, plasmid

pHC514 was used in the subsequent analysis to represent the complete *exoR* promoter region.

4.3.4 Mutations in the *exoS* gene blocks the upregulation of the *exoR* promoter in the *exoR95* mutant background

Both the *exoR95exoS** mutant and the *exoR95supA* mutant have a spontaneous point mutation in the *exoS* gene (as described in Chapter 3). The point mutation in either strain suppressed the *exoR95* mutation, so that succinoglycan production was decreased and the swarming ability was restored. To examine the *exoR* promoter activities in the two mutants, plasmid pHC514 was introduced into them and the GFP fluorescence intensities of each strain were determined. As shown in Figure 4-3, compared with 87.56 ± 4.40 from *exoR95*(pHC514), *exoR95exoS**(pHC514) and *exoR95supA*(pHC514) produced much lower levels of GFP fluorescence, 32.26 ± 2.59 and 32.57 ± 3.48 , respectively, which were similar to the Rm1021(pHC514). The fact that the mutations in the *exoS* gene blocked the upregulation of the *exoR* promoter in *exoR95* mutant background raises the possibility of the involvement of ExoS in regulating the *exoR* gene expression.

4.3.5 ExoS regulates the *exoR* gene expression

To determine whether ExoS is involved in the *exoR* gene expression, plasmid pHC560, carrying the *exoS* gene under the inducible *lac* promoter, was introduced into different *S. meliloti* backgrounds carrying pHC514. The vector used for constructing

plasmid pHC560 was IncP-derived pSW213, and it is compatible with the vector pMB393 (derived from pBBR) used for constructing P_{exoR} -*gfp* fusions.

As shown in Figure 4-3, the levels of GFP fluorescence for Rm1021(pHC514+ pHC560) were 30.10 ± 5.82 , which was similar to Rm1021(pHC514), suggesting expression of more ExoS protein does not affect the *exoR* promoter activity in the wild type strain. However, in the *exoR95*(pHC514+ pHC560) strain, the levels of GFP fluorescence increased to 123.92 ± 3.51 , while in the *exoS96*(pHC514+ pHC560) strain, the levels of GFP fluorescence decreased to 37.80 ± 6.42 . This suggests that more ExoS protein can suppress the upregulation of the *exoR* promoter activity in the *exoS96* background, but more ExoS protein will further increase the *exoR* promoter activity in the *exoR95* mutant. Expression of ExoS also significantly increased the GFP fluorescence levels produced by *exoR95exoS** and *exoR95supA* mutants, for which 88.98 ± 8.60 and 85.69 ± 0.52 were detected, respectively. These data suggest that the suppressions of the upregulation of the *exoR* promoter in the *exoR95* background by mutations in the *exoS* gene can be complemented by the wild type *exoS*.

Altogether these results suggest that the *exoR* promoter activity is regulated by the ExoS protein.

4.3.6 The *exoR* gene expression is regulated by the ExoR protein

To determine whether ExoR regulates its own expression, the effects of ExoR and ExoR without its signal peptide (ExoR Δ SP) were examined by expressing them on plasmids pHC530 and pHC556, respectively, in different *S. meliloti* backgrounds carrying pHC514. As shown in Figure 4-4, expression of ExoR Δ SP did not change the

GFP fluorescence intensities in any backgrounds. Expression of the full-length ExoR significantly decreased the levels of GFP fluorescence produced in the *exoR95* background to 25.66 ± 5.59 , which were similar to the wild type levels. Expression of the full-length ExoR also decreased the *exoR* promoter activities slightly in the wild type strain, the *exoS96*, *exoR95exoS** and *exoR95supA* mutants. These data suggest that the *exoR* promoter activity is repressed by full-length ExoR protein, not by an ExoR Δ SP protein which stays in cell cytoplasm. Therefore, the ExoR protein must be transported into periplasm first and then regulate its own promoter activity through the ExoS/ChvI signal transduction pathway.

4.3.7 Effects of ExoR overexpression on succinoglycan production in free living *S. meliloti*

To determine the affects of overexpression of ExoR during free-living, the *exoR* gene was expressed from inducible promoters on plasmid pHC530 in the wild type Rm1021 and the *exoR95* mutant. Succinoglycan produced in bacterial liquid culture was measured quantitatively using anthrone-H₂SO₄ method. As shown in Table 4-3, Rm1021 produced low levels of succinoglycan, 1.45 ± 0.25 pg/cell, while the *exoR95* mutant produced high levels of succinoglycan, 23.44 ± 2.32 pg/cell. When ExoR was expressed from plasmid pHC530 in Rm1021 and the *exoR95* mutant, succinoglycan production decreased to 0.62 ± 0.11 and 1.52 ± 0.22 pg/cell, respectively. These data suggest that overexpressing ExoR decreases succinoglycan production.

To determine the target of ExoR regulation on succinoglycan production, the *exoY* gene promoter activities (from plasmid pHC77) in Rm1021 and the *exoR95* mutant were

also monitored under the same conditions. In wild type Rm1021, ExoR overexpression reduced the *exoY* gene promoter activities from 67.2 ± 2.9 to 49.6 ± 4.5 . In the *exoR95* mutant, ExoR overexpression reduced the *exoY* gene promoter activities from 370.3 ± 23 to 46.5 ± 6.3 . The *exoY* promoter activities were consistent with the succinoglycan production level, suggesting that ExoR negatively regulates succinoglycan production by repressing the *exoY* gene expression.

4.3.8 Effects of overexpression of ExoR during *S. meliloti*-alfalfa symbiosis

To determine the affects of overexpression of ExoR during *S. meliloti*-alfalfa symbiosis, the *exoR* gene was expressed from its own promoter on plasmid pHC510 in the wild type Rm1021 and the *exoR95* mutant. Alfalfa plants inoculated with these strains were examined after 28 days post inoculation (shown in Figure 2-4 and in Table 4-4). Alfalfa plants inoculated with Rm1021 were very healthy (tall and green) with mainly pink nodules, and the average wet weight for plants shoots was 51.3 mg/plant. Alfalfa plants inoculated with the *exoR95* mutant were not healthy (shorter plants with leaves turning yellow) with mainly white nodules, and the average wet weight for plants shoots was 23.2 mg/plant. Alfalfa plants inoculated with Rm1021(pHC510) were not very healthy and a few leaves were turning yellow. A few white nodules were formed on the plants roots and the average wet weight for these plants shoots was 37.9 mg/plant. Alfalfa plants inoculated with *exoR95*(pHC510) were healthy with mainly pink nodules and the wet weight for plants shoots was 47.4 mg/plant. These results suggest that the ExoR protein is required for *S. meliloti* to establish a successful symbiosis with alfalfa, and expression of ExoR can rescue the symbiosis defect of the *exoR95* mutant. But

overexpression of ExoR in the wild type strain can also impair the symbiosis at a certain level, suggesting the expression of the *exoR* gene has to be controlled during symbiosis.

4.4 DISCUSSION

The activities of the *exoR* promoter in different backgrounds were analyzed in this work. Based on the results of promoter analysis, the model that the ExoR functions upstream of ExoS (as discussed in Chapter 3), and the recent finding that ExoR directly interacts with ExoS (19), I propose that the ExoR protein auto-regulates its own expression through the ExoS/ChvI 2CRS (Figure 4-5). In *S. meliloti*, ExoS most likely functions in dimeric form as some sensor kinases in other two-component regulatory systems (21). When activated by environmental signals, it phosphorylates ChvI, the response regulator, which in turn binds to the promoter regions of its target genes and either activates or suppresses their expression (21).

In this model, the wild type ExoR protein represses the ExoS protein and prevents it from being active. Therefore, the activated ChvI form, ChvI-P, is low in cell cytoplasm and the *exoR* promoter activity is kept at low levels in the wild type strain Rm1021 (Figure 4-5, Panel A). But in the loss-of-function *exoR95* mutant, the interaction between ExoR and ExoS is disrupted. Without the repression of ExoR, ExoS is activated and phosphorylates ChvI which results in the upregulation of the *exoR* gene in the *exoR95* mutant (Figure 4-5, Panel B). When extra ExoS is expressed in the *exoR95* background, more active ExoS is formed which results in more ChvI-P, therefore, the *exoR* gene expression is further upregulated, as seen in *exoR95*(pHC514+ pHC560). When the wild-

type ExoR is expressed from a plasmid in the *exoR95* mutant, the ExoR protein is translocated into periplasm and interacts with ExoS and keeps it inactivated. Therefore, the *exoR* promoter activity is brought back to the wild type levels, as shown in the *exoR95*(pHC514+ pHC530) strain.

In the *exoS96* mutant, the ExoS96 protein, a constitutively active form of ExoS, is able to keep phosphorylating ChvI, which results in upregulation of the *exoR* gene (Figure 4-5, Panel C). When a wild-type ExoS is expressed from a plasmid in the *exoS96* mutant, ExoS may form dimers with itself and ExoS96, thus reducing the active ExoS96-ExoS96 dimers. As a result, less ChvI-P is formed and the expression of the *exoR* promoter is decreased in the *exoS96*(pHC514+ pHC560) strain.

In the *exoR95exoS** mutant, the point mutation in ExoS has blocked the upregulation of the *exoR* promoter activity. The ExoS* is recessive to ExoS, though its nature remains unknown (as discussed in Chapter 3). When the wild type ExoS is expressed in the *exoR95exoS**, ExoS-ExoS dimers (and/or ExoS*-ExoS perhaps) are able to phosphorylate ChvI, which results in the upregulation of the *exoR* expression, as shown in *exoR95exoS**(pHC514 + pHC560). The same mechanism may apply to the *exoR95supA* mutant as well (Figure 4-5, Panel D).

The *S. meliloti* symbiosis-important ExoR protein is a periplasmic protein which regulates both bacterial succinoglycan and flagellum production (117, 120). Previous reports have shown that the *exoR95* mutant produces large amount of succinoglycan and the mutant exhibits great nodulation deficiency (27). In this work, I have shown for the first time that extra copies of ExoR reduced succinoglycan production in the wild type strain and impaired symbiosis between the bacterium and alfalfa host. My experimental

results support the idea that the *exoR* promoter activity is kept at a certain level by auto-regulation through ExoS/ChvI 2CRS.

Microarray data have indicated that the ExoR protein also affects hundreds of genes expression and may be involved in several metabolic pathways (117). The finding that the expression of the *exoR* gene is auto-regulated has great significance.

Environmental factors, such as temperature, pH, osmotic pressure, and types of nutrient all affect bacterial metabolism. The *S. meliloti* ExoR protein may serve a role in sensing certain environmental stimuli and changing the cellular gene expression profile correspondingly. ExoR autoregulation is able to provide stability and more importantly to bring changes of gene expression caused by environmental stimuli back to a constant and sustainable level to maintain the balance of metabolisms.

Future work including finding how ChvI interacts with and regulates the *exoR* promoter and what triggers the ExoR signal transduction pathway will definitely help understand the complexity of the gene regulatory network in *S. meliloti*.

Table 4-1. Stains and plasmids.

Strain or plasmid	Relevant properties	References
<i>E. coli</i>		
DH5 α	General purposed strain	(45)
MT616	MT607, pRK600, Cm ^R	(32)
<i>S. meliloti</i>		
Rm1021	Wild type, Sm ^R	(60)
Rm7095	Rm1021 <i>exoR</i> 95::Tn5, Nm ^R	(27)
Rm7096	Rm1021 <i>exoS</i> 96::Tn5, Nm ^R	(27)
SmHC20	<i>exoR</i> 95 <i>exoS</i> *; Rm7095 with A76V mutation in ExoS	(91)
SmHC21	<i>exoR</i> 95 <i>supA</i> ; Rm7095 with T150A mutation in ExoS	This work
Plasmids		
pJK19-1	GFP-expression plasmid, Ap ^R	Lab stock
pMB393	Cloning vector, pBBR-derived, Sp ^R	(36)
pHC505	pMB393 carrying P _{<i>exoR</i>} - <i>gfp</i> fusion, P _{<i>exoR</i>} region is from -20 to -1	This work
pHC501	pMB393 carrying P _{<i>exoR</i>} - <i>gfp</i> fusion, P _{<i>exoR</i>} region is from -662 to -1	This work
pHC514	pMB393 carrying P _{<i>exoR</i>} - <i>gfp</i> fusion, P _{<i>exoR</i>} region is from -325 to -1	This work
pHC548	pMB393 carrying P _{<i>exoR</i>} - <i>gfp</i> fusion, P _{<i>exoR</i>} region is from -662 to -353 and -20 to -1	This work
pSW213	Cloning vector, IncP-derived, <i>lacI</i> ^Q , P _{<i>lac</i>} <i>lacZ</i> , Tc ^R	(75)
pHC530	pSW213 carrying the <i>exoR</i> gene in control of an inducible promoter	This work
pHC556	pSW213 carrying signal sequence-lacking <i>exoR</i> gene in control of an inducible promoter	This work
pHC560	pSW213 carrying the <i>exoS</i> gene in control of an inducible promoter	This work
pRK600	Helper plasmid, Cm ^R	(32)

Table 4-2. Primers used for plasmid constructions.

Plasmid	Primers	Sequence of primers
pHC501	<i>exoRf-662h</i>	CCCAAGCTTCATCGGCCACAAGTTCAACG
	<i>exoRr3n</i>	CTAGCTAGCCATAACAATCAGTTTCTTTTCGTTCG
	<i>gfp3n</i>	GGCATGGCTAGCAAAGGAGAAGAAC
	<i>gfpr877x</i>	CCGCTCGAGCACACCCGTCCTGTGGATAT
pHC505	<i>exoRf-20h</i>	CCCAAGCTTAACGAAAGAACTGATTGTTA
	<i>gfpr877x</i>	CCGCTCGAGCACACCCGTCCTGTGGATAT
pHC514	<i>exoRf-325h</i>	CCCAAGCTTGTATCTGGTAACCACGCTTAT
	<i>gfpr877x</i>	CCGCTCGAGCACACCCGTCCTGTGGATAT
pHC548	<i>exoRf-662h</i>	CCCAAGCTTCATCGGCCACAAGTTCAACG
	<i>exoRr-353-20</i>	TAACAATCAGTTTCTTTTCGTTGGAGTAATTGCCGAGAGG
	<i>exoRf-353-20</i>	CCTCTCGGCAATTACTCCAACGAAAGAACTGATTGTTA
	<i>gfpr877x</i>	CCGCTCGAGCACACCCGTCCTGTGGATAT
pHC530	<i>exoRf-20x</i>	CCGCTCGAGTAACGAAAGAACTGATTGTTATG
	<i>exoRr807k</i>	GGGGTACCTATCAATCGTCGTCGTTCTGC
pHC556	<i>exoRfATG91x</i>	CCGCTCGAGTAACGAAAGAACTGATTGTTATGT TCGATCCCGGAGCCGGCGTGA
	<i>exoRr807k</i>	GGGGTACCTATCAATCGTCGTCGTTCTGC
pHC560	<i>exoSf1-atgb</i>	CGGGATCCATGGTAGAAGTCTTGCAG
	<i>exoSr1818k</i>	GGGGTACCGGTCCCGTGGACATTGA

Table 4-3. Succinoglycan production and the *exoY* gene promoter activities in different *S. meliloti* strains.*

Strain	Succinoglycan (pg/cell)						$P_{exoY-gfp}$ (pHC77)**					
	None			+ ExoR (pHC530)			None			+ ExoR (pHC530)		
Rm1021 (wild type)	1.45	±	0.20	0.62	±	0.11	67.22	±	2.93	49.57	±	4.47
Rm7095 (<i>exoR95</i>)	23.44	±	2.32	1.52	±	0.22	370.25	±	22.73	46.52	±	6.34

* *S. meliloti* strains were cultured in Z-MGS medium for 50 hours. IPTG (0.8 mM) was used to induce the *exoR* expression. The results are the average value of two independent experiments and the corresponding standard deviations are indicated.

** The *exoY* promoter activities are represented by normalized GFP fluorescence in strains carrying plasmid pHC77, the *exoY* promoter-*gfp* fusion.

Table 4-4. Weights of alfalfa plants inoculated with different *S. meliloti* strains. *

	Rm1021(wild type)		Rm7095 (<i>exoR95</i>)	
	None	+ ExoR (pHC510)	None	+ ExoR (pHC510)
Wet weight of plants shoots (mg/plant)	51.3	37.9	23.2	47.4

* Alfalfa plants inoculated with *S. meliloti* cells were grown in Jensen's nitrogen-free agar medium for 28 days. The results are the average of 8 plants grown on one square Petri dish per bacterial strain.

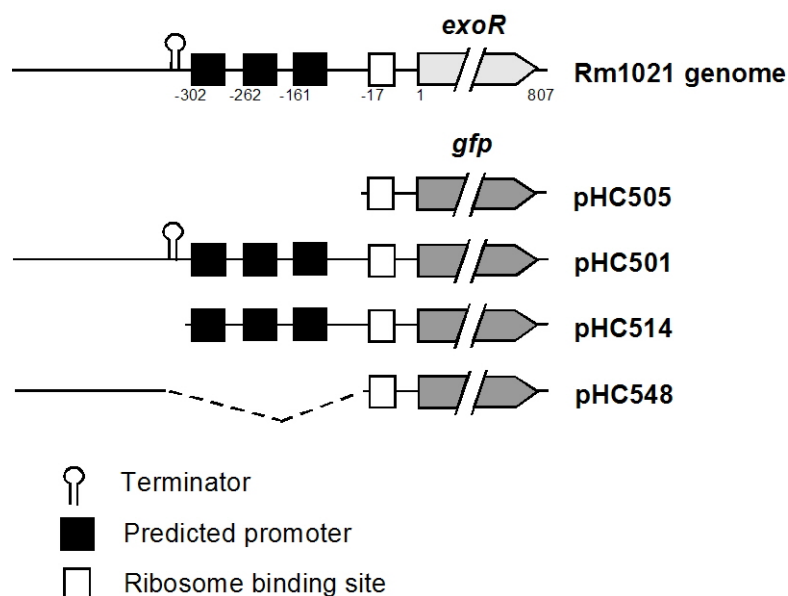


Figure 4-1. Construction of a set of plasmids carrying the *exoR* gene upstream sequence fused with the *gfp* gene. The positions of the *exoR* promoters predicted with Prokaryote Promoter Prediction Program on Berkeley Drosophila Genome Project website are marked as short solid boxes. A putative ribosome binding site (open box) and a putative transcription terminator (hairpin) of *exoR* upstream gene were indicated. The solid lines are used to represent the regions of the *exoR* upstream sequence carried on each of the plasmids. The dotted lines represent regions that are deleted.

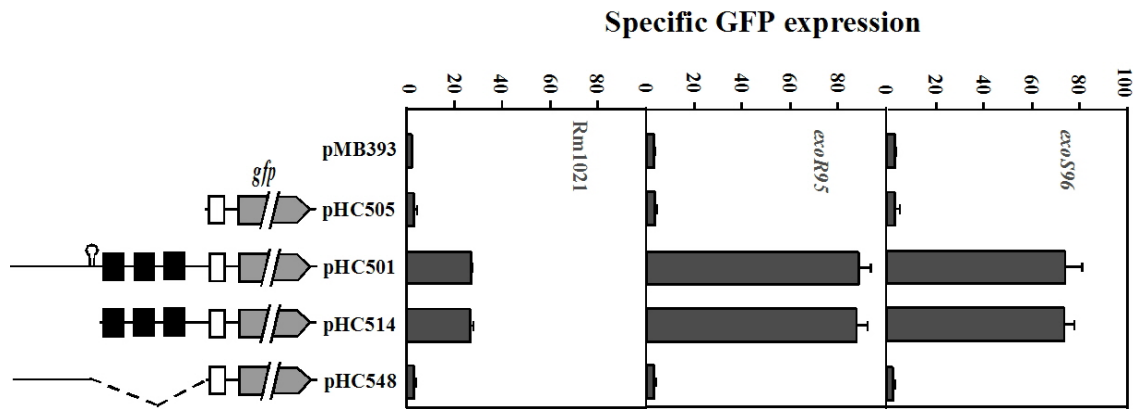


Figure 4-2. The *exoR* promoter activities are upregulated in the *exoR95* and the *exoS96* mutants. *S. meliloti* strains were cultured in LB/MC for 15 hours. Activities of the *exoR* promoters from different constructs in different genetic backgrounds are represented by normalized GFP fluorescence. The results are the average value of two independent experiments and the corresponding standard deviations are indicated.

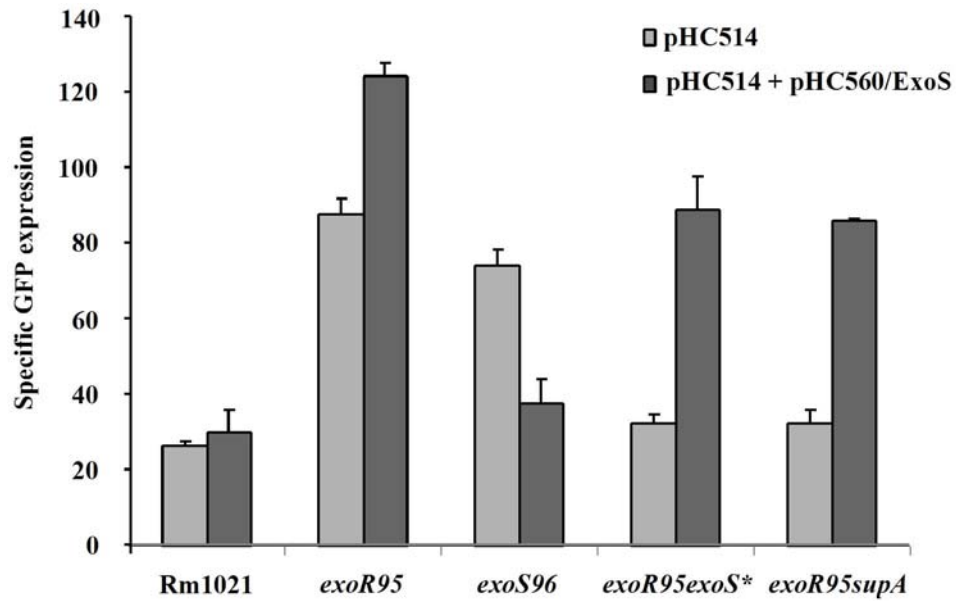


Figure 4-3. The ExoS protein is involved in the regulation of the *exoR* expression. *S. meliloti* strains were cultured in LB/MC for 15 hours. Plasmid pHC514 carries the entire *exoR* promoter region fused with *gfp*. The *exoR* promoter activities were shown downregulated in the *exoR95exoS** and *exoR95supA* mutants. When the *exoS* gene was expressed, the *exoR* promoter activities were significantly upregulated in the *exoR95*, *exoR95exoS** and *exoR95supA* mutants, and downregulated in the *exoS96* mutant. The results are the average value of two independent experiments and the corresponding standard deviations are indicated.

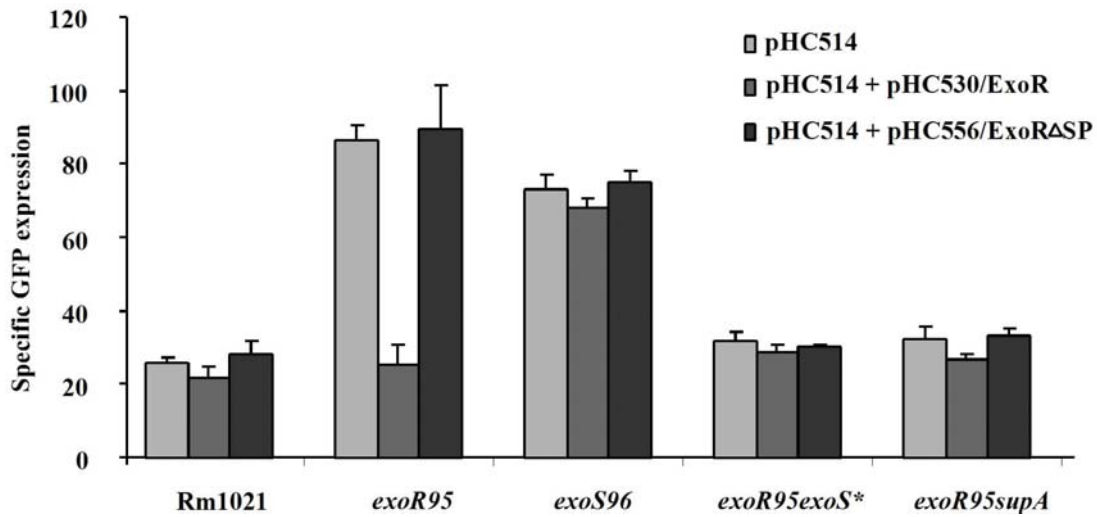


Figure 4-4. The ExoR protein auto-regulates its own expression. *S. meliloti* strains were cultured in LB/MC for 15 hours. Plasmid pHC514 carries the entire *exoR* promoter region fused with *gfp*. When the full-length ExoR was expressed, the *exoR* promoter activity was significantly downregulated in the *exoR95* mutant. The expression of an altered ExoR without signal peptide (from pHC556) did not affect the *exoR* promoter activities. The results are the average value of two independent experiments and the corresponding standard deviations are indicated.

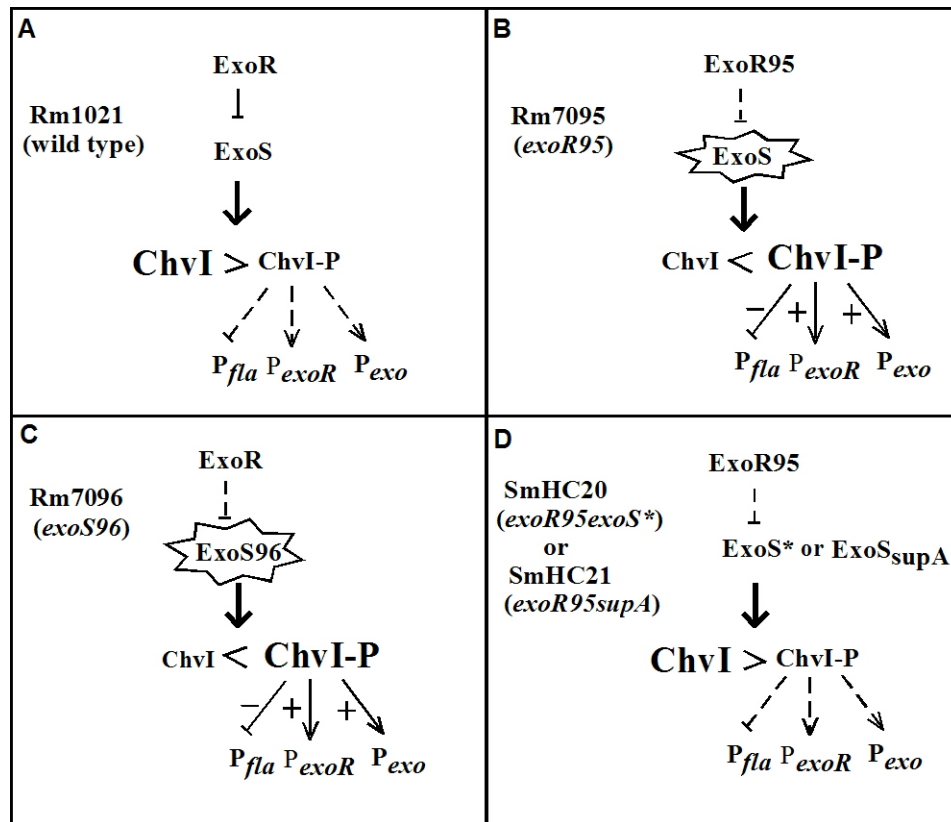


Figure 4-5. Model of the expression of the *exoR* gene in different *S. meliloti* backgrounds. Arrows and bar-ending arrows indicate activation and repression, respectively. Dotted lines indicate the repressions or activations are not in effect. Star-rings indicate proteins are active.

5 CHAPTER V THE C-TERMINUS OF THE *S. MELILOTI* EXOR PROTEIN

5.1 INTRODUCTION

The *exoR* gene was discovered as one of *S. meliloti* succinoglycan regulatory genes in a screen using transposon Tn5 mutagenesis (27). In the genome of the *exoR95* mutant, the Tn5 was predicted to be inserted at a site very close to the end of the *exoR* ORF and caused overproduction of succinoglycan (27, 98). The ExoR protein also plays important role in regulating flagellum production since the *exoR95* mutant formed only small size colonies on 0.3% soft agar (non-swarming phenotype) (120). The *exoR95sup7* mutant was previously isolated from one of the few pink nodules on alfalfa elicited by the *exoR95* mutant and *exoR95sup7* produced less succinoglycan than the *exoR95* mutant, suggesting the *sup7* mutation suppressed the *exoR95* mutation. In this chapter, I have further characterized the nature of the two mutants and analyzed the functions of mutated ExoR proteins with different C-termini.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this project are listed in Table 5-1. *E. coli* strains used in this study were grown in Luria-Bertani (LB) medium at 37°C (104). *S. meliloti* was grown in LB medium supplemented with 2.5 mM MgSO₄ and 2.5 mM CaCl₂ (LB/MC) at 30°C (60). Agar (1.5%) was used to solidify media. A modified M9 minimal medium supplemented with 0.1% ammonium chloride (NH₄Cl), biotin and thiamine was

used to grow bacteria to examine succinoglycan production in liquid medium (104).

When required, IPTG (isopropyl- β -d-thiogalactopyranoside) was added to induce gene expression. The following antibiotics were used at the concentrations indicated:

chloramphenicol (Cm), 10 μ g/mL; gentamycin, 50 μ g/mL; kanamycin (Km), 25 μ g/mL; neomycin (Nm), 200 μ g/mL; spectinomycin (Sp), 100 μ g/mL; streptomycin (Sm), 500 μ g/mL; and tetracycline (Tc), 10 μ g/mL.

5.2.2 Construction of the *exoR108* mutants

To make a plasmid insertion mutation in the *exoR* gene, a DNA fragment containing the middle part of the *exoR* gene ORF (nucleotide 88-433) was obtained in PCR using *S. meliloti* Rm1021 genomic DNA as template and two PCR primers: *exoRf88h* and *exoRr433b*. The PCR product was first treated with restriction enzymes *Hind*III and *Bsr*GI, and ligated with similarly treated suicide vector pK19mob2 Ω HMB to generate plasmid pHC508. The resulting plasmid was then conjugated into wild type *S. meliloti* Rm1021 using helper MT616 in a triparental mating. A few single colonies arose from the selective medium containing streptomycin and neomycin after 6 days. Genomic DNA was then isolated from one of the purified mutant using a Wizard genomic DNA purification kit (Promega) and used as a template in PCR to detect the juncture of the insertion. Primers used were *pK19Mp* which annealed to the suicide plasmid and *exoRf-193h* which annealed to the promoter region of the *exoR* gene (Figure 5-1). PCR results with the expected product confirmed that the plasmid insertion occurred in the *exoR* ORF. The constructed mutant was named *exoR108i*.

Phage lysis of *exoR108i* was generated and used to transduce the mutation into the wild type Rm1021 by selecting colonies which were able to grow on neomycin containing medium. But only one transductant was obtained, and the transductant was named *exoR108t* (Strain SmHC18).

Genomic DNA of *exoR108t* mutant was isolated and the *exoR* gene in this background was sequenced at the Albert Einstein College of medicine DNA facility.

5.2.3 Construction of a plasmid expressing the *exoR108* ORF

Based on the sequencing results of the *exoR* gene in the *exoR108t* mutant background, two primers (Table 5-2) were used to clone the *exoR108* ORF from the mutant genomic DNA in a PCR reaction. The PCR product was first treated with restriction enzymes *HindIII* and *XhoI*, and ligated with similarly treated vector pMB393 to generate plasmid pHC654.

5.2.4 Construction of plasmids which express mutated *exoR* ORFs encoding ExoR proteins with different C-termini

Mutated *exoR* ORFs encoding ExoR protein with different C-termini along with *exoR* promoter region were amplified from genomic DNA of different *S. meliloti* strains, Rm1021, the *exoR95sup7* mutant, and the *exoR95* mutant. Primers used for constructing each plasmid are listed in Table 5-2. Each of the PCR products was first treated with restriction enzymes *HindIII* and *XhoI*, and ligated with similarly treated vector pMB393.

5.2.5 Sequencing the *exoS* gene and the *chvI* gene in the *exoR108* mutants

The genomic DNA of the *exoR108* mutants was isolated and used as the template in PCR with two primers, *exoSf-61* and *exoSr1918* to get a DNA product covering the *exoS* ORF. This DNA product was then sequenced using primers *exoSr407*, *exoSr829*, *exoSf653*, *exoSf1098* and *exoSr1912*.

To sequence the *chvI* gene, the genomic DNA of the *exoR108* mutants was used as the template in PCR with two primers, *chvIf-27* and *chvIr759* to get a DNA product covering the *chvI* ORF. This DNA product was then sequenced using primers *chvIf293* and *chvIr419*.

5.2.6 Exopolysaccharide detection

To examine succinoglycan production by *S. meliloti* grown on solid medium, calcofluor white M2R (Fluorescent Brightener 28, Sigma, St. Louis, MO) was added to a final concentration of 0.02% in LB/MC agar, which was buffered to pH 7.4 with 10 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) (60). To measure succinoglycan production, bacteria were first grown in LB/MC liquid medium to mid-log phase, washed with M9 medium and then resuspended to approximately OD₆₀₀ of 0.3 in M9 medium. The cells were then incubated at 30°C with shaking for 48 hours. Cell cultures were diluted and centrifuged at 13,000 g for 15 minutes at 4°C. Culture supernatants were used to measure succinoglycan production with anthrone-H₂SO₄ method as described earlier (66).

5.2.7 Alignment of the ExoR proteins in different bacteria

Protein sequences of ExoR homologues were obtained from NCBI and alignment was performed using Vector NTI software (Invitrogen, Carlsbad, CA).

5.2.8 Cell motility

Refer to chapter 2.

5.2.9 Alfalfa nodulation assays

Refer to chapter 2.

5.3 RESULTS

5.3.1 Phenotypes of the *exoR95sup7* mutant

The *exoR95* mutant is very ineffective in nodulating alfalfa, thus the majority alfalfa root nodules elicited are white and defective in nitrogen fixation (27). However, the *exoR95* mutant can still invade nodules successfully at a low efficiency and elicit a few pink nodules. An isolate, named *exoR95sup7* (lab collection), was recovered previously from one pink nodule elicited by the *exoR95* mutant. Unlike most bacterial cells recovered from the *exoR95* elicited pink nodules, the *exoR95sup7* mutant produced less succinoglycan than the *exoR95* mutant on LB/MC/CF medium. Therefore, it was further characterized in my work to determine if it carries any suppressor mutation of the *exoR95* mutation.

When the *exoR95sup7* mutant was used to inoculate alfalfa, it showed higher efficiency than *exoR95*. The percentage of pink nodules on alfalfa inoculated by Rm1021,

the *exoR95* mutant and the *exoR95sup7* mutant were $94.15\% \pm 5.58\%$, $15.74\% \pm 4.03\%$ and $71.25\% \pm 3.18\%$, respectively. When the *exoR95sup7* mutant was streaked on LB/MC/CF medium, it formed colonies less bright than the *exoR95* mutant under UV light, but a little brighter than the wild type Rm1021 (Figure 5-2). When grown in M9 liquid medium, the levels of succinoglycan (OD_{620}/OD_{600}) by Rm1021, the *exoR95* mutant and the *exoR95sup7* mutant were 0.10, 19.70, and 0.68, respectively, as measured using anthrone- H_2SO_4 method.

Previous report has shown that ExoR is involved in regulating succinoglycan as well as flagellum production (120), which can be examined easily by the size of swarming colonies formed on 0.3% agar by different strains. As shown in Figure 5-3, while Rm1021 formed a large swarming colony and the *exoR95* mutant formed a small non-swarming colony, the *exoR95sup7* mutant formed a colony with a medium size.

Altogether, these results suggest that the *exoR95sup7* mutant very likely contains a suppressor mutation, *sup7*, in the genome and presence of the *sup7* suppressor mutation suppresses the succinoglycan production and motility phenotypes of the *exoR95* mutation.

5.3.2 Identification of the *sup7* suppressor mutation

As the first the step towards identifying the *sup7* mutation, an effort was made to confirm that the original *exoR95::Tn5* mutation has not been changed and that Tn5 is inside the *exoR* gene in the *exoR95sup7* mutant. To amplify the region covering the junction of the *exoR* gene and Tn5, genomic DNA of the *exoR95* mutant and the *exoR95sup7* mutant was used in PCR reactions using primer *exoRf-78* which anneals to *exoR* promoter region and primer *Pis50* which anneals to the Tn5 IS50 region (Figure 5-

4). While a PCR product around 1 Kb was detected from the *exoR95* mutant, no PCR product was detected from the *exoR95sup7* mutant (data not shown), which raised the possibility that this mutant might have lost part or entire Tn5 in *exoR*.

To further understand the possible change of Tn5 in the *exoR* gene in the *exoR95sup7* mutant, a new primer, *Pneo*, which anneals to the neomycin resistance gene in the middle of Tn5, was used to repeat the same set of PCR (Figure 5-4). This time a shorter DNA fragment (around 1 Kb) was detected from the *exoR95sup7* mutant (Lane D, Figure 5-5), compared to the longer DNA fragment (around 2.5 Kb) from the *exoR95* mutant (Lane C, Figure 5-5). The size difference (1.5 Kb) between the two PCR products suggested that the Tn5 inserted into the *exoR* gene in the *exoR95sup7* mutant might have lost one IS50 region.

The PCR products covering the juncture of *exoR* and Tn5insertion from both *exoR95* and *exoR95sup7* mutants were purified and sequenced. Analysis of the DNA sequencing results revealed that the site of Tn5 insertion in the *exoR* gene of the *exoR95sup7* mutant remains the same as the *exoR95* mutation, which is before the 746th bp of the *exoR* ORF, but the Tn5 inserted into the *exoR* gene was incomplete in the *exoR95sup7* mutant, where almost the entire IS50 region in Tn5 was missing (Figure 5-6).

Before further analysis of the *exoR95sup7* mutant, the *exoR95sup7* mutation was transduced by phage ΦM12 into the wild type strain Rm1021 to regenerate the mutant. Once the mutation in the *exoR* gene was successfully transduced, the transductant was tested for succinoglycan production, swarming ability and symbiosis efficiency. The new transductant exhibited the same phenotypes as that of original strain (data not shown),

which demonstrated that the phenotypes of *exoR95sup7* mutant resulted only from a mutated Tn5 form inserted into the *exoR* gene.

5.3.3 The ExoR95sup7 protein is a mutated ExoR with a different C-terminus

The wild type *exoR* gene ORF in *S. meliloti* has 807 bps encoding a protein with 268 amino acid residues. In the genome of the *exoR95* mutant, Tn5 has inserted in front of the codon for the 249th amino acid so that the *exoR* ORF extends into IS50L to generate a 257 amino acid ExoR95 protein. The last 20 amino acid of the ExoR C-terminus (C_{ExoR20}, DDRVAITMSQNMHLQNDDD) is replaced by the ExoR95 C-terminus of 9 amino acids (C_{R95}, ADSYTQVAS). In the *exoR95sup7* mutant, Tn5 insertion has resulted in a 256 amino acid ExoR95sup7 protein. Its last 8 C-terminal amino acids (C_{sup7}, GMRIVSHD) are coded by the mutated Tn5, replacing the original last 20 amino acids of the ExoR C-terminus. So the *sup7* mutation leads to the generation of a mutated ExoR protein with a C-terminus that is different from the wild type ExoR and the mutated ExoR95 protein (Figure 5-7).

Noticeably, the ExoR95 C-terminus only shares the same amino acid at position 250 (aspartic acid) with the ExoR protein, while the ExoR95sup7 C-terminus shares the same amino acids at positions 251 (arginine) and 253 (valine) with the ExoR protein. In addition, the secondary structures of the three proteins were predicted (Figure 5-7). All three proteins are predicted to form a β -sheet by their last few amino acids. These results raised the possibility that one or a few amino acid residues in the ExoR C-terminal may play an important role in maintaining the function of the protein.

5.3.4 Functional analysis of ExoR, ExoR95, and ExoR95sup7

To analyse the function of the ExoR95sup7 protein, *exoR95sup7* was cloned and expressed with native *exoR* promoter in plasmid pHC511. The *exoR* and the *exoR95* were similarly cloned to generate plasmids pHC510 and pHC513, respectively. These plasmids were transferred to the *exoR95* mutant. Succinoglycan production by all the strains grown on LB/MC/CF medium was determined. As shown in Figure 5-2, expression of ExoR95sup7 in the *exoR95* mutant brought the levels of calcofluor fluorescence down to levels comparable to that of the wild type Rm1021, suggesting more ExoR95sup7 proteins act as efficient as the wild type ExoR protein in reducing the levels of succinoglycan production by the *exoR95* mutant. Interestingly, expressing more ExoR95 also reduced the levels of succinoglycan production (shown as the calcofluor fluorescence) by the *exoR95* mutant a little when compared to the *exoR95* mutant itself, but it was far less effective than the wild type.

When cell motility of each strains was examined on swarming plates (Figure 5-3), the presence of ExoR95sup7 was as efficient as the wild type ExoR in restoring the motility of the *exoR95* mutant to allow the formation of swarming colonies as big as the wild type strain. Surprisingly, the presence of ExoR95 was also almost as effective as the wild type ExoR in restoring the motility of the *exoR95* mutant, suggesting extra copies of ExoR95 do affect cell motility.

These findings suggest that the C-terminus of the ExoR protein plays an important role in its regulatory function since the three proteins differ only in their C-termini.

5.3.5 ExoR functions without C-terminal 20 amino acids

To test the hypothesis that the C_{ExoR20} might play an essential role in the function of ExoR, a set of new mutated ExoR proteins with progressively longer C-terminal deletions was generated (Figure 5-8). The five mutated ExoR proteins, ExoR258, ExoR248, ExoR238, ExoR223, and ExoR198, missing C-terminal 10, 20, 30, 45, and 70 amino acids, were expressed in pH537, pH512, pH538, pH535, and pH536, respectively. The plasmids were transferred to the *exoR95* mutant and each constructed strain was tested for the succinoglycan and cell motility. As shown in Figure 5-2 and 5-3, the presence of ExoR258 and ExoR248 reduced the levels of calcofluor fluorescence of the *exoR95* mutant to that of the wild type strain and enabled the *exoR95* mutant to form similar size swarming colonies to the wild type. But none of the ExoR238, ExoR223, or ExoR198 proteins was able to complement the *exoR95* mutation.

Altogether, these results suggest that the presence of the C_{ExoR20} is not essential to the regulatory function of the ExoR protein. But if more than 30 amino acids are removed from its C-terminus, the ExoR will completely lose its function.

5.3.6 The comparative analysis of ExoR C-termini

Even though all three mutated ExoR proteins, ExoR-20, ExoR95, and ExoR95sup7 share the common N-terminal 248 amino acids as in ExoR, only ExoR-20 and ExoR95sup7 exhibited the wild type ExoR function. To further assess the effects of different ExoR C-termini on the regulatory function of the ExoR proteins, a new set of mutated ExoR proteins was generated (Figure 5-8). The 9 amino acid ExoR95 C-terminus (C_{R95}) was added to the C-terminus of ExoR to generate ExoRC_{R95} protein

expressed in plasmid pHC539, and also used to replace the C-terminal 9 amino acids of ExoR to generate ExoR259C_{R95} protein expressed in pHC541. The 8 amino acid ExoR95sup7 C-terminus (C_{sup7}) was added to C-terminus of ExoR to generate ExoRC_{sup7} protein expressed in plasmid pHC540, and also used to replace the C-terminal 8 amino acids of ExoR to generate ExoR260C_{sup7} protein expressed in pHC542.

The four plasmids were transferred into the *exoR95* mutant background individually to examine the function of the four new hybrid ExoR proteins, ExoRC_{R95}, ExoR259C_{R95}, ExoRC_{sup7}, and ExoR260C_{sup7}. The presence of all four new hybrid ExoR proteins was able to bring the calcofluor fluorescence of the *exoR95* mutant down to the levels of the wild type strain (Figure 5-2), suggesting that these four hybrid proteins were as effective as the wild type ExoR protein in regulating succinoglycan production in the *exoR95* mutant background. The presence of all four new hybrid ExoR proteins also enabled the *exoR95* mutant to form swarming colonies similar to that formed by the wild type Rm1021 (Figure 5-3), suggesting that these four hybrid ExoR were as effective as the wild type ExoR in regulating motility in the *exoR95* mutant background.

All these findings suggest that the 9 amino acid ExoR95 C-terminus is able to diminish the function of the ExoR95 protein which can also be considered as the N-terminal 248 amino acids of ExoR (ExoR248) fused with the 9 amino acid ExoR95 C-terminus (C_{R95}). But with more N-terminal amino acids of the ExoR protein present, like in the hybrid proteins ExoRC_{R95} and ExoR259C_{R95}, the 9 amino acid C_{R95} does not interfere with the regulatory functions of ExoR.

5.3.7 The *exoR* gene cannot be deleted from the wild type strain from the wild type strain using plasmid insertion mutation method

The *exoR108* mutant was constructed by inserting a suicide plasmid in the middle of the *exoR* ORF after the 433rd bp as detailed in the methods. As a control, with extra copies of wild type *exoR* gene present, the suicide plasmid was transferred into Rm1021 (pHC510) strain easily. However, the conjugation efficiency for the suicide plasmid to be transferred into the wild type strain was extremely low and only very few colonies were obtained by conjugation. The colonies had very mucoid appearance similar to the *exoR95* mutant. One such single colony, named *exoR108i* was kept. The mutation was transduced into wild type strain to ensure that it is the result of the suicide plasmid insertion, but only one transductant were obtained, which was named *exoR108t*.

Sequencing results of the *exoR* gene in *exoR108t* mutant revealed that *exoR108* encodes a 195 amino acid protein, which is the fusion of ExoR N-terminal 144 amino acids and 51 amino acids from the suicide vector (Figure 5-9). When expressed from plasmid pHC564 in the *exoR95* mutant, the ExoR108 could not change either succinoglycan or motility phenotype of the mutant (Figure 5-10), which demonstrated that it is a non-functional protein. As a control, the phenotypes of the *exoR95* mutant can be suppressed by expressing the wild type ExoR from plasmid pHC510.

The phenotypes of both *exoR108i* and *exoR108t* were also tested. Similar to the *exoR95* mutant, the *exoR108i* overproduced succinoglycan on LB/MC/CF medium. But unlike the *exoR95* mutant, the *exoR108i* has a normal swarming ability and formed a large size colony similar to the wild type on soft agar. To our surprise, although *exoR108t*, the re-transduced mutant, overproduced succinoglycan, it failed to form large swarming

colony, which is similar to the *exoR95* mutant. Like the *exoR95* mutant, the succinoglycan overproduction of both *exoR108i* and *exoR108t* could be repressed by the presence of pHC510, which carries the wild type *exoR* ORF with its native promoter. The swarming deficiency phenotype of *exoR108t* can also be repressed by the presence of pHC510 (Figure 5-10).

Because of the extremely low efficiency of conjugation and transduction and also the phenotype difference between *exoR108i* and *exoR108t*, it is highly possible that there is a secondary mutation in both backgrounds. Therefore, both *exoS* and *chvI* genes in both mutant backgrounds were sequenced. Sequencing results only indicated a point mutation from G to A at the 325th nucleotide in the *chvI* gene in the *exoR108t* background, which results in the substitution of valine at the 109th position in ChvI by a methionine (V109M). But it remains possible that there are mutations somewhere else in both backgrounds.

5.4 DISCUSSION

5.4.1 Is *exoR95* a complete loss-of-function mutation?

The *S. meliloti* *exoR95* mutation was believed to be a complete loss-of-function mutation, since it changed the bacterial succinoglycan and flagellum production, and also caused dramatic deficiency in nodulating alfalfa (27, 98, 120). Another *exoR* mutation named *exoR99*, constructed by inserting a cassette before nucleotide 299 in the *exoR* ORF, also caused succinoglycan overproduction which could be complemented by a cosmid carrying the *exoR* gene (98). This also implies that both mutations could be complete loss-of-function mutations, since their sites of Tn5 insertion were very different (98). When the ExoR95 protein was overexpressed in the *exoR95* mutant, to our surprise, it

reduced the production of succinoglycan at a limited degree and restore swarming ability, suggesting that it may have a low level of regulatory function.

To determine whether *exoR95* is a complete loss-of-function mutation, plasmid insertion mutation method was used to interrupt the *exoR* gene in the middle to generate a complete loss of function mutation. But the difficulties in making this plasmid insertion mutation and also different phenotypes of the *exoR108i* and *exoR108t* mutants made us suspect that *exoR* could be an essential gene and cannot be deleted unless secondary mutations occur in the genome. Indeed, a point mutation in *chvI* was found in *exoR108t* mutant. However, it remains possible that there is undetected mutation(s) in other genes in both *exoR108i* and *exoR108t* mutants, which could be further studied in the future. Therefore, *ExoR95* should not be tested in either *exoR108i* or *exoR108t* mutant backgrounds.

However, the hybrid *ExoR108* protein itself should be a complete loss-of-function protein. Different from *ExoR95*, expressing *ExoR108* in the *exoR95* mutant did not change any phenotypes of the mutant. Therefore, we conclude that the *exoR95* mutation is not a complete loss-of-function mutation, and that most likely the *exoR* gene could not be completely deleted.

5.4.2 Does the ExoR C-terminus play an important role in the function of the protein?

The *exoR95sup7* mutant was previously isolated from a pink nodule on alfalfa root induced by the *exoR95* mutant and produced less succinoglycan than *exoR95*. When this *exoR95sup7* mutant was further characterized, the *sup7* mutation was found to be a

sequence change in the Tn5 inserted into the *exoR* gene. The two mutated proteins, ExoR95 and ExoR95sup7, share the common N-terminal 248 amino acids as the wild type ExoR, and they differ only in the last several amino acids at their C-termini. It is possible that one or a few amino acids in C_{ExoR20} may play an important role in maintaining the ExoR function and they are conserved in C_{sup7}, but not in C_{R95}. However, expressing either ExoR without the last 10 amino acids (ExoR258) or 20 amino acids (ExoR248) in the *exoR95* mutant was able to complement the mutation, suggesting the conserved amino acids found in C_{sup7} are not necessary to be essential to the ExoR protein. These data suggest that the presence of C_{ExoR20} is not essential to maintain the function of the ExoR protein.

The ExoR95 can be considered as a fusion of ExoR248 (the one without the C_{ExoR20}) and C_{R95}. The ExoR248 protein is functional, but why would the addition of C_{R95} to ExoR248 diminish its regulatory function? And why was the presence of C_{R95} not able to diminish the function of ExoR when placed at other locations in the protein, such as in ExoRC_{R95}, ExoR259C_{R95}? To answer these questions, I compared the ExoR C-terminal 20 amino acids in ExoR homologues in different bacteria and found that amino acids at positions 250(D), 251(R), 252(R), 254(A), 255(I), and 259(Q) are well conserved (Figure 5-11). This raised the possibility that those amino acids, although not essential to the ExoR protein, may help to maintain ExoR function by stabilizing the structure of ExoR or the interaction between ExoR and ExoS.

That replacing the C_{ExoR20} of ExoR with C_{sup7} slightly reduced the function is probably due to a small change in the structure. This functional deficiency of ExoR95sup7 can easily be compensated by increasing the amount of the protein. In

comparison, replacing the C-terminus of ExoR with C_{R95} may induce a specific structure change and therefore diminish its function more dramatically, but not completely yet. The fact that expressing more ExoR95 in the *exoR95* mutant background was able to decrease the succinoglycan production to a limited degree and restore the swarming ability could be explained by increased amount of stable ExoR95 protein or chance of it to interact with ExoS.

Analysis of *S. meliloti* ExoR sequence has shown that it has four Sell repeats (at positions: 43-74, 75-110, 121-159 and 161-196) (81) and amino acids between 38 and 248 are also predicted to form tetratricopeptide-like superhelix (tetratricopeptide repeat, TPR), suggesting ExoR might rely on protein-protein interactions with ExoS (and/or other intermediate proteins) to regulate gene expression indirectly. The structure predictions seem to be consistent with my findings that the deletion of C_{ExoR20} did not disrupt the function of ExoR, but deleting more amino acids from its C-terminus, 30, 45, and 70, definitely made it completely nonfunctional. Future study of *S. meliloti* ExoR protein structure using biochemical and biophysical methods should reveal its topological features and help us to understand how it works.

Table 5-1. Strains and plasmids.

Strain or plasmid	Relevant properties	References
<i>E. coli</i>		
DH5 α	General purposed strain	(45)
MT616	MT607, pRK600, Cm ^R	(32)
<i>S. meliloti</i>		
Rm1021	SU47, Sm ^R	(60)
Rm7095	Rm1021 <i>exoR95::Tn5</i> , Nm ^R	(27)
Rm7096	Rm1021 <i>exoS96::Tn5</i> , Nm ^R	(27)
Rm7210	Rm1021 <i>exoY210::Tn5</i> , Nm ^R	(60)
SmHC18	Rm1021 <i>exoR108::pK19mob2</i> Ω HMB, transduced	This work
SmHC19	<i>exoR95sup7</i> , Nm ^R	G.C. Walker
Plasmids		
pK19mob2 Ω HMB	A suicide plasmid, Km ^R	(70)
pMB393	Cloning vector, Sp ^R	(36)
pHC108	pK19mob2 Ω HMB carrying a 300 bp <i>exoR</i> gene fragment, Km ^R	This work
pHC510	pMB393 carrying the <i>exoR</i> gene, <i>exoR</i> promoter	This work
pHC511	pMB393 carrying a mutated <i>exoR</i> gene encoding ExoR95sup7, <i>exoR</i> promoter	This work
pHC512	pMB393 carrying a mutated <i>exoR</i> gene encoding ExoR which missing the C-terminal 20 aa, <i>exoR</i> promoter	This work
pHC513	pMB393 carrying a mutated <i>exoR</i> gene encoding ExoR95, <i>exoR</i> promoter	This work
pHC535	pMB393 carrying a mutated <i>exoR</i> gene encoding ExoR without its C-terminal 45 aa, <i>exoR</i> promoter	This work
pHC536	pMB393 carrying a mutated <i>exoR</i> gene encoding ExoR without its C-terminal 70 aa, <i>exoR</i> promoter	This work
pHC537	pMB393 carrying a mutated <i>exoR</i> gene encoding ExoR without its C-terminal 10 aa, <i>exoR</i> promoter	This work
pHC538	pMB393 carrying a mutated <i>exoR</i> gene encoding ExoR without its C-terminal 30 aa, <i>exoR</i> promoter	This work
pHC539	pMB393 carrying a mutated <i>exoR</i> gene encoding ExoR with extra 9 aa from C _{R95} , <i>exoR</i> promoter	This work
pHC540	pMB393 carrying a mutated <i>exoR</i> gene encoding ExoR with extra 8 aa from C _{sup7} , <i>exoR</i> promoter	This work
pHC541	pMB393 carrying a mutated <i>exoR</i> gene encoding ExoR with its c-terminal last 9 aa replaced by C _{R95} , <i>exoR</i> promoter	This work
pHC542	pMB393 carrying a mutated <i>exoR</i> gene encoding ExoR with its C-terminal last 8 aa replaced by C _{sup7} , <i>exoR</i> promoter	This work
pHC564	pMB393 expressing the <i>exoR108</i> ORF, <i>exoR</i> promoter	This work
pRK600	Helper plasmid, Cm ^R	(32)
Phage		
ϕ M12	Generalized transducing phage for <i>S. meliloti</i>	(31)

Table 5-2. Primers used in plasmid constructions.

Plasmid	Primer	Primer sequence
pHC510	<i>exoRf-662h</i> <i>exoRr807x</i>	CCCAAGCTTCATCGGCCACAAGTTCAACG CCGCTCGAGTCATCAATCGTCGTCGTTCTGC
pHC564	<i>exoRf-662h</i> <i>exoR108r588x</i>	CCCAAGCTTCATCGGCCACAAGTTCAACG CCGCTCGAGTCAGGCTGCGCAACTGTTGGGA
pHC511	<i>exoRf-662h</i> <i>ExoR95sup7r771x</i>	CCCAAGCTTCATCGGCCACAAGTTCAACG CCGCTCGAGTCATCAATCATGCGAAACGATCC
pHC512	<i>exoRf-662h</i> <i>exoRr744x</i>	CCCAAGCTTCATCGGCCACAAGTTCAACG CCCCTCGAGTCATTCCGGTGGCGAGCGAGAA
pHC513	<i>exoRf-662h</i> <i>ExoR95r774x</i>	CCCAAGCTTCATCGGCCACAAGTTCAACG CCGCTCGAGTCATCAGGACGCTACTTGTGTAT
pHC535	<i>exoRf-662h</i> <i>exoRr669x</i>	CCCAAGCTTCATCGGCCACAAGTTCAACG CCCCTCGAGTCACGCCGCCGTCATGTATGC
pHC536	<i>exoRf-662h</i> <i>exoRr594x</i>	CCCAAGCTTCATCGGCCACAAGTTCAACG CCCCTCGAGTCAGCCGGCATGACCATTCTT
pHC537	<i>exoRf-662h</i> <i>exoRr774x</i>	CCCAAGCTTCATCGGCCACAAGTTCAACG CCCCTCGAGTCAGGACATCGTTATCGCTACCC
pHC538	<i>exoRf-662h</i> <i>exoRr714x</i>	CCCAAGCTTCATCGGCCACAAGTTCAACG CCCCTCGAGTCACATGGCCTGGAGCCAGGG
pHC539	<i>exoRf-662h</i> <i>R w R95rx</i>	CCCAAGCTTCATCGGCCACAAGTTCAACG CCGCTCGAGTCAGGACGCTACTTGTGTATAAGAGTCAGC ATCGTCGTCGTTCTGCAGAT
pHC540	<i>exoRf-662h</i> <i>R w sup7rx</i>	CCCAAGCTTCATCGGCCACAAGTTCAACG CCGCTCGAGTCAATCATGCGAAACGATCCTCATCCCATC GTCGTCGTTCTGCAGAT
pHC541	<i>exoRf-662h</i> <i>R/R95rx</i>	CCCAAGCTTCATCGGCCACAAGTTCAACG CCGCTCGAGTCAGGACGCTACTTGTGTATAAGAGTCAGC CTGGGACATCGTTATCGCTA
pHC542	<i>exoRf-662h</i> <i>R/sup7rx</i>	CCCAAGCTTCATCGGCCACAAGTTCAACG CCGCTCGAGTCAATCATGCGAAACGATCCTCATCCCGTT CTGGGACATCGTTATCGC
pHC108	<i>exoRf88h</i> <i>exoRr433b</i>	GCCAAGCTTTCGATCCCGGAGC GGCTGTACAGGTCGGACCTTACCG

Table 5-3. Primers used in sequencing.

Primer	Primer sequence
<i>pK19Mp</i>	TCCCGCTTCAGTGACAACG
<i>exoRf-193h</i>	CCCAAGCTTTAGGGACGAAGGCTAGTCGA
<i>exoRr-1</i>	AACAATCAGTTTCTTTCGTTTCG
<i>exoRf-78</i>	CAGGAGCCAGTGCGTACA
<i>exoRf128</i>	CCTTCGCGCTCTTCAAGTTC
<i>exoRr433b</i>	GGCTGTACAGGTCGGACCTTACCG
<i>exoSf-61</i>	ACGGGCGAATGCGACAGG
<i>exoSr1918</i>	CCCCGGTGCCTCACTTCC
<i>exoSr407</i>	CGCTCCTGAATGATCGGGAAT
<i>exoSr829</i>	CGGCATGGACGATCTTGTCG
<i>exoSf653</i>	CGATCTATCCGGAGGTGATGAAC
<i>exoSf1098</i>	GAGCCACGAACCTCAAGAACCC
<i>exoSr1912</i>	TGCCTCACTTCCGAAAGACAG
<i>chvIf-27</i>	CCGGGCCTCGAAACGGAGTA
<i>chvIr759</i>	GGCCACGAGTGATCCGCAAT
<i>chvIf293</i>	TCATCACCAAGCCCTTCTCACAG
<i>chvIr419</i>	GGGAAGGCGTGTCGGCATT
<i>Pneo</i>	GAGCAGCCGATTGTCTGTTGTG
<i>Pis50</i>	CGATGAAGAGCAGAAGTTATCA

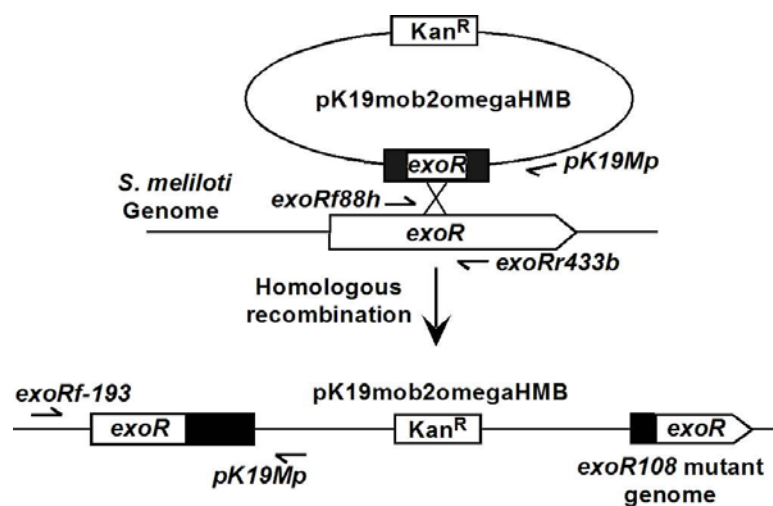


Figure 5-1. Schematic representation of the strategies for plasmid insertion into the *exoR* gene in *S. meliloti* genome. The locations of primers used to amplify the middle part of *exoR* and the junction between *exoR* and the suicide plasmid are indicated.

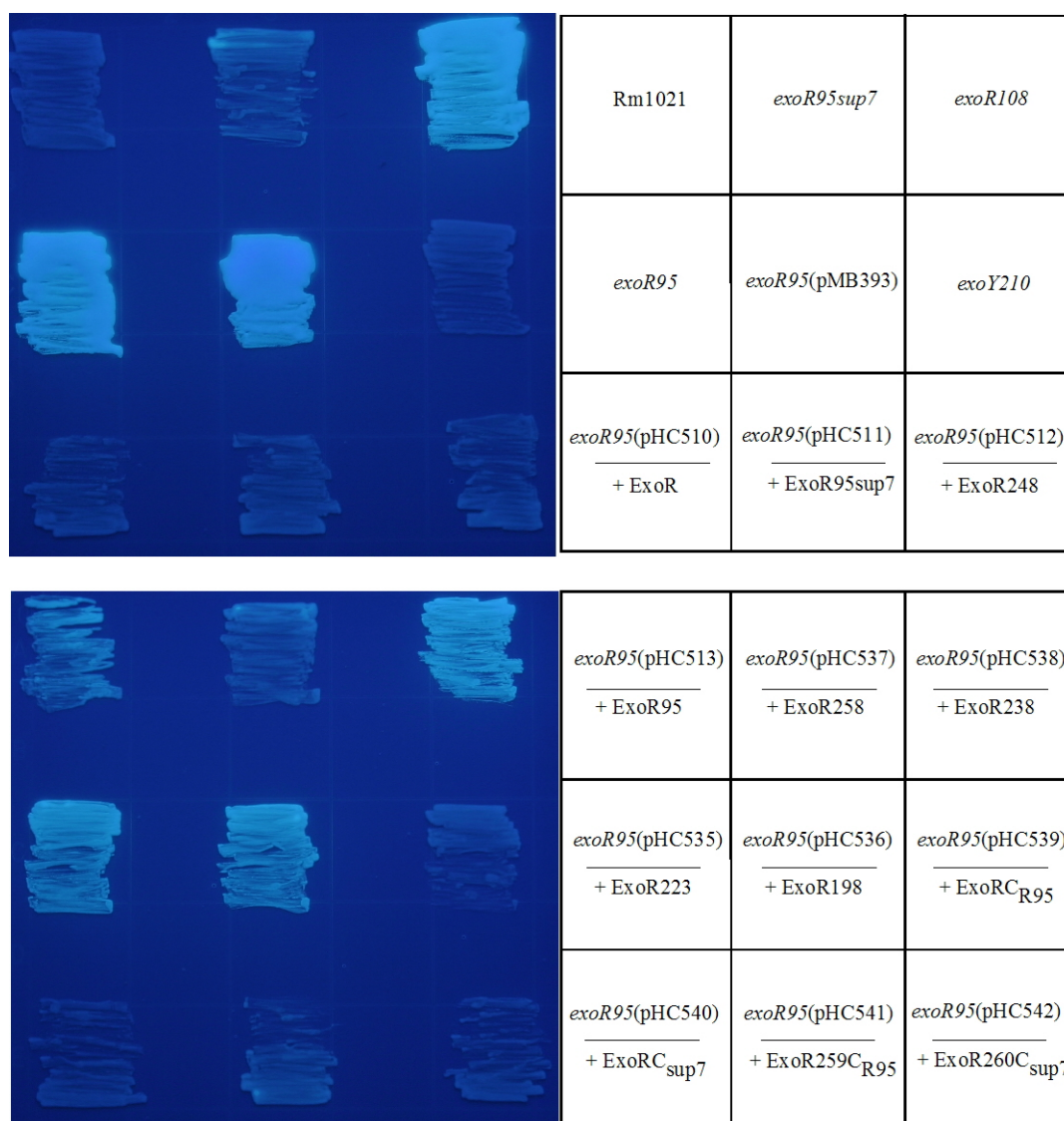


Figure 5-2. Effects of C-terminus of ExoR on regulation of succinoglycan production. The calcofluor fluorescence represents the levels of succinoglycan produced by different *S. meliloti* strains on LB/MC/CF plate.

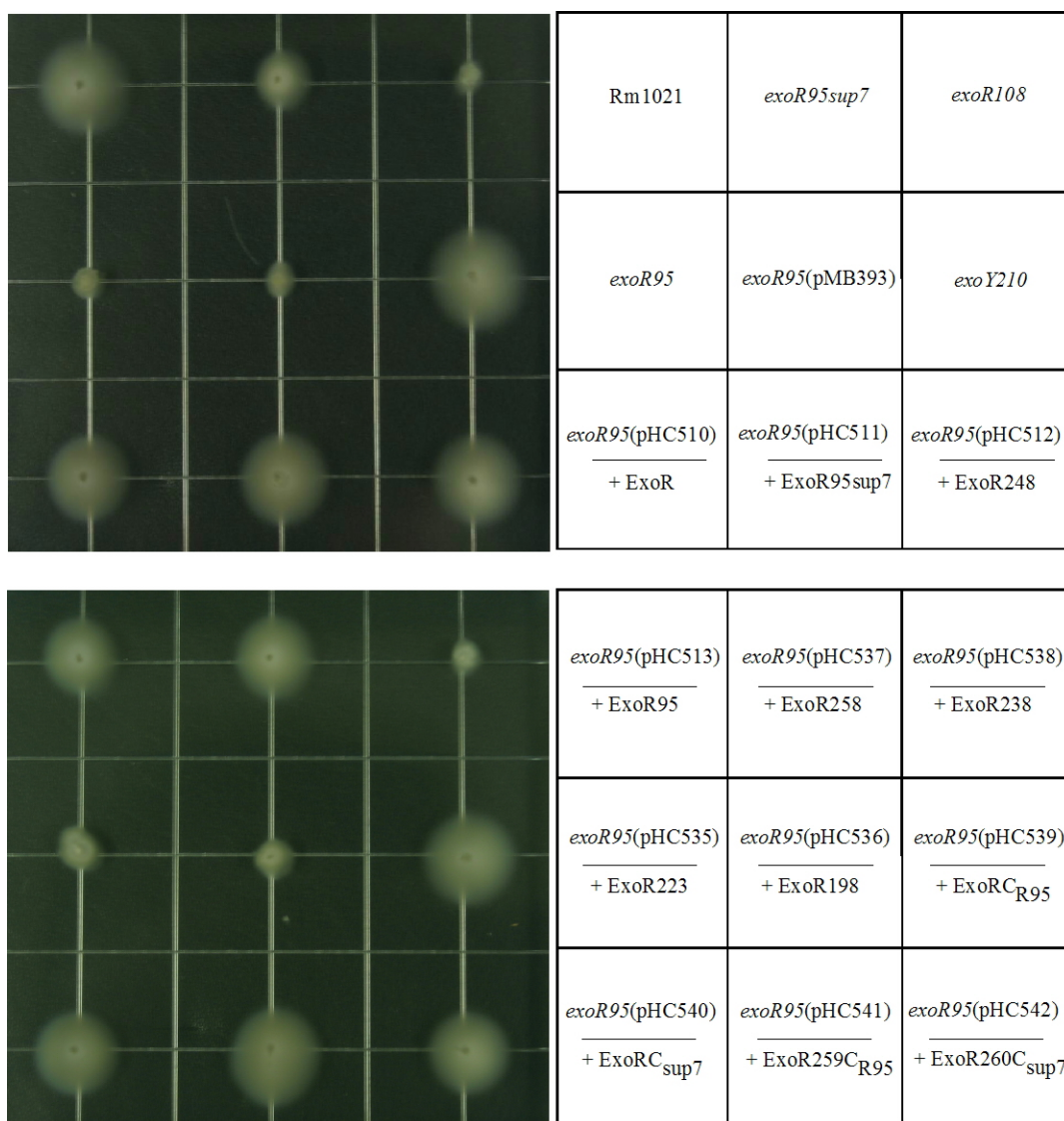


Figure 5-3. Effects of C-terminus of ExoR on regulation of swarming motility. Colonies of different size were formed by different *S. meliloti* strains on LB/MC soft agar (0.3%) medium.

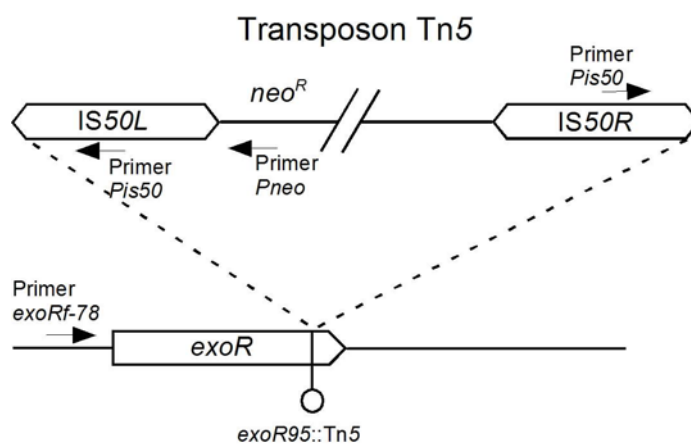


Figure 5-4. Schematic representation of transposon Tn5 insertion in the *exoR* gene that formed *exoR95::Tn5* mutation. The primers used in PCR reactions to confirm the insertion of Tn5 are also shown.

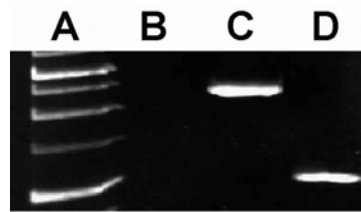


Figure 5-5. Detecting Tn5 insertion in the *exoR* gene using PCR. Agarose gel picture showing the size of DNA fragments amplified, using primers *exoRf-78* and *Pneo*, and genomic DNA from the wild type strain Rm1021 (B), the *exoR95* (C) and the *exoR95sup7* mutants (D). DNA ladder is shown in Lane A.

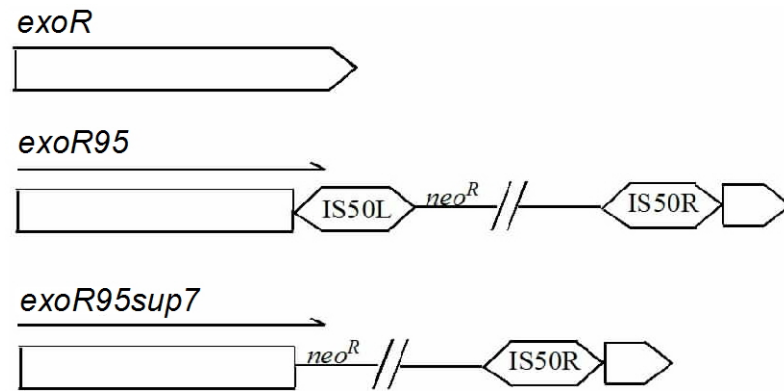


Figure 5-6. Tn5 insertion in the *exoR* gene in the *exoR95* and *exoR95sup7* mutants. The *exoR* ORF extends into the Tn5 due to the transposon insertion in the *exoR95* and *exoR95sup7* mutants. In the *exoR95sup7* mutant, almost the entire IS50L region of Tn5 is missing.

ExoR:	249	<u>DDRRVAITMSQNMHLQ</u> <u>NDDD</u>	268
ExoR95:	249	AD <u>SY</u> TO <u>V</u> AS	257
		* • ::	
ExoR:	249	<u>DDRRVAITMSQNMHLQ</u> <u>NDDD</u>	268
ExoR95sup7:	249	<u>G</u> <u>MRIV</u> <u>SHD</u>	256
		• * *	
ExoR:	249	<u>DDRRVAITMSQNMHLQ</u> <u>NDDD</u>	268
ExoR95sup7:	249	G----- <u>MRIV</u> <u>SHD</u>	256
		• * :: • • *	
Legend:			
* Fully conserved residue			
: Conservation of strong groups			
• Conservation of weak groups			

Figure 5-7. Protein alignment results for ExoR, ExoR95 and ExoR95sup7. Residues underlined are predicted to form β -sheet secondary structure. Residues in plain font are predicted to form random coil. Analysis is done by using the Biology Workbench tool.

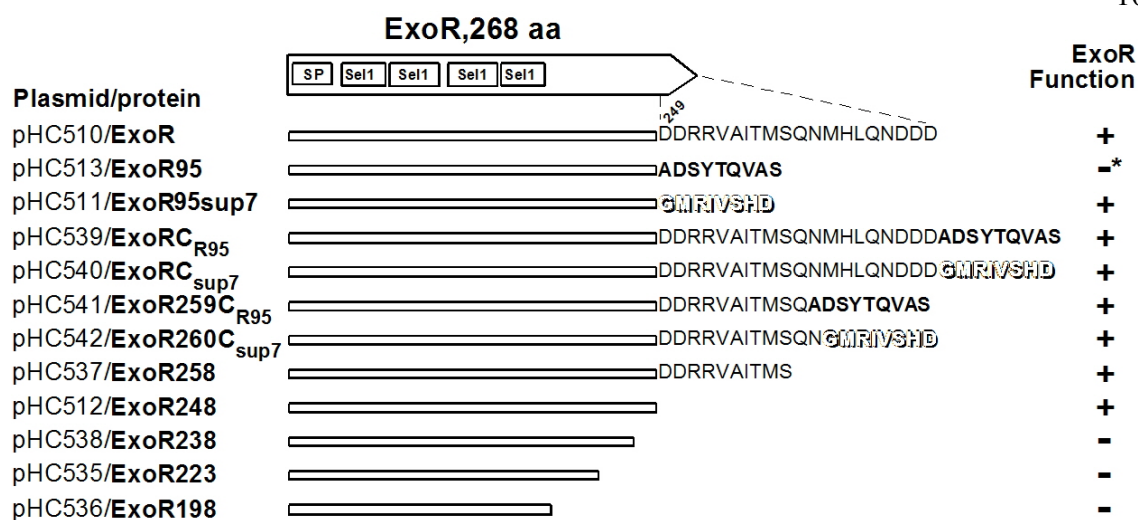


Figure 5-8. Comparison of C-terminal amino acids of mutated ExoR proteins produced from different plasmids. Signal peptide (SP) and the 4 Sel1 domains are indicated in wild type ExoR protein. * Extra copies of ExoR95 affect both succinoglycan and motility at a limited degree.

M R A G E L K S L R V A V L G M S L A V

```

1  TGGGGTCGGCGTGGCCGGCTCGAACGAAAGAACTGATTGTTATGCGCGCGGGTGAATTGAAGTCGCTTAGGGTTGCGGTGCTTGGCATGTCGCTGGCGG
   G A T A A G P A R A F D P G A G V T K E S G P F A L F K F G F S A
101 TTGGCGCAACTGCGGCCGGGCCAGCACGGGCTTTTCGATCCCGGAGCCGGCGTGACCAAGGAGTCAGGCCCTTCGCGCTCTTCAAGTTCGGTTTTTCCGC
   Y K S G R K D E A V E A Y R Y A A E K G H T G S R W A L A N M Y A
201 CTATAAGAGCGGCCGGAAGACGAAGCGGTAGAGGCTACCGGTATGCGGCCGAAAAGGGGCACACGGGCTCTCGCTGGGCGCTCGCCAATATGTACGCC
   Y G D G V A E N D L E A F K I Y S E I A Q Q G V E P G S E D T G Y F
301 TATGGCGATGGGGTCGCGGAGAACGATCTCGAAGCGTTCAGATCTACAGCGAAATCGCACACAGGGCGTCGAGCCCGGCTCGGAAGATACCGGCTACT
   V N A L I S L A G Y Y R R G I P D T P V R S D L Y R I H W P S F Y
401 TCGTCAATGCATTGATTTGCTTGCCGGCTACTACCGGCGCGGCATTCCCGACCCCCGGTAAGGTCGACCTGTACAGAAITCACTGGCCGTCGTTTTA
   N V V T G K T L A L P N L I A L Q H I P L S P A G V I A K R P A P
501 CAACGTCGTGACTGGGAAAACCCCTGGCGTTACCCAACTTAATCGCCTTGACGACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCG
   I A L P N S C A A *
601 ATCGCCCTTCCCAACAGTTGCGCAGCCTGATGCGGAATGGCGATAAGCTAGCTTCACGCTGCGCAAGCACTCAGGGCGCAAGGGCTGCTAAAGGAAGC
701 GGAACACGTAGAAAGCCAGTCCGCAGAAACGGTGCTGACCCCGGATGAATGTGAGCTACTGGGCTATCTGGACAAGGAAAACGCAAGCGCAAAGAGAAAG
801 CAGTAGCTTGCA

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Figure 5-9. Sequencing result of the *exoR* gene region in the *exoR108* mutant background. Shown is a translation of the *exoR108* ORF with the start codon and the stop codon boxed. Inserted sequence from the plasmid is underline.

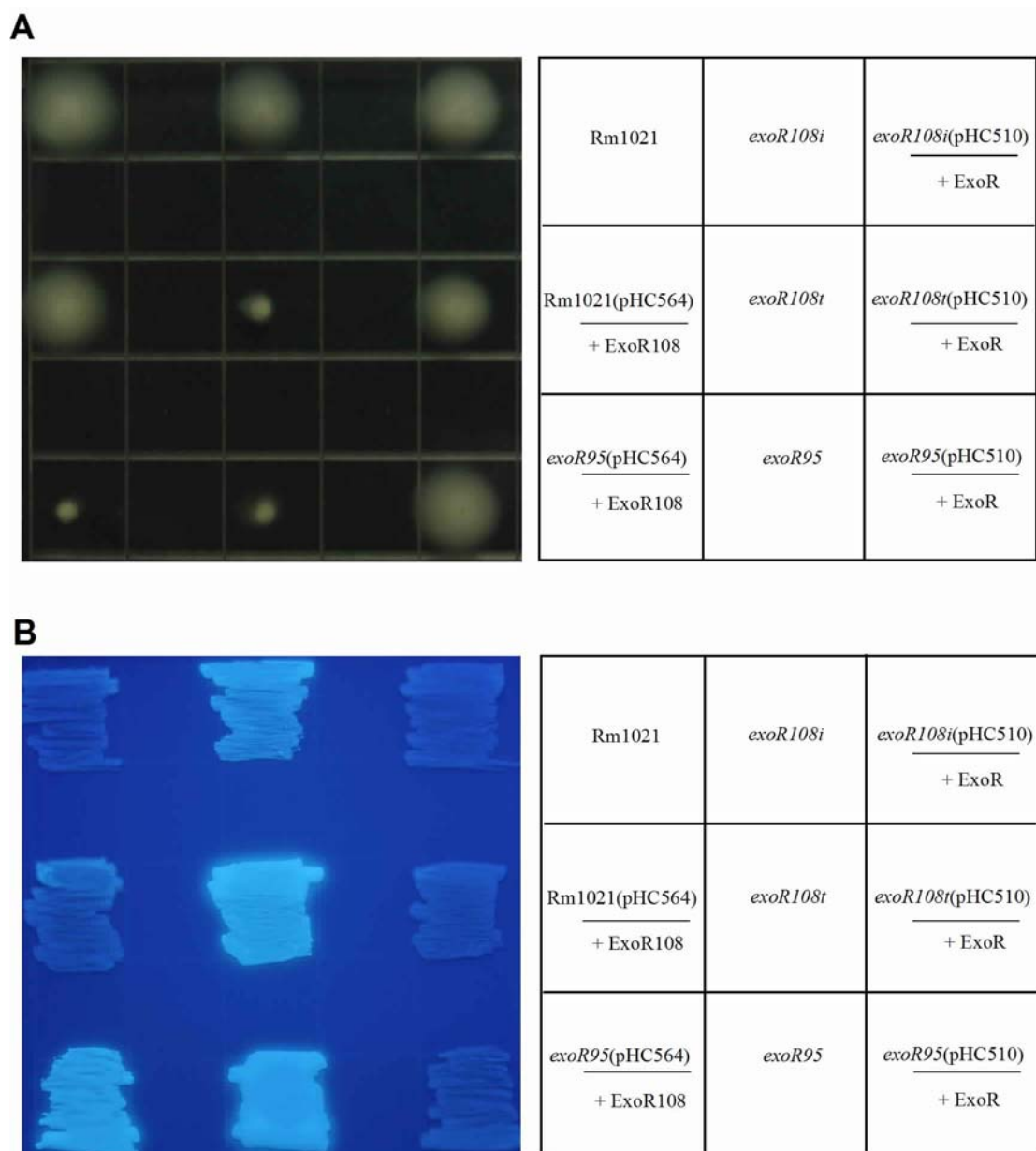


Figure 5-10. Phenotypes of *exoR108* mutants and complementary tests. Panel A, *S. meliloti* colonies on LB/MC plate with 0.3% agar. Panel B, Calcofluor fluorescence image showing the levels of succinoglycan produced by different *S. meliloti* strains on LB/MC/CF plate. Plasmid pHC510 carries the wild type *exoR*; plasmid pHC564 carries the *exoR108* ORF.

<i>Sinorhizobium meliloti</i> 1021 <i>Rhizobium leguminosarum</i> bv. <i>trifolii</i> WSM1325 <i>Bradyrhizobium</i> sp. ORS278 <i>Mesorhizobium</i> sp. BNC1 <i>Agrobacterium tumefaciens</i> str. C58 <i>Brucella melitensis</i> 16M <i>Ochrobactrum anthropi</i> ATCC 49188 <i>Hoeflea phototrophica</i> DFL-43 Consensus	(1)	MRAGELKSRVAVLHSHAVG---ATAAGTARADPGAAVTH---KSGDHALFKKFGSAJKS---RHEFAVAYRYAAE*****
	(1)	MVTSEFKLFKFMHSHSIVA---LALSFCRAPIIKGVSH---KSGDHLFFKFKATKTH---QHEFAVAYRYAAE*****
	(1)	-----MAGPQALAGALIRSVPAATTAATNPTOSLTLQYAAE*****
	(1)	-----MRISQIIRLJGALLFGLETAALIEKAVVQPSRN---LAVFRFPFATHRE---QFAVAYRYAAE*****
	(1)	MLKCEANVLKPLMHHLAS---LAMVLPIIHACVTH---KSGDHLFFKFKATKTH---RHEFAVAYRYAAE*****
	(1)	-----MRSRFSFCSALAHAGLVLTALCHSTFPHLDDSEH---SRSPETFFKFGFATHHGHKAKLELYAAE*****
	(1)	-----MRIRFSYSALAHAG---LAIHCNSFAPHLDDSEH---SRSPETFFKFGFATHHGHKAKLELYAAE*****
	(1)	-----MRISDRTRTSIFCAVHAAA---IGVGTPQALIPHSVSR---KSGDHLFFKFGFATHHGHKAKLELYAAE*****
	(1)	*****K K SLIGMALA A A GPA AFDINAGVSK ESGPFDLFKFGFSAYKNG KDEAVEAYRYAAE*****
	(1)	KUTESEFALANHYAYTRGVAMHULEFAKLYETIAQGVF---FGSEFTGCFYVHALISLAGYRRGIDTIVRSDL*****
<i>Sinorhizobium meliloti</i> 1021 <i>Rhizobium leguminosarum</i> bv. <i>trifolii</i> WSM1325 <i>Bradyrhizobium</i> sp. ORS278 <i>Mesorhizobium</i> sp. BNC1 <i>Agrobacterium tumefaciens</i> str. C58 <i>Brucella melitensis</i> 16M <i>Ochrobactrum anthropi</i> ATCC 49188 <i>Hoeflea phototrophica</i> DFL-43 Consensus	(72)	KUTESEFALANHYAYTRGVAMHULEFAKLYETIAQGVF---FGSEFTGCFYVHALISLAGYRRGIDTIVRSDL*****
	(72)	KUTESEFALANHYAYTRGVAMHULEFAKLYETIAQGVF---FGSEFTGCFYVHALISLAGYRRGIDTIVRSDL*****
	(37)	ELUPVQOKLGRVYAAKGVARDIMRADYFERIANAHARDSAPQAQIVAMAFVAGLYLSIINTKVKEDP*****
	(70)	NQVBARWKLARHYAEIRGVAKULFEFAVADWQ---FGREESTVSDRIVAVGNTLRRGITGSPVAAANP*****
	(69)	KUTESEFALANHYAYTRGVAMHULEFAKLYETIAQGVF---FGSEFTGCFYVHALISLAGYRRGIDTIVRSDL*****
	(72)	RUTQAKKRLARHYAEIRGVARDIYEAYEIEFEKIVREISE---FGFNAPYVADALVALAGYVKNGITGSPVQANP*****
	(68)	RHQCAKRLARHYAEIRGVARDIYEAYEIEFEKIVREISE---FGFNAPYVADALVALAGYVKNGITGSPVQANP*****
	(70)	KGPANALANHYAYTRGVAMHULEFAKLYETIAQGVF---FGSEFTGCFYVHALISLAGYRRGIDTIVRSDL*****
	(76)	KGH GARWKLARMYADGVAENDYEAKIFSETAQGVF PGSETCGVVNAVALA YYK GIPGSPVKANP*****
	(76)	*****K K SLIGMALA A A GPA AFDINAGVSK ESGPFDLFKFGFSAYKNG KDEAVEAYRYAAE*****
<i>Sinorhizobium meliloti</i> 1021 <i>Rhizobium leguminosarum</i> bv. <i>trifolii</i> WSM1325 <i>Bradyrhizobium</i> sp. ORS278 <i>Mesorhizobium</i> sp. BNC1 <i>Agrobacterium tumefaciens</i> str. C58 <i>Brucella melitensis</i> 16M <i>Ochrobactrum anthropi</i> ATCC 49188 <i>Hoeflea phototrophica</i> DFL-43 Consensus	(145)	SQFOLTFQAACTHGVAMHULEFAKLYETIAQGVF---FGFNAPYVADALVALAGYVKNGITGSPVQANP*****
	(145)	SQFOLTFQAACTHGVAMHULEFAKLYETIAQGVF---FGFNAPYVADALVALAGYVKNGITGSPVQANP*****
	(112)	DRHEMFSAAYKNGDAQYDIAHYIKTADASRDDDFRYGARWIGIAQKQHOAAALLOMLFNGDRLPRIPA*****
	(143)	TILLEYTRAAAYRNADAQYDIAHYIKTADASRDDDFRYGARWIGIAQKQHOAAALLOMLFNGDRLPRIPA*****
	(142)	SQFOLTFQAACTHGVAMHULEFAKLYETIAQGVF---FGFNAPYVADALVALAGYVKNGITGSPVQANP*****
	(145)	NMABELVPAANRGDSIRQFELGKMLD---FGERNVAVAAARFQIAARKNTAAAMLMIMLFQ---AKTV*****
	(141)	GMHNLVPAANRGDSIRQFELGKMLD---FGERNVAVAAARFQIAARKNTAAAMLMIMLFQ---AKTV*****
	(143)	GAFOLTFQAACTHGVAMHULEFAKLYETIAQGVF---FGFNAPYVADALVALAGYVKNGITGSPVQANP*****
	(151)	S ARQLYFQAASTFGVAEQF LARMLLA GEGG VQQAKKWLNLRKKGH GAQAVLGNMLFQ EGQTV*****
	(151)	S ARQLYFQAASTFGVAEQF LARMLLA GEGG VQQAKKWLNLRKKGH GAQAVLGNMLFQ EGQTV*****
<i>Sinorhizobium meliloti</i> 1021 <i>Rhizobium leguminosarum</i> bv. <i>trifolii</i> WSM1325 <i>Bradyrhizobium</i> sp. ORS278 <i>Mesorhizobium</i> sp. BNC1 <i>Agrobacterium tumefaciens</i> str. C58 <i>Brucella melitensis</i> 16M <i>Ochrobactrum anthropi</i> ATCC 49188 <i>Hoeflea phototrophica</i> DFL-43 Consensus	(215)	KCLAYHTAAIGQS---KRPFIQAMKQAFSLATDIEHVAITMSNMHLQNDDD*****
	(215)	KCLAYHTAAIGQS---KRPFIQAMKQAFSLATDIEHVAITMSNMHLQNDDD*****
	(187)	KCLAYHTAAIGQS---KRPFIQAMKQAFSLATDIEHVAITMSNMHLQNDDD*****
	(213)	KCLAYHTAAIGQS---KRPFIQAMKQAFSLATDIEHVAITMSNMHLQNDDD*****
	(212)	QCLAYHTAAIGQS---KRPFIQAMKQAFSLATDIEHVAITMSNMHLQNDDD*****
	(215)	KCLAYHTAAIGQS---KRPFIQAMKQAFSLATDIEHVAITMSNMHLQNDDD*****
	(211)	KCLAYHTAAIGQS---KRPFIQAMKQAFSLATDIEHVAITMSNMHLQNDDD*****
	(213)	KCLAYHTAAIGQS---KRPFIQAMKQAFSLATDIEHVAITMSNMHLQNDDD*****
	(226)	RGLAMTAAIDRC P DC WIRAMQQAFAFSLAGEADRRRTAALAAQDYL G D*****
	(226)	RGLAMTAAIDRC P DC WIRAMQQAFAFSLAGEADRRRTAALAAQDYL G D*****

Figure 5-11. Alignment of ExoR homologues in different bacteria. Amino acids that are identical and conserved are highlighted with the darkest grey shade, and the weakly conserved ones are with lighter grey shade. The four predicted Sel1 domains in *S. meliloti* ExoR are indicated as *, +, >, and <.

6 CHAPTER VI THE PROCESSING OF THE *S. MELILOTI* EXO R PROTEIN

6.1 INTRODUCTION

The *S. meliloti* ExoR protein and the ExoS/ChvI system regulate the production of succinoglycan and flagellum (120). As shown in our own work and in another group's publications that the ExoR protein is located in the periplasm of *S. meliloti* cells, and the ExoR suppressors have been found in both ExoS and ChvI (117). In the wild type *S. meliloti* cells, the ExoR protein is translated into a 29 KDa precursor form based on sequence analysis, and a 26 KDa mature ExoR, ExoRm, is predicted to form by the removal of its N-terminus 30 amino acid signal peptide. ExoRm appears to interact directly with the ExoS protein and this interaction can be blocked by single amino acid changes in either ExoR or the periplasmic domain of the ExoS protein (19). The ExoR-ExoS interaction may suppress ExoS and the disassociation of ExoR from ExoS should lead to ExoS activation by unknown environmental signals. For ExoR to exert the regulatory function through the interaction with the ExoS/ChvI 2CRS, it is possible that both the expression of the ExoR protein is regulated (as described in Chapter 4) and also the ExoR protein itself is modified to become activated or deactivated. In this work, I report my effort to understand the *in vivo* processing of the ExoR protein.

6.2 METHODS

6.2.1 Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this project are listed in Table 6-1. *E. coli* strains used in this study were grown in Luria-Bertani (LB) medium at 37°C (104). *S. meliloti* was grown in LB medium supplemented with 2.5 mM MgSO₄ and 2.5 mM CaCl₂ (LB/MC) at 30°C (60). Agar (1.5%) was used to solidify media. For detecting alkaline phosphatase activity, 5-bromo-4-chloro-3-indolyl phosphate (XP) was added at 40 µg/mL to solid medium. When required, IPTG (isopropyl-β-d-thiogalactopyranoside) was added to induce gene expression. The following antibiotics were used at the concentrations indicated: chloramphenicol (Cm), 10 µg/mL; gentamycin, 50 µg/mL; kanamycin (Km), 25 µg/mL; neomycin (Nm), 200 µg/mL; spectinomycin (Sp), 100 µg/mL; streptomycin (Sm), 500 µg/mL; and tetracycline (Tc), 10µg/mL.

6.2.2 Construction of *exoR*-expressing plasmids using vector pMB393

Refer to Chapter 2 for description.

6.2.3 Construction of the *exoR-phoA* fusions

Refer to Chapter 2 for description.

6.2.4 Construction of *exoR108* mutant

Refer to Chapter 5 for description.

6.2.5 Protein electrophoresis and western blot

To detect the ExoR protein and the ExoR-PhoA fusions, total cellular proteins were run on 7.5% and 15% SDS-PAGE gels, respectively, using Bio-Rad (Hercules, CA)Mini-PROTEAN Electrophoresis System, according to manufactory instructions. Proteins were transferred onto PVDF membranes using Bio-Rad Mini Trans-Blot cell.

A His-tagged *S. meliloti* ExoR protein was expressed and purified in this lab and sent to Pacific Immunology Company (Ramona, CA) to raise ExoR specific polyclonal antibodies in Rabbits.

To detect the ExoR protein, the PVDF membrane was incubated first with rabbit anti-ExoR polyclonal antibodies and then goat anti-rabbit IgG alkaline phosphatase (AP) - conjugated secondary antibody (Novagen). The membrane was treated finally with Sigma NBT/BCIP (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) to detect alkaline phosphatase activities.

To detect the ExoR-PhoA fusions, the PVDF membrane was incubated first with mouse monoclonal anti-bacterial alkaline phosphatase antibody (Sigma). Then, goat anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP) was used as the secondary antibody (Sigma). The ExoR-PhoA fusions were identified using Sigma DAB (3, 3'-Diaminobenzidine) as a precipitating substrate for the localization of peroxidase activities.

6.2.6 Determination of the apparent molecular weight of a protein

The apparent molecular weight (MW) of a sample protein is determined by comparing it to pre-stained protein markers (Bio-Rad). For each SDS-PAGE gel,

migration distances of the standards within the linear range were measured (25 KDa and 37 KDa bands for 15% gel, and 50 KDa and 75 KDa bands for 7.5% gel). A formula for $\log_{10}$ of MW vs. migration distance was generated in the form of $\log_{10}MW=a + bf$, with f being the migration distance (116). Then the formula was used to calculate the MW of the unknown protein bands based on their migration distance. There could be 10% difference between the apparent MW and the actual MW of a protein.

6.3 RESULTS

6.3.1 The ExoR signal peptide can be cleaved from the ExoR-PhoA fusion protein

The ExoR protein is predicted to have a cleavable signal peptide. The conserved cleavage site is between amino acids at position 30 (alanine) and 31 (phenylalanine). As described in chapter 2, three different ExoR-PhoA fusion proteins were constructed to study the location of ExoR. The constructs are 1) PhoA with full length ExoR (ExoR-PhoA) on plasmid pHC528, 2) PhoA with ExoR without its N-terminal signal peptide (ExoR Δ SP-PhoA) on plasmid pHC555, and 3) PhoA with only the N-terminal ExoR (SP_{ExoR}-PhoA) signal peptide on plasmid pHC533.

When the three fusions were expressed in the *phoA*⁻ strain Rm8002 and tested on culture media containing XP, Rm8002(pHC528) and Rm8002(pHC533) cells were blue, and Rm8002(pHC555) cells were white (Figure 2-1). Since both of the ExoR protein and the ExoR signal peptide were sufficient to bring the PhoA protein to the periplasm, and that no other transmembrane domain is predicted in ExoR, we conclude that the ExoR protein is most likely located in cell periplasm. The ExoR Δ SP protein, lacking of ExoR signal peptide, is most likely remained in the cytoplasm.

To further test whether the ExoR signal peptide will be cleaved after the protein is translocated in periplasm, specific anti-PhoA monoclonal antibody was used to detect the ExoR-PhoA fusions. As shown in Figure 6-1, no endogenous PhoA was detected from the control strain Rm8002 (Lane B). In Lane A, two bands were clearly detected from Rm8002 (pHC533), where SP_{ExoR}-PhoA was expressed. Based on the protein standards, protein molecular weight can be calculated using the formula $\log_{10}M=5.2557-0.0221f$. The upper band in Lane A represents fusion of ExoR signal peptide with PhoA protein (PhoAp), which has a MW of 49.49 KDa, and the lower band, which has a MW of 46.59 KDa represents only the PhoA part after the ExoR signal peptide has been cleaved (PhoAm). This suggests that the ExoR signal peptide is cleavable from the SP_{ExoR}-PhoA fusion.

6.3.2 ExoRm-PhoA fusion protein is further digested after the signal peptide is cleaved

When total proteins of Rm8002 strain expressing the full length ExoR-PhoA fusion were resolved and probed with the anti-PhoA monoclonal antibody in the same western blot (Figure 6-1, Lane C), four bands were clearly detected. The sizes of the four proteins are 75.8, 72.9, 65.1 and 48.0 KDa.

The 75.8 KDa protein is most likely the full length precursor of the ExoR-PhoA protein (ExoRp-PhoA), and the 72.9 KDa protein appears to be the mature form of ExoR-PhoA (ExoRm-PhoA) after the ExoR signal peptide has been cleaved. These results confirmed again that the signal peptide is cleavable. The 65.1 and 48.0 KDa proteins represent two degraded ExoR-PhoA proteins containing PhoA. Both bands have a larger

size than the mature form of PhoA (46.59 KDa), with 18.5 KDa and 1.4 KDa more, respectively, suggesting that the ExoRm-PhoA was further digested in periplasm after the signal peptide is cleaved from the ExoR protein.

6.3.3 The processing of wild type ExoR protein

In order to detect ExoR protein directly, ExoR specific polyclonal antibodies were generated by cloning and expressing a His-tagged ExoR protein as the elicitor in rabbits. However, when total proteins from Rm1021 were resolved and probed in a western, a lot of bands were detected (data not shown). To have a clearer background to compare the different forms of the ExoR protein, the *exoR108t* mutant (Chapter 5) was used as a control in western blot. This *exoR108t* mutant was constructed by inserting a suicide plasmid in the middle of the *exoR* ORF to generate ExoR108, which is the fusion of ExoR N-terminal 144 amino acids and C-terminal 51 amino acids from the suicide vector. The predicted ExoR108p has a MW of 21 KDa, and if the signal peptide is cleaved, the ExoR108m will be 18 KDa.

In a western blot as shown in Figure 6-2 which used anti-ExoR antibodies as the primary antibody, five bands were detected for Rm1021, which were the exact the same as for the *exoS96* mutant (Line A and Line C, respectively). Based on the protein standards, formula $\log_{10}M=5.2557-0.0221f$ was generated to calculate the molecular weights of these unknowns. Using the formula, the sizes of the five Rm1021 proteins (and *exoS96* mutant proteins) were calculated as 29, 26, 24, 19, and 18 KDa.

In the *exoR108t* mutant background, both 24 and 19 KDa proteins were found, suggesting the two bands could be non-specific proteins. In *exoR108t*(pHC510) which

expressed the wild type ExoR, the 29, 26, and 18 KDa bands were detected with stronger intensity, but not the 24 and 19 KDa proteins. The 29 and 26 KDa Rm1021 proteins are most likely ExoRp and ExoRm, which match their molecular weights predictions very well. These data suggest that the signal peptide is cleaved from the wild type full length ExoR protein and the ExoR protein is also digested to yield an 18 KDa fragment.

6.3.4 The processing of the ExoR95 protein

The ExoR95 is predicted to have 257 amino acids with a unique 9 amino acid terminus (C_{R95}) replacing the 20 amino acid C-terminus of the wild type ExoR protein due to Tn5 insertion in the *exoR* gene (Chapter 5). In the same western shown in Figure 6-2, four bands were detected for the *exoR95* mutant (Line B). The sizes of them are 28, 25, 24, and 19 KDa. Compared with the control, the 24 and 19 KDa proteins should be non-specific proteins; the other two bands, the 28 and 25 KDa, should represent the ExoR95p and ExoR95m, respectively, which match their predicted molecular weights very well. This result suggests that the signal peptide is also cleavable from the ExoR95 protein.

When more ExoR95 proteins were expressed in *exoR108t*(pHC513), the ExoR95p and ExoR95m proteins were also detected. But one more protein, a very faint band slightly smaller than 18 KDa was also detected, which was not seen in the *exoR95* mutant background. This finding suggest that although the wild type ExoR protein is digested to yield an 18 KDa fragment which appears to be stable, the ExoR95 protein is not equally sensitive to the same proteolysis.

6.3.5 The proteolysis of ExoR takes place in periplasm requires an intact signal peptide

To determine the cellular location of the 18 KDa protein detected in the wild type Rm1021 background, an ExoR protein without its signal peptide, ExoR Δ SP, was expressed from plasmid pHC557 in *exoR108t* and used in western blot (Figure 6-3). In addition to the ExoRm protein and a slightly smaller protein, only a very faint ExoR18 protein was detected from this strain (Lane C). Because the mutated ExoR Δ SP should stay in cytoplasm, these data suggest that the proteolysis of ExoR takes place in periplasm.

6.3.6 The origin of the ExoR18 fragment from the ExoR protein

The 18 KDa ExoR fragment resulted from proteolysis could represent its N-terminus, C-terminus, or a middle part of the ExoRm protein. To determine its origin, total proteins from *exoR108t* (pHC539) and *exoR108t* (pHC540) expressing two mutated but functional ExoR proteins with different C-termini were resolved and probed in a western blot. As shown in Figure 6-4, compared to the vector control (Lane B), three strong bands were detected in *exoR108t* (pHC539) which expressed the ExoR with extra 9 amino acids from the C-terminus of ExoR95 (ExoR_{C95}) (Lane D). By calculating the molecular weight against standards, the first band is most likely the full length ExoR_{C95} protein (30 KDa), the second band could be the mature form of the ExoR_{C95} protein (27 KDa), and the lowest band (the smallest size) represents a digested ExoR_{C95} protein (19 KDa). Similarly, in *exoR108t*(pHC540) which expressed the ExoR with extra 8 amino

acids from the C-terminus of ExoR95sup7 (ExoRC_{sup7}), three strong bands representing the full length ExoRC_{sup7} protein, the mature form of the ExoRC_{sup7} protein, and a digested ExoRC_{sup7} protein were detected (Lane E), with each of them a slightly smaller in size than its corresponding band in the *exoR108t*(pHC539) background.

Compared with the three forms of ExoR detected from the wild type background, with the molecular weights of the precursor and the mature form of ExoR increased in each mutant, the molecular weight of the proteolytic product also increased. This finding suggests that the ExoR protein is recognized at a specific site by a periplasmic protease and that the detectable proteolytic product of ExoR, the ExoR18 fragment, was from the C-terminus of the protein.

6.4 DISCUSSION

I have shown previously that the ExoR protein is mostly likely a periplasmic protein by using ExoR-PhoA fusions, which was confirmed later in another group's publications (117). Since ExoR contains a conserved cleavage site at the end of the signal peptide, it is very likely that the signal peptide will be cleaved from the ExoR protein before it is released into periplasm. By using monoclonal anti-PhoA antibody, I showed that the ExoR signal peptide is cleavable from the SP_{ExoR}-PhoA fusion. Meanwhile, the ExoR-PhoA fusion was also expressed from *S. meliloti phoA*⁻ mutant and used in western blot. Surprisingly, in addition to the precursor and the mature form of the fusion protein, two other bands with smaller molecular weights were also detected. The results suggest that ExoRm-PhoA could be further digested. It is very likely that the protease digestions are on the ExoR part in the ExoR-PhoA fusion because the PhoA alone was shown to be

stable in SP_{ExoR}-PhoA. However, it is not possible to determine whether the sites of digestion in the ExoR protein are conserved, because the fusion protein may have affected the folding of the ExoR part which makes it susceptible to protease digestions or makes the sites changed. Therefore, it is necessary to use anti-ExoR antibody to detect ExoR protein in *S. meliloti* cells.

When the polyclonal antibodies against ExoR were used in western blot, a lot of bands were detected. The *exoR108t* mutant, resulted from a plasmid insertion mutation in the *exoR* gene (Chapter 5) made it possible to have a clearer background, even though a point mutation in the *chvI* gene was found in this mutant. Three major ExoR derivative bands were detected in the wild type Rm1021, the 29 KDa ExoRp, the 26 KDa ExoRm and an 18 KDa proteolytic product. These results not only demonstrate that the signal peptide of the ExoR protein is cleaved before the protein is transported into cell periplasm, but also suggest that the wild type ExoR is subject to further proteolysis. The finding of the 18 KDa proteolytic form of ExoR is very important because it presents a possible way to regulate the ExoR protein.

Comparison of the protein profiles between the *exoR95* mutant and the wild type strain led to a surprising finding that no proteolytic product around 18 KDa was detected in the *exoR95* mutant. Noticeably, when the ExoR95 was overexpressed in the *exoR108t* background, a very faint band with a molecular weight slightly smaller than 18 KDa was detected, suggesting a very small percentage of ExoR95 protein could be digested similarly. Previously I have found that the C-terminus of ExoR95 (C_{R95}) disrupts the function of the protein, and this functional deficiency could be compensated partially by overexpressing ExoR95 in the *exoR95* mutant (Chapter 5), suggesting that a very small

percentage of ExoR95 protein might be able to interact with ExoS. This small percentage of ExoR95 protein may fold correctly and act like the wild type ExoR, and can be digested like the wild type ExoR. Therefore, it is possible that the ExoR proteolysis is structural specific and the ExoR95 protein is not susceptible to the same protease digestion due to a dramatic structure change. However, without crystal structure of ExoR being revealed, it is difficult to determine the biochemical features of the ExoR95 protein.

To better understand the proteolysis of ExoR, it is important to determine the cellular location of this protease digestion and also the origin of the 18 KDa proteolytic product. In western blot, only a very faint band around 18 KDa was detected from the ExoR Δ SP compared to a strong band detected from the wild type ExoR, suggesting that the specific proteolysis mainly occurs in periplasm. By analyzing two other mutated but functional ExoR proteins with longer C-termini (ExoR_{C_{R95}} and ExoR_{C_{sup7}}) in western, I found that the 18 KDa proteolytic product represents the C-terminus of the ExoR_m protein.

Taken together, the ExoR protein is likely processed in two steps: the removal of the signal peptide to generate a 26 KDa ExoR_m by signal peptide peptidase, and then in the periplasm the digestion of ExoR_m to yield the C-terminal 18 KDa fragment (ExoR_{c18K}) by unknown protease. It is possible that the other 8 KDa ExoR fragment is further degraded into smaller fragments.

Gram-negative bacteria comprise a large collection of proteins responsible for many vital functions in their periplasm (29). Among those periplasmic proteins, proteases play important roles in bacterial sensing of environmental signals including temperature,

osmotic stress, and pH changes (30). My finding that ExoR, the key regulator of ExoS/ChvI signal transduction pathway, is subject of proteolysis in the periplasm reveals a novel regulatory mechanism in *S. meliloti* which integrates activities of protease in periplasm into two-component regulatory system.

Table 6-1. Strains, plasmids and phage.

Strain or plasmid	Relevant properties	References
<i>E. coli</i>		
DH5 α	General purposed strain	(45)
MT616	MT607, pRK600, Cm ^R	(32)
<i>S. meliloti</i>		
Rm1021	SU47, Sm ^R	(60)
Rm7095	Rm1021 <i>exoR</i> 95::Tn5, Nm ^R	(27)
Rm8002	Rm1021 <i>phoA</i> ⁻	(38)
SmHC18	<i>exoR108t</i> ; Rm1021 <i>exoR108</i> ::pK19mob2 Ω HMB, Nm ^R	This work
Plasmids		
pSW213	Cloning vector, <i>lacI</i> ^Q , P _{lac} <i>lacZ</i> , Tc ^R	(75)
pHC528	pSW213, expressing <i>exoR-phoA</i> fusion under an inducible promoter, Tc ^R	This work
pHC533	pSW213, expressing <i>phoA</i> with <i>exoR</i> signal peptide under an inducible promoter, Tc ^R	This work
pMB393	Cloning vector, Sp ^R	(36)
pHC510	pMB393 carrying the <i>exoR</i> gene, <i>exoR</i> promoter	This work
pHC513	pMB393 carrying a mutated <i>exoR</i> gene encoding ExoR95, <i>exoR</i> promoter	This work
pHC539	pMB393 carrying a mutated <i>exoR</i> gene encoding ExoR with extra 9aa from the C-terminus of ExoR95, <i>exoR</i> promoter	This work
pHC540	pMB393 carrying a mutated <i>exoR</i> gene encoding ExoR with extra 8aa from the C-terminus of ExoR95sup7, <i>exoR</i> promoter	This work
pRK600	Helper plasmid, Cm ^R	(32)
Phage		
ϕ M12	Generalized transducing phage for <i>S. meliloti</i>	(31)

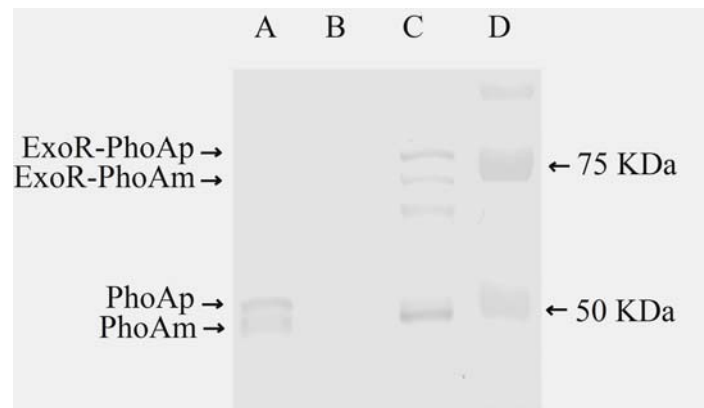


Figure 6-1. ExoR-PhoA fusion protein profiles of *S. meliloti* *phoA*⁻ mutant strain in western blot using PhoA specific monoclonal antibody. Lane A, Rm8002(pHC533) expressing the SP_{ExoR}-PhoA fusion (PhoA fused with the ExoR signal peptide); Lane B, Rm8002(pSW213), the vector control; Lane C, Rm8002(pHC528) expressing the ExoR-PhoA fusion (PhoA fused with the full length ExoR); Lane D, Pre-stained protein marker. Two forms of the PhoA protein were detected in Rm8002(pHC533): precursor (PhoAp), mature (PhoAm). In the Rm8002(pHC528) background, precursor (ExoRp-PhoA), mature (ExoRm-PhoA) and two main cleavage products were detected.

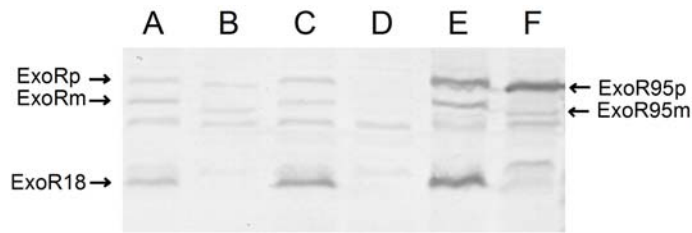


Figure 6-2. ExoR protein profiles of different *S. meliloti* strains in western blot using ExoR specific polyclonal antibodies. Lane A, the wild type *S. meliloti* Rm1021; Lane B, the *exoR95* mutant; Lane C, the *exoS96* mutant; Lane D, the *exoR108t* mutant; Lane E, *exoR108t*(pHC510) expressing the wild type ExoR; Lane F, *exoR108t*(pHC513) expressing the ExoR95. Three forms of the ExoR protein were detected in the wild type background: precursor (ExoRp), mature (ExoRm), and main cleavage product ExoR18. In the *exoR95* mutant background, two smaller ExoR proteins were detected, ExoR95p and ExoR95m, and a weakly detected cleavage product with a size small than ExoR18.

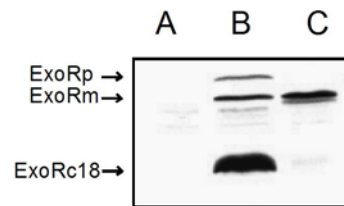


Figure 6-3. Western blot of ExoR protein from *S. meliloti* *exoR108t* mutant expressing an ExoR without signal peptide. Lane A, *exoR108t*(pHC393), the vector control; Lane B, *exoR108t*(pHC510), expressing the wild type ExoR; Lane C, *exoR108t*(pHC557), expressing ExoRΔSP.

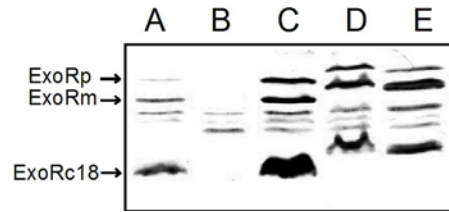


Figure 6-4. Western blot of ExoR protein from *S. meliloti* *exoR108t* mutant expressing mutated ExoR proteins with longer C-termini. Lane A, Rm1021; Lane B, *exoR108t*(pMB393), the vector control; Lane C, *exoR108t*(pHC510), expressing the wild type ExoR; Lane D, *exoR108t*(pHC539), expressing ExoRC_{R95} protein; Lane E, *exoR108t*(pHC540), expressing ExoRC_{sup7} protein.

7 CHAPTER VII SUMMARY AND PERSPECTIVES

The primary purpose of my research was to examine how *S. meliloti* ExoR protein regulates bacterial succinoglycan and flagella production. I have carried out extensive genetic and biochemical analyses of the ExoR protein, focusing mainly on its cellular location, the ExoR and ExoS regulatory pathway, the *exoR* gene expression and the *in vivo* processing of the ExoR protein.

My biochemical analysis of the ExoR-PhoA fusions suggests that the ExoR protein has a cleavable signal peptide and is most likely a periplasmic protein. The genetic analysis of my collection of *exoR*, *exoS*, and *chvI* mutants suggests that ExoR functions upstream as a negative regulator of the ExoS/ChvI two-component regulatory system. This conclusion was further supported by the analysis of the *exoR* promoter activities in different genetic backgrounds, which lead to the conclusion that the ExoR/ExoS/ChvI pathway is regulated by ExoR through feedback. The combination of biochemical and genetic analyses suggest that ExoR may function in periplasm through interaction with the sensing domain of ExoS to keep ExoS in the off state. Using ExoR specific polyclonal antibodies, I found the wild type ExoR protein is subject to protease digestion. Based on my own findings and other group's data, one likely model is that the mature form of ExoR, ExoR_m, binds to the ExoS and keeps it in the off state. The proteolysis of ExoR reduces the amount of ExoR_m which interacts with ExoS, thereby leading to the release and activation of ExoS. Activated ExoS phosphorylates ChvI which then upregulates the expression of the *exo* genes and the *exoR* gene and downregulates the expression of the *fla* genes (Figure 7-1).

Bacterial periplasmic proteins and two-component regulatory systems play important roles in protein folding and secretion and in sensing environmental signals (1, 71, 76, 82, 123). The ExoR protein appears to integrate the activities of periplasmic protease into the ExoS/ChvI pathway. Further investigation to find the site of proteolytic digestion and then to determine whether the proteolytic product is functional will provide more insights into the interaction between ExoR and ExoS. Understanding the proteolysis of ExoR will also help to find the protease, and the conditions that trigger the ExoR proteolysis which ultimately affects transcription of hundreds of genes.

Another related research direction on ExoR should focus on identifying environmental factors, such as stresses and nutrient types, which could influence the *exoR* gene expression. Most importantly, the *exoR* gene expression during symbiosis, especially at the early stages during the establishment of symbiosis should be characterized.

The *S. meliloti* ExoR protein represents a novel type of proteins, solenoid proteins, which consist of Sel1 domains involved in protein-protein interactions. Recently, another research group has shown that the ExoR protein interacts directly with the ExoS protein using immunoprecipitation and that point mutations in the Sel1 domain of ExoR interrupted its binding to ExoS (19). To date, only a few bacterial Sel1 proteins have been studied. Therefore, further biochemical studies on ExoR-ExoS interaction are necessary to reveal the underlying molecular mechanisms.

The regulatory mechanism of ExoR in *S. meliloti* has a great similarity to the CpxP protein in *E. coli*. The CpxP protein is part of the *E. coli* stress response system that regulates the expression of several dozens of genes involved in resistance to hostile

conditions, mobility, adherence factors and metabolism (119). The inner membrane sensor CpxA and the response regulator CpxR form a two-component regulatory system that regulates the expression of the Cpx regulon (28, 33, 97). In the absence of environmental stress, CpxP interacts with CpxA and keeps CpxA in the off state (33, 96, 97). Under environmental stress, such as pH change, CpxP interacts with misfolded periplasmic proteins and forms protein complexes with them that will be degraded by DegP protease in periplasm (14, 49). Released from CpxP suppression in the CpxP-CpxA complex, CpxA is activated and phosphorylates CpxR, the cognate response regulator of the two-component regulatory system in cytoplasm, which in turn acts as a transcriptional regulator to regulate downstream genes expression (14, 49).

The ExoR protein and also the ExoS/ChvI 2CRS close homologues have been found in other bacteria that interact with both plant and animal hosts including *Agrobacterium tumefaciens* and *Brucella abortus*. My findings will contribute to a better understanding of the microbe-host interactions in general.

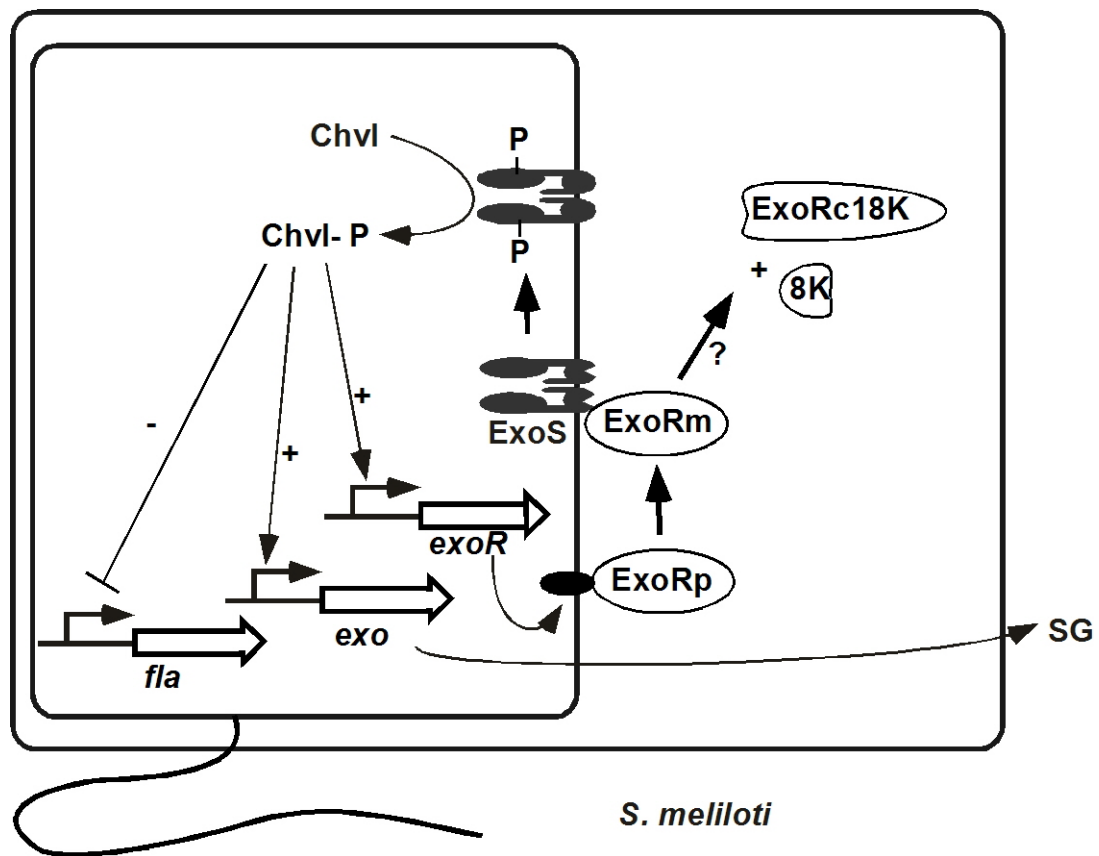


Figure 7-1. Model for the ExoR/ExoS/ChvI signal transduction pathway. The mature form of ExoR, ExoRm, interacts with ExoS and keeps it in the off state. Proteolysis of ExoRm results in free and activation of ExoS. Activated ExoS phosphorylates ChvI which then regulates the expression of downstream genes, including the *exoR*, *fla*, and *exo* genes.

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