The *Sinorhizobium meliloti* ExoS/ChvI Two-Component Regulatory System

by

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Exopolysaccharides, either succinoglycan or galactoglucan, are essential for the establishment of the symbiosis between *Sinorhizobium meliloti* and *Medicago sativa* (alfalfa). The ExoS/ChvI two-component regulatory system is known as a regulator of succinoglycan production but the genes that are directly regulated by ChvI have not been determined. Difficulty isolating *exoS* and *chvI* null mutants has prompted the suggestion that these genes are essential for *S. meliloti* viability. We have successfully isolated *exoS* and *chvI* null mutants using a merodiploid facilitated strategy. We present evidence that the *S. meliloti* ExoS/ChvI two-component regulatory system is essential for symbiosis with alfalfa. Phenotypic analyses of *exoS* and *chvI* null mutant strains demonstrate that ExoS/ChvI controls both succinoglycan and galactoglucan production and is required for growth on over 21 different carbon sources. These new findings suggest that the ExoS/ChvI regulatory targets might not be the *exo* genes that are specific for succinoglycan biosynthesis but rather genes that have common influence on both succinoglycan and galactoglucan production. To obtain further insight into the nature of the ChvI regulon, we obtained a purified His•Tag-ChvI and used it to perform modified electrophoretic mobility shift assays. These assays were done using genomic DNA and were followed by cloning of DNA fragments having the highest affinity for ChvI. Sequencing of these fragments revealed that ChvI has a diverse regulon, it affects transcription of genes encoding enzymes that are involved in

different pathways. Transcriptional gene fusion assays confirmed that ChvI is important for the activation of the transcription of the *msbA2* operon, as well as repression of the transcription of the rhizobactin 1021 operon and genes SMc00262-61. ChvI-regulation of genes that are part of the connected thiamine and histidine biosynthesis pathways suggest that ChvI could act in a concerted manner to avoid limitation of important intermediates in these pathways. Addition of uracil or proline in MM9-succinate defined growth media permitted the growth in liquid of Rm1021 *chvI* mutant strains and is additional evidence that the ExoS/ChvI system has an impact on central metabolites and thus the regulation of exopolysaccharides could be a side effect rather than a targeted one. This study presents for the first time genes directly regulated by ChvI and this includes none of the *exo* genes. This work opens new avenues in the understanding of the global regulatory role of the symbiotically important ExoS/ChvI two-component regulatory system but could also help in understanding the role of the orthologous systems found to be important for the pathogenesis of *Brucella abortus* and *Agrobacterium tumefaciens*.

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List of Abbreviations

ABC	ATP binding cassette
Ac	acetyl
AICAR	aminoimidazole carboxamide ribonucleotide
AIR	5-aminoimidazole ribonucleotide
Ala	alanine
Amp	ampicillin
AMP	adenosine-5'-monophosphate
AP	acetylphosphate
APHI	alpha-proteobacteria host interaction
Asp	aspartate
ATP	adenosine-5'-triphosphate
bp	base pair
BSA	bovine serum albumin
CAIR	4-carboxyaminoimidazole ribonucleotide
ChIP	chromatin-immunoprecipitation
Cm	chloramphenicol
CoA	coenzyme A
DHAP synthase	2-dehydro-3-deoxyphosphoheptonate aldolase

DNA	deoxyribonucleic acid
dTDP	thymidine-5'-diphosphate
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
EPS	exopolysaccharide
EPS I	succinoglycan
EPS II	galactoglucan
EPSb	galactoglucan
FAICAR	phosphoribosyl-formamido-carboxamide
FGAM	5-phosphoribosyl-N-formylglycineamidine
FGAR	5'-phosphoribosyl-N-formylglycineamide
Gal	galactose
GAR	5-phospho-ribosyl-glycineamide
GD	genomic DNA
Glc	glucose
Glu	glutamic acid
Gly	glycine
Gm	gentamicin
His	histidine
HMP-P	hydroxymethylpyrimidine phosphate
HMP-PP	4-amino-5-hydroxymethyl-2-methylpyrimidine-pyrophosphate
HMW	high-molecular-weight
IGP	D-erythro-imidazole-glycerol-phosphate
IMP	inosine-5'-phosphate
IS	insertion sequence

kb	kilobase
kDa	kiloDalton
Km	kanamycin
LB	Luria-Bertani
LMW	low-molecular-weight
LPS	lipopolysaccharide
Mb	megabase
MBN	mung bean nuclease
MM9	M9 with metals
NAD	nicotinamide adenine dinucleotide
NDP	nucleoside-5'-diphosphate
Nm	neomycin
Nod	nodulation
OMP	orotidine-5'-phosphate
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidylcholine
PCR	polymerase chain reaction
PD	plasmid DNA
PEP	phosphoenolpyruvate
P_i	inorganic phosphate
PRA	5-phospho-β-D-ribosyl-amine
PR-AMP	phosphoribosyl-AMP
PR-ATP	phosphoribosyl-ATP
PRFAR	phosphoribulosylformimino-AICAR-P

Pro	proline
PRoFAR	phosphoribosylformiminoAICAR-P
PRPP	5-phosphoribosyl 1-pyrophosphate
PTS	phosphotransferase system
Pyr	pyruvyl
REC	receiver domain
RNA	ribonucleic acid
SAICAR	5'-phosphoribosyl-4-(N-succinocarboxamide)-5-aminoimidazole
SB	sodium boric acid
SD	standard deviation
SDS	sodium dodecyl sulfate
SHOPS	show operon structures
Sm	streptomycin
Sp	spectinomycin
Succ	succinyl
TBE	tris-borate-EDTA
Tc	tetracycline
TCA	tricarboxylic acid
TCRS	two-component regulatory system
Thy	thymine
THZ-P	4-methyl-5-(β-hydroxyethyl)thiazole phosphate
TRAP	tripartite ATP-independent periplasmic
TY	tryptone-yeast extract
UDP	uridine-5'-diphosphate
UMP	uridine-5'-monophosphate

UTP	uridine-5'-triphosphate
UV	ultraviolet
WT	wild-type

Claims of Contributions to Knowledge

1. I constructed a *Sinorhizobium meliloti* Rm1021 derivative strain with a functional *expR* gene (SmUW6). This strain was then used to create a SmUW6 derivative with an *exoY*$^{-}$ mutation (SmUW24). *Medicago sativa* cv. *Iroquois* inoculated with SmUW6 gave higher shoot weights than those inoculated with Rm1021 after 40 days of incubation.

2. I tested SmUW6 and SmUW24 for growth on MM9-minimal media agar-plates with each containing a different carbon source. These strains grew on all carbon sources tested that allow growth of Rm1021. SmUW6 and SmUW24 were highly mucoid compared with Rm1021 on most carbon sources tested except for nucleosides, dipeptides, most amino acids, three carboxylic acids, and six sugars.

3. I tested the addition of different metals in Calcofluor-M9 medium agar-plates. Addition of zinc in the medium increased the fluorescence of Rm1021 and SmUW6 and suggests that this metal has an impact on succinoglycan production. Addition of cobalt had the opposite effect with complete loss of fluorescence. Copper had an intermediate effect by presenting lower fluorescence than without any metal but

Rm1021 colonies were not completely dark.

4. I isolated a *S. meliloti* Rm1021 *chvI*::*nptII* mutant strain (SmUW38) by selecting for the mutation in a merodiploid strain followed by a transduction to obtain the haploid strain. I also obtained a *S. meliloti* SmUW6 *chvI*::*nptII* mutant strain (SmUW40). Using SmUW38 and SmUW40 strains, I present evidence that a *chvI* null mutation prevents the growth of *S. meliloti* on complex media TY and LB.

5. I obtained a *S. meliloti* Rm1021 $\triangle exoShprK$ mutant strain (SmUW37) and a *S. meliloti* SmUW6 $\triangle exoShprK$ mutant strain (SmUW39) by continuing the work started by others in the laboratory. These strains were also unable to grow on complex media and it presents *in vivo* evidence that ExoS and ChvI act together as has been suggested by *in vitro* experiments.

6. I performed phenotypic analyses of *S. meliloti* SmUW37, SmUW38, SmUW39, and SmUW40 strains. These *exoS* and *chvI* mutant strains had a large number of growth defects: sensitivity to SDS, inability to grow in liquid M9-succinate and M9-glucose media, and ability to grow on M9-minimal agar media only when particular carbon sources were provided.

7. Further phenotypic analyses performed using strains SmUW37, SmUW38, SmUW39, and SmUW40 present evidence that ExoS and ChvI are required for *S. meliloti* to produce succinoglycan and galactoglucan and thus all these strains were symbiotically inefficient.

8. All phenotypes were restored to a wild-type level by merodiploid complementation of mutant strains using either *chvI* or *exoS* from *A. tumefaciens* or *S. meliloti*.

9. I developed a method that uses the electrophoretic mobility shift assay to isolate

DNA fragments from a large set of DNA fragments, for example restricted genomic DNA or restricted plasmid DNA, that have the highest affinity for a DNA-binding protein. These DNA fragments can be subsequently cloned and sequenced to identify them. I named this method GD.EMSA when it uses genomic DNA and PD.EMSA when it uses plasmid DNA.

10. I report for the first time the binding to DNA of a purified *S. meliloti* His•Tag-ChvI protein. I cloned, sequenced and identified 28 different DNA fragments that had binding affinity for ChvI. This binding depended upon the presence of Mg^{2+} but not acetylphosphate.

11. I made a series of Rm1021 and SmUW38 derivative strains, each containing a gene fusion to the *gusA* reporter gene to test for the effect of ChvI on transcription of previous GD.EMSA identified genes.

12. Transcription of the SMb21189-90*msbA2* operon depended upon the presence of ChvI to be activated. Our results suggest that ChvI binds within SMb21188 to activate the transcription of the downstream genes.

13. Transcription of the SMc00262-61 operon depended upon the presence of ChvI to be repressed. Our results suggest that ChvI binds within SMc00262 to repress or attenuate the transcription of these genes.

14. Transcription of the *rhtXrhbABCDEF* operon depended upon the presence of ChvI to be repressed. Our results suggest that ChvI binds within *rhtX* in at least two different places to repress or attenuate the transcription of these genes.

15. Results from the GD.EMSA experiment suggest that thiamine and histidine biosynthesis could be regulated by ChvI and if this is the case ChvI could have an indirect

regulatory effect on metabolically linked pathways. I tested this hypothesis and found that the growth of the SmUW38 strain depended upon the presence of proline or uracil in M9-succinate liquid media. I suggest that null mutation of *chvI* creates an auxotrophic strain by changing metabolic fluxes in thiamine and histidine biosynthesis pathways. The ability to grow the *chvI* mutant strain in liquid media is important because more analyses could be envisaged with *exoS* and *chvI* mutant strains to better understand the role of the ExoS/ChvI two-component regulatory system.

Chapter 1

Literature Review

At a time when the international scientific community acknowledges that our planet is facing the prospect of climate change, every advance towards a better understanding of natural processes that may alleviate the pressure humanity has put upon the earth's ecosystems is a step in the right direction for future generations. One of the problems we are facing is the always increasing release of reactive nitrogen to the biosphere. An alternative to the application of reactive nitrogen in agriculture is the use of nitrogen-fixing microorganisms. These microorganisms, in some cases, have the ability to form symbiosis with some legume plants and release only a limited amount of reactive nitrogen that is rapidly assimilated by the plant. This kind of symbiosis overcomes the need for external addition of nitrogen fertilizer and represents one solution, among others, to sustainable agriculture.

1.1 Rhizobiales

The Rhizobiales, an order of the α-proteobacteria, are comprised of diverse families (Bisby *et al.*, 2009). Among them the *Bradyrhizobiaceae*, *Phyllobacteriaceae* and *Rhizobiaceae* include numerous legume-associated bacteria that have the ability to trigger the formation of nodules on their host, invade these nodules and fix nitrogen in a symbiotic process. These bacterial symbionts are referred to as *Rhizobia*. Although the majority of known and well-studied *Rhizobia* belong to these three families of Rhizobiales, they are not exclusive to them. *Rhizobia* are also found in the *Brucellaceae* (some *Ochrobactrum* species), in the *Hyphomicrobiaceae* (some *Azorhizobium* species), and in the *Methylobacteriaceae* family (*Methylobacterium nodulans*) (Ngom *et al.*, 2004; Trujillo *et al.*, 2005; de Souza Moreira *et al.*, 2006; Jourand *et al.*, 2004). Interestingly, *Rhizobia* are not restricted to the α-proteobacteria as some species are found in the *Burkholderiaceae* and *Oxalobacteraceae* families of the β-proteobacteria class (Menna *et al.*, 2006; Valverde *et al.*, 2003).

Sinorhizobium meliloti is a *Rhizobia* belonging to the *Rhizobiaceae* family. This family also includes *Agrobacterium* species. *A. tumefaciens* is well-known for its ability to transfer DNA into its plant-host and causes crown gall. This particular ability of *Agrobacterium* has led to its use as an important tool in plant genetic engineering. This has also generated much interest in understanding the mechanisms and pathways involved in *A. tumefaciens* phytopathogenicity (McCullen and Binns, 2006). Although *S. meliloti* and *A. tumefaciens* are genetically very closely related organisms, their impact on plants is different, one being beneficial and the other causing a disease.

Brucella from the *Brucellaceae* family are other famous Rhizobiales because they are the causative agent of the *brucellosis*, a zoonotic disease endemic in some parts of the world (Gorvel, 2008). These mammalian pathogens have a very different lifestyle than *Sinorhizo-*

bium and *Agrobacterium* as they survive better inside host cells. These different capacities of Rhizobiales to adapt to environmental changes are correlated in their respective genome sizes. *Brucella* genomes sequenced to date are about 3.3 Mb, *S. meliloti* Rm1021 genome is 6.69 Mb in size and *Agrobacterium tumefaciens* C58 is 5.67 Mb (Halling *et al.*, 2005; Galibert *et al.*, 2001; Wood *et al.*, 2001). The lifestyles of these bacteria are quite different but as genetically close relatives they also have a great deal in common. In fact, this study is about ExoS/ChvI, a particular two-component transduction system that is found to be important for the bacterial-host interaction in all these three species of bacteria. Genes coding for this regulatory system and neighboring genes are well conserved among α-proteobacteria (Figure 1.1).

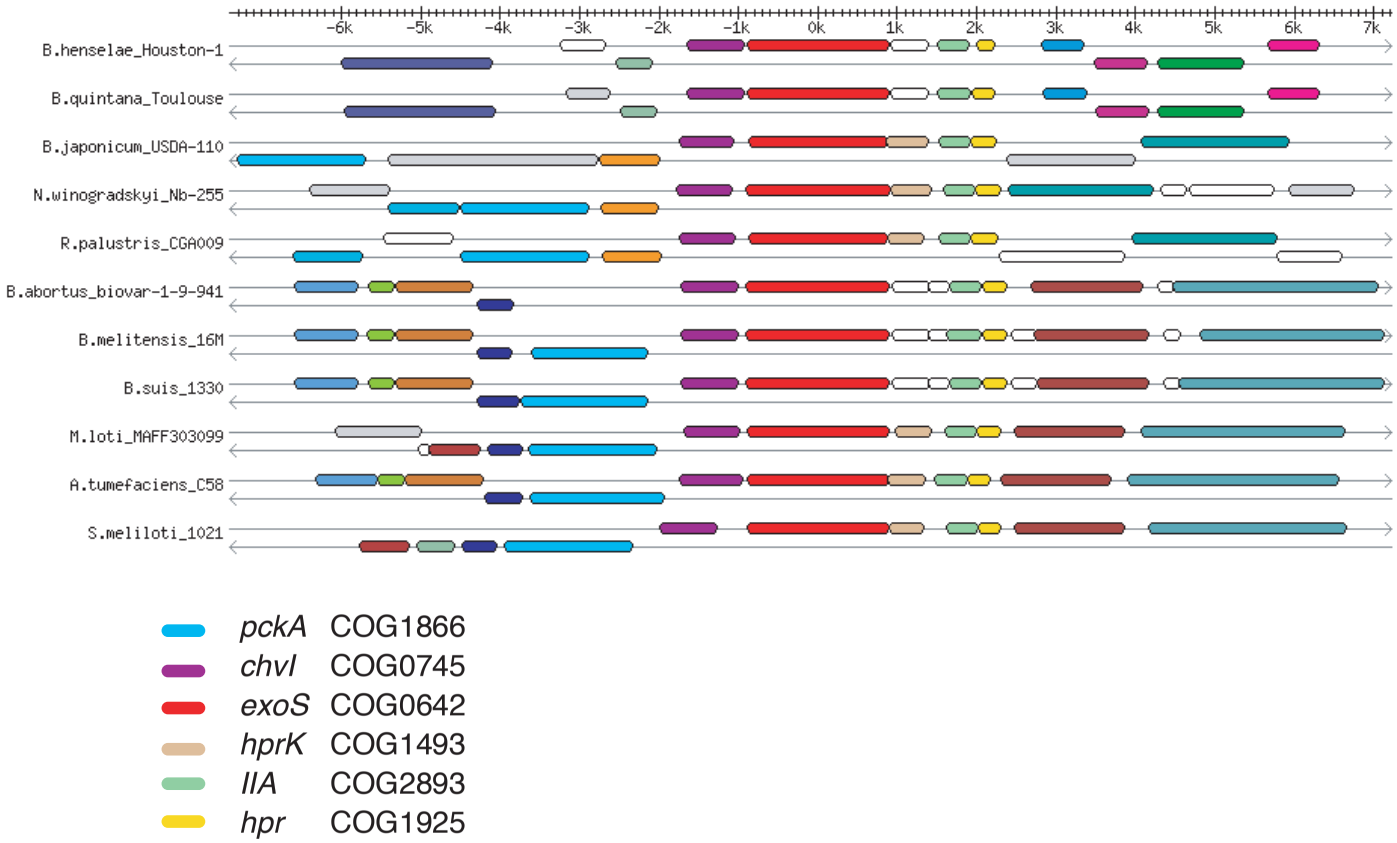

Figure 1.1: Homologs of *exoS* and *chvI* genes and their surrounding genes in other Rhizobiales. Designed using SHOPS web application (van Bakel *et al.*, 2004)

1.2 *S. meliloti* symbiosis with alfalfa

S. meliloti is a symbiotic bacterium inducing the formation of nodules on specific plant roots. *Medicago sativa* (alfalfa) and *M. truncatula* (barrel medic) are the plant-model organisms used to uncover most of the knowledge we have about the interaction between *S. meliloti* and its legume host, however *S. meliloti* is able to nodulate other legumes such as *Melilotus* and *Trigonella* (Spaink *et al.*, 1998).

While triggering the formation of alfalfa nodules, *S. meliloti* cells invade these nodules. Once inside plant cells, they differentiate into a specialized form called the bacteroid that reduces dinitrogen to biologically available ammonia (Jones *et al.*, 2007; Gibson *et al.*, 2008). This process is sustained by the provision of *S. meliloti* with carbon and energy generated by alfalfa through photosynthesis. This symbiosis involves a complex developmental pathway depicted in (Figure 1.2). It starts with the secretion of plant flavonoid molecules in the rhizosphere. Bacteria respond with the production of lipochitin oligosaccharide signaling molecules referred to as Nod factors. Root cortical cell division that leads to nodule formation is then initiated while an infection thread, inside a root hair, facilitates the bacterial invasion. During progression of the infection thread down the root hair and through cortical cells, bacteria divide until their release in the nodule primordium at the tip of the thread. Once they have entered nodule cells, bacteria differentiate into bacteroids that express genes needed for nitrogen reduction. In this complex cross-talk between *S. meliloti* and its host plant, the presence of bacterial exopolysaccharides (EPS) is essential for the development of the infection thread and therefore nodule invasion to occur (Fraysse *et al.*, 2003; Gage, 2004).

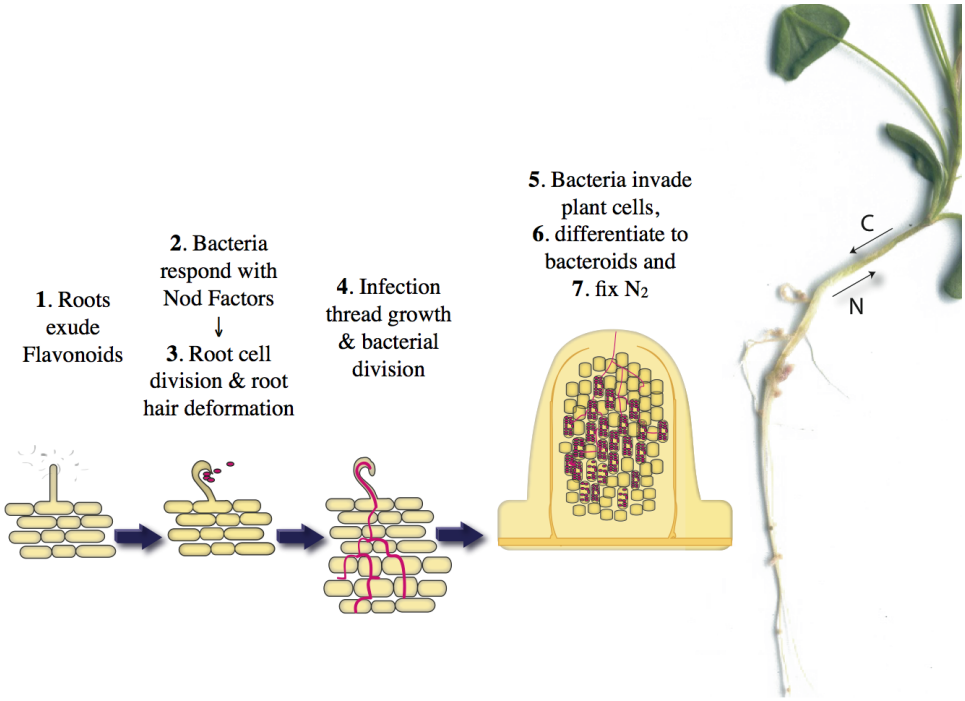

Figure 1.2: Steps leading to a successful symbiosis between *S. meliloti* and *M. sativa*. Exopolysaccharides are crucial during step 4. Green leafs and pink nodules indicate that bacteria have efficiently fixed nitrogen.

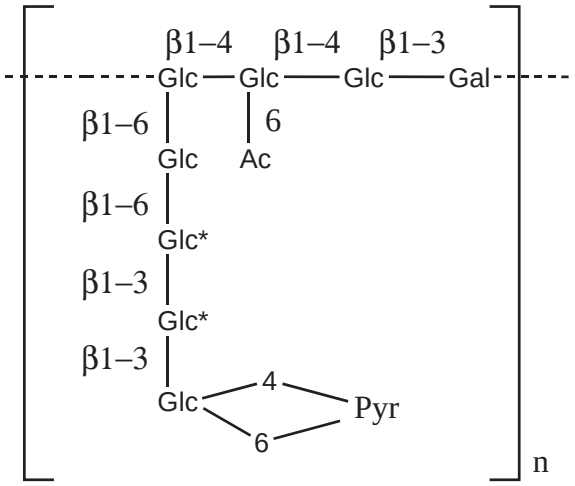

Figure 1.3: Molecular schema of the substituted octasaccharide unit forming *S. meliloti* succinoglycan (EPS I). * represents the possible positions of succinylation as a succinyl group can be found at one or both of these sites. (Wang *et al.*, 1999)

1.3 Exopolyssacharides

Two symbiotically important exopolysaccharides are biosynthesized by *S. meliloti* Rm1021: succinoglycan (EPS I) and galactoglucan (EPS II). Either one of these can mediate nodule invasion. Succinoglycan, made of octasaccharide repeating units (Figure 1.3), was the first *S. meliloti* extracellular polysaccharide to have its structure described (Jansson *et al.*, 1977). This polysaccharide is thus often referred to as EPS I and it is the major EPS produced by *S. meliloti* Rm1021. The octasaccharide unit made of one galactose and seven glucose molecules has acetyl, pyruvyl and succinyl substitutions (Reinhold *et al.*, 1994).

A second exopolysaccharide, galactoglucan or EPS II, is also excreted by *S. meliloti*. Galactoglucan is made of disaccharide repeating units (Figure 1.4). The disaccharide

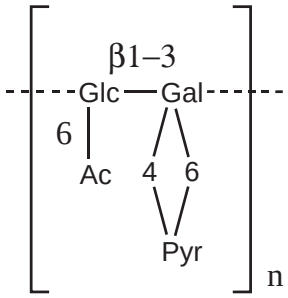

Figure 1.4: Molecular schema of the substituted disaccharide unit forming *S. meliloti* galactoglucan (EPS II or EPSb). (Chandrasekaran *et al.*, 1994)

is composed of glucose and galactose (1:1) that have acetyl and pyruvyl substitutions respectively (Her *et al.*, 1990). Both EPS can be found excreted as polymers of low- or high-molecular-weight.

The *S. meliloti* pathway for succinoglycan biosynthesis is well known (Figure 1.5) (Jones *et al.*, 2007). The production and export of succinoglycan requires over twenty genes and most of them have been identified and named as *exo* and *exs* (Glucksmann *et al.*, 1993; Becker *et al.*, 1995b). The genes are clustered on the pSymB plasmid (Finan *et al.*, 2001). The polysaccharide polymerization takes place in the cytoplasm and starts with the transfer of galactose from UDP-galactose to an undecaprenyl-phosphate. Polymerization is then obtained by subsequent glucose transfers at the non-reducing end of the lipid-linked intermediates. The lipid-linked polysaccharide is then exported. The mechanism by which the export happens and the involvement of transporters is still ill defined but a number of proteins (ExoP, ExoQ, ExoT, TolC) are known to be involved (González *et al.*, 1998; Cosme *et al.*, 2008).

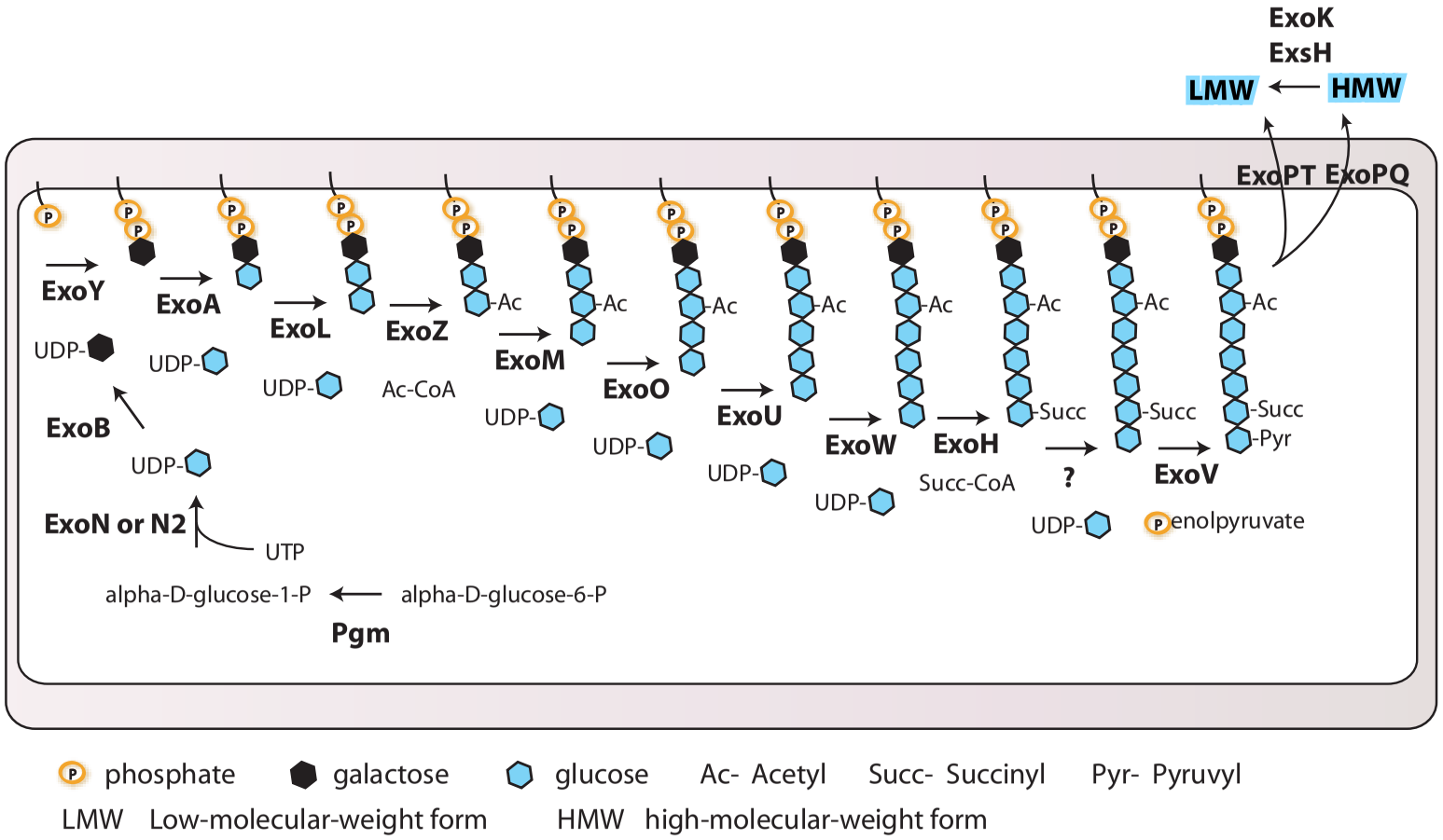

Figure 1.5: Pathway for succinoglycan production.

The galactoglucan biosynthesis pathway is not as well understood as the succinoglycan biosynthesis pathway. A 32-kb cluster of genes responsible for the production and export of galactoglucan is also found on the pSymB megaplasmid (Becker *et al.*, 1997). The 21 genes were originally named *exp* but have recently been renamed *wga* to *wgg* representing five transcripts (Bahlawane *et al.*, 2008a). Functions for most of the *wg* genes have been assigned based on homology of predicted proteins but functional analyses are missing. While we know that succinoglycan is biosynthesized from the NDP-sugars, UDP-glucose and UDP-galactose, it is unclear which NDP-sugars are used for the biosynthesis of galactoglucan. Sequence homology analyses suggest that dTDP-glucose may be used and converted to dTDP-rhamnose (Becker *et al.*, 1997). The role for dTDP-rhamnose in galactoglucan biosynthesis, if there is one, is elusive because the excreted polymer is only composed of glucose and galactose.

1.3.1 Importance and role of EPS in the establishment of the symbiosis

The exact role exopolysaccharides play during symbiosis has not yet been defined but there is evidence suggesting that EPS is needed during the initiation of the infection process. *S. meliloti* strains unable to produce EPS do not initiate infection thread formation or are blocked in the middle of the infection thread and do not further invade plant nodule cells (Cheng and Walker, 1998). It is unknown whether EPS acts as a signaling molecule or in a mechanistic role by protecting bacterial cells against plant defence reactions. In fact, studies have pointed in both directions and perhaps a dual role may be the case. *S. meliloti* mutants unable to produce EPS only need a very low concentration of purified low-molecular-weight EPS to successfully invade nodule cells (González *et al.*, 1996). More-

over, a lack of succinoglycan production seems to be associated with a higher sensitivity to hydrogen peroxide (H_2O_2) (Davies and Walker, 2007) and the antimicrobial peptide protamine (Nogales *et al.*, 2006). The exact purpose of EPS during symbiosis is still a matter of debate among scientists but the necessity for EPS during the invasion of nodules is an established fact. This is true not only for *S. meliloti* but also for *Rhizobium leguminosarum* bv. *trifolii* and *viciae* as well as for *Rhizobium* sp. strain NGR234 (Wielbo *et al.*, 2004; van Workum *et al.*, 1998; Djordjevic *et al.*, 1987; Staehelin *et al.*, 2006).

Another phenomenon observed by many investigators is that *S. meliloti* mutants unable to produce succinoglycan but producing galactoglucan are proficient in invading nodule cells (Glazebrook and Walker, 1989; Zhan *et al.*, 1989; González *et al.*, 1996; Pellock *et al.*, 2000). Although these two EPS have very different composition of their repeating units, they are able to perform the same task. It is noteworthy that this is true for the symbiosis between *S. meliloti* and *Medicago sativa* (alfalfa) but not when the plant host is *Medicago truncatula*, *Medicago caerulea*, *Melilotus alba*, or *Trigonella foenum-graecum* (fenugreek) (Glazebrook and Walker, 1989; Zhan *et al.*, 1989). It was also reported that galactoglucan is less efficient than succinoglycan at promoting nodule invasion (Pellock *et al.*, 2000). Another important fact to consider when trying to understand the role of EPS is that although *S. meliloti* can produce low- and high-molecular-weight forms of both succinoglycan and galactoglucan, only low-molecular-weight forms are successful at promoting the nodule invasion (Battisti *et al.*, 1992; González *et al.*, 1996).

1.3.2 Regulation of EPS production

Several genes are involved in the production and the export of exopolysaccharides. Because the production of these polymers is metabolically demanding and important for *S. meliloti*

to establish a symbiosis with its host, it is unsurprising to find many other genes that have a regulatory effect on EPS production. Most EPS regulator genes have been identified through a genetic approach rather than a functional one. This may have led to genes being assigned to EPS regulation because they cause shifts in metabolites or catabolites required for the EPS biosynthesis pathways. Therefore, it is important to identify the regulatory role of EPS regulator genes.

An important regulator of *S. meliloti* EPS is MucR. This DNA-binding protein has a zinc-finger motif and is homologous to *A. tumefaciens* Ros protein. MucR is a regulator of both succinoglycan and galactoglucan production (Keller *et al.*, 1995). MucR influences the production of succinoglycan by binding DNA upstream of *exoH* and *exoY*. The transcription of *exoHK* is repressed by MucR while the transcription of *exoYFQ* is increased by the binding of MucR (Bertram-Drogatz *et al.*, 1998). The result is that MucR is important for the production of high-molecular-weight succinoglycan. When *mucR* is mutated, only low-molecular-weight succinoglycan is found in the culture supernatant (Bertram-Drogatz *et al.*, 1998). MucR is also known to be a repressor of galactoglucan production which was shown by an increased production of galactoglucan once mutated (Zhan *et al.*, 1989). A recent study showed that this repression is probably done through the binding of MucR on the distal part of the intergenic region upstream of *wgaA*, *wgeA*, *wgdA*, and *wggR* (Bahlawane *et al.*, 2008a). MucR also has an influence on the molecular-weight form of galactoglucan. A *S. meliloti* Rm1021 derivative having double mutations *mucR/exoY* is unable to invade nodules because of the lack of low-molecular-weight galactoglucan produced in addition to its lack of succinoglycan production (González *et al.*, 1996). Aside from modulating EPS production, MucR is also a regulator of motility by repressing *rem* transcription, which leads to a repression of motility (Bahlawane *et al.*, 2008b). This latest study has led to the development of a model for the MucR regulatory role, linking the

regulation of EPS biosynthesis to the regulation of motility.

Two other transcriptional regulators WggR (previously ExpG), a MarR-like regulator, and PhoB, a OmpR-like response regulator, are also involved in the regulation of galactoglucan biosynthesis. Together they induce the transcription of *wga*, *wgd*, and *wge* operons under low inorganic phosphate conditions (Bahlawane *et al.*, 2008a). The fact that these operons are induced under low inorganic phosphate conditions is important because it is the only currently known condition to induce the production of galactoglucan in *S. meliloti* Rm1021. When grown in low inorganic phosphate media, *S. meliloti* Rm1021 produces galactoglucan of high-molecular-weight form, which is the symbiotically inactive form. Thus, *S. meliloti* Rm1021 relies only on its succinoglycan production to invade root nodule cells.

The finding that galactoglucan can suppress the nodulation defect caused by a lack of succinoglycan production is the result of the isolation of a spontaneous mutant of *S. meliloti* Rm1021 (Glazebrook and Walker, 1989). This mutation, called *expR101*, is unusual because it does not inactivate a gene but rather restores the function of an inactive gene in *S. meliloti* Rm1021. The Rm1021 strain, widely used as the wild-type strain and whose genomic sequence is entirely known, has an insertion sequence (IS*Rm*2011-1) in the *expR* gene (Pellock *et al.*, 2002). In *S. meliloti* Rm8530, a functional *expR* is present and it leads to a higher mucoidy phenotype due to greater production of galactoglucan. In this case, the galactoglucan produced is in its low-molecular-weight form and is effective in alfalfa nodule invasion (González *et al.*, 1996). ExpR is a LuxR homolog, part of a quorum-sensing regulatory system, and it controls the expression of *wgcA* (formerly *expC*) and *wgeB* (formerly *expE2*) in a density-dependent manner (Pellock *et al.*, 2002).

SyrA is a small hydrophobic protein (81 amino acids, 9 kDa) with no currently identified

domain (Barnett *et al.*, 1998). This small protein affects EPS production but its function is unknown. SyrA has some sequence similarities with ExoX, another small protein that influences EPS I production. Mutation of *exoX* causes an increase in EPS I production (Reed *et al.*, 1991). Both SyrA and ExoX do not present any similarity to known DNA-binding domains and possibly act at a post-transcriptional level. SyrA promotes the transcriptional regulation of *lpsS* (sulfotransferase modifying lipopolysaccharides) and *exo* genes but most likely indirectly and this regulation is dependent upon the ExoS/ChvI two-component regulatory system (Keating, 2007a). A LysR-type regulator SyrM, is known to activate *syrA* expression in free-living conditions and thus is also identified as an EPS regulator. However, *in planta* SyrM does not influence *syrA* expression, under these conditions *syrA* transcription is rather directed by the upstream operon *nifHDKE*, which is comprised of genes required for nitrogen fixation (Barnett *et al.*, 1998).

ExoD is a putative membrane associated protein that confers resistance to alkaline conditions (Reed and Walker, 1991a). Mutation of *exoD* alters the amount of EPS I production but this defect does not seem to be the primary cause of this mutant symbiotic deficiency (Reed and Walker, 1991b).

Other genes reported to influence EPS production are *exsA* and *exsB*. The latter gene is intriguing because of its similarity to queuosine biosynthesis protein. ExsB is found to negatively regulate EPS I biosynthesis which does not occur at a transcriptional level (Becker *et al.*, 1995a). ExsA is possibly involved in the transport of EPS I because of its significant homology to ATP binding cassette (ABC) transporter proteins and its influence on the ratio of high-molecular-weight to low-molecular-weight EPS I produced (Becker *et al.*, 1995a). However, the functions and respective roles in EPS I production still remain to be identified for both ExsA and ExsB proteins.

The calcofluor-bright regulator A (CbrA) was identified by a *cbrA*::Tn*5* mutation that caused an overproduction of succinoglycan (Gibson *et al.*, 2006). This increase in succinoglycan production was observed during stationary-phase growth and was probably due to an observed increase in transcript levels for *exoB, exoH, exoT* and *exoK* (Gibson *et al.*, 2006). CbrA, a putative two-component histidine kinase sensor, is required for symbiosis and affects many components of the cell envelope apart from its regulation of succinoglycan (Gibson *et al.*, 2007). The CbrA partner that acts as a response regulator and modulates gene transcription has not been identified and this is important to understand the main role for this two-component regulator.

ExoR/ExoS/ChvI is another important regulatory system for succinoglycan production. The isolation of two succinoglycan over-producing mutants carrying either *exoS*::Tn*5* or *exoR*::Tn*5* led to the first publication mentioning the *exoS* gene (Doherty *et al.*, 1988). The *exoS*::Tn*5* mutant has been extensively used to investigate the role of succinoglycan and its biosynthesis (Reuber and Walker, 1993; Glucksmann *et al.*, 1993). The *S. meliloti exoS* gene and the adjacent *chvI* gene were characterized as a two-component regulatory system (TCRS) (Østerås *et al.*, 1995). The study of the ExoS protein revealed that the mutant carrying *exoS*::Tn*5* (*exoS96*), which was initially assumed to be a null allele, actually produces a N-terminal truncated ExoS that is missing 45 amino acids (5 kDa smaller) (Cheng and Walker, 1998). ExoS was originally identified as a repressor of succinoglycan biosynthesis because of the overproduction phenotype of the *exoS96* mutant, however the study by Cheng and Walker (1998) changed the perception about ExoS and it is now considered an activator of succinoglycan. The *exoS96* mutant has been shown to up-regulate the expression of *exoY* but it is suggested that the ExoS/ChvI system does not interact directly with the *exoY* promoters (Cheng and Yao, 2004).

1.3.3 Environmental regulation of EPS production

Many genes are identified as regulators of EPS production but only one of them, PhoB, has its activity influenced by a known external factor, phosphate. Stimuli activating all other regulators mentioned in the previous section are unknown. Although many regulators, *exoS* and *cbrA* for example, are genetically identified as sensors and should be activated by environmental factors, by analogy with other sensor proteins, no one has yet identified what stimulates them. However, a number of studies have determined different environmental factors influencing EPS production. Moreover, certain environmental conditions are known to be detrimental to *S. meliloti* strains unable to produce EPS.

The most well-known environmental parameter that is important in determining *S. meliloti* EPS production is the concentration of inorganic phosphate (P_i) in media. *S. meliloti* Rm1021 can produce HMW galactoglucan but not LMW galactoglucan and this only happens when the concentration of P_i is low in the growth medium (Zhan *et al.*, 1991; Mendrygal and González, 2000). However, when bacteria of this same strain are in the presence of high P_i concentrations, the production of succinoglycan is stimulated instead (Mendrygal and González, 2000). Media lacking ammonia increase the production of succinoglycan by *S. meliloti* and interestingly this is dependent upon the presence of the *exoR* gene (Doherty *et al.*, 1988). When using M9-glucose media, Leigh *et al.* (1985) found that succinoglycan is produced in liquid medium only in the absence of nitrogen, sulfur, or phosphorus; conditions that are not required on solid agar media. In other *S. meliloti* isolates, salt (NaCl) influences the production of EPS. Strain EFB1, a halotolerant strain, produces galactogucan constitutively unless NaCl is added to the medium. NaCl significantly lowers the expression of *wgdA* (fomerly *expE*) and the production of galactoglucan (Lloret *et al.*, 1998). The production of galactoglucan by *S. meliloti* Rm1021 $expR^+$ is

also affected by salts. High concentration of NaCl is detrimental to both that strain and *S. meliloti* Rm1021 *exoF1*$^-$ strain. Moreover, the presence of either Mg^{+2} or K^+ in the media can lower the galactoglucan production of Rm1021 *expR*$^+$ strain (Miller-Williams *et al.*, 2006).

A number of microarray analyses have also shown differential transcription of genes required for EPS production depending upon growth conditions. Because most of these studies are done using *S. meliloti* Rm1021 — a strain lacking a functional ExpR regulator — it is no surprise to see wider transcriptional changes for succinoglycan biosynthesis genes compared to galactoglucan biosynthesis genes. Transcription of *exo/exs* genes is higher in M9 succinate or glucose minimal media than in LB complex medium (Barnett *et al.*, 2004). Higher transcription of these genes also occur in acidic conditions (Hellweg *et al.*, 2009), as a consequence of an osmotic upshift (Domínguez-Ferreras *et al.*, 2006), and in conditions of iron limitation (Chao *et al.*, 2005). Addition of elevated concentration of zinc in the growth medium increases the transcription of *exoK*, an endoglycanase that depolymerizes nascent succinoglycan chains, however change in transcription of other *exo* genes is not reported under these conditions (Rossbach *et al.*, 2008).

1.4 Two-component regulatory systems

Bacteria live in precarious environments. They are continuously subjected to rapid and drastic changes in their surroundings. Nutrient and toxin levels, acidity, temperature, osmolarity, humidity, and many other conditions can change rapidly and unexpectedly. Their survival is linked to their ability to sense and respond quickly to environmental changes. Cells have sensory proteins on their cell surfaces. These biosensors detect environmental signals and transmit this critical information to activate appropriate cellular responses.

Bacteria possess many regulatory systems involved in responding to extracellular compounds and signals. Two-component regulatory systems are one type of regulatory system often involved in the control of nutrient acquisition, virulence, antibiotic resistance, and numerous other pathways in diverse bacteria (Hoch and Silhavy, 1995). Typical two-component regulatory systems are comprised of a transmembrane "sensory kinase" protein and a cytosolic partner "response regulator" protein (Stock *et al.*, 2000). The histidine kinase senses an environmental signal that triggers its autophosphorylation. It then transmits the signal to its partner response regulator via a phosphotransfer reaction. The sensory kinase is responsible for the regulated phosphorylation of the response regulator and, in some cases, also plays a role in its regulated dephosphorylation. The important characteristic of two-component systems is the covalent modification (phosphorylation/dephosphorylation) of a response regulator by a histidine kinase/phosphatase. These systems are an extremely fast and effective means of communication and accordingly large families of homologous sensory histidine kinase proteins and response regulatory proteins have been identified (D'Souza *et al.*, 2007).

Apart from bacteria, many archaea and eukarya such as plants, fungi and yeasts use two-component regulatory systems for many of their signaling processes (Klumpp and Krieglstein, 2002; Puthiyaveetil and Allen, 2009). However, there is no evidence of the presence of two-component proteins in animals and these proteins are not found encoded by the human, fly or worm genomes (West and Stock, 2001).

1.4.1 Protein domains and activities

Two-component signal transduction proteins possess a modular architecture. They have conserved domains arranged in different ways within proteins. This allows fast evolutionary

adaptation to respond to the specific regulatory needs of many different signaling systems (Babu *et al.*, 2006).

Typically, the histidine kinase proteins possess a sensor domain that detects a stimulus and regulates the histidine kinase activity. This protein also possesses a dimerization domain which contains the autophosphorylated histidine residue. The autophosphorylation is done in an ATP-dependent manner. Catalysis of the autophosphorylation reaction is facilitated by the ATP-binding domain, which contains conserved sequence motifs (N, G1, F and G2) (West and Stock, 2001). The dimerization and the ATP-binding domains are sometimes identified in the literature as one domain called the transmitter or the autokinase domain. Therefore, it is probably more appropriate to identify the dimerization and the ATP-binding domain as subdomains of the transmitter or the autokinase domain. Autokinase domains have high similarities in length and amino acid motifs, whereas signal detection domains show considerable variations in length and amino acid sequences, reflecting the diversity of signals (Varughese, 2002).

Response regulators possess two domains: the regulator and the effector domains. These proteins have a receiver also called a regulatory domain that contains a phospho-accepting aspartate residue. This aspartate residue can acquire the phosphoryl-group from the phospho-histidine of its sensory partner. Phosphorylation of this conserved regulatory domain activates an effector domain that triggers the specific output response (West and Stock, 2001). The phosphoryl transfer from the phospho-histidine of the histidine kinase protein to the conserved Asp residue of the response regulator appears to be catalyzed by the response regulator, since many response regulators have been shown to become phosphorylated in the presence of acetyl phosphate in the absence of histidine kinase protein (McCleary and Stock, 1994).

The majority of response regulators, for example 25 out of 32 in *E. coli*, are transcription factors with DNA-binding effector domains (Mizuno, 1997). Their role is to activate and/or repress transcription of specific genes. Others, like *E. coli* CheY, consist only of a regulatory domain and these response regulators associate with an effector protein in an intermolecular interaction (Welch *et al.*, 1993). DNA-binding response regulators are identified based on their sequence similarity to three well-characterized response regulators: OmpR, NarL, and NtrC. Other families of response regulators have been more recently identified but these three families still represent almost 60% of all response regulators (Galperin, 2006). Although these subdivisions are determined based on sequence similarities, considerable complexity and differences in their function as transcription factors exist within these families of response regulators (Mizuno, 1997; Stock *et al.*, 2000).

The *E. coli* OmpR acts as both a transcriptional activator and a repressor of genes encoding outer membrane porin proteins. In an unphosphorylated state caused by low osmotic strength, OmpR activates *ompF* transcription. However, in high osmolarity conditions, OmpR becomes phosphorylated and activates the transcription of *ompC* while repressing the transcription of *ompF*. The OmpR activation of transcription is also dependent upon its direct interaction with the α subunit of RNA polymerase (Pratt and Silhavy, 1994). *E. coli* PhoB, a response regulator of the OmpR family, binds to *pho* promoters and activates expression of genes belonging to the phosphate regulon (Shindoh *et al.*, 2002). In this latter case, the gene expression activation depends on the PhoB interaction with the σ^{70} subunit of RNA polymerase (Kumar *et al.*, 1994).

1.4.2 *S. meliloti* two-component regulatory systems

S. meliloti has 57 predicted response regulators: 16 are part of the OmpR family, 12 are in the NarL family, and the NtrC family has 5 representatives. Another important protein family in this bacteria with 15 members is REC, a group of response regulators composed of only a receiver domain. A complete census of prokaryotic response regulators can be found online (Jenal and Galperin, 2009; Galperin, 2006). A genome-wide comparison of all histidine kinase sensors and response regulators of *Mesorhizobium loti*, *Bradyrhizobium japonicum*, and *S. meliloti* has been published to improve our understanding of their functions in symbiosis (Hagiwara *et al.*, 2004). However, the regulatory functions of most *S. meliloti* two-component transduction systems are still unknown. PhoB is a well-studied OmpR-response regulator which has its regulon defined (Yuan *et al.*, 2006). CtrA, an essential OmpR-response regulator, is involved in cell cycle regulation (Barnett *et al.*, 2001). Another identified response regulator of the OmpR family is FeuP. This regulator acts with the histidine kinase FeuQ to regulate the export of cyclic β-glucan (Griffitts *et al.*, 2008). FixJ, of the NarL family, is the response regulator for the histidine kinase FixL and together they are part of a larger regulatory network that controls genes involved in nitrogen-fixation (Bobik *et al.*, 2006). DctD, a member of the NtrC family, along with its cognate sensor DctB activates transcription of *dctA* in a σ^{54}-dependent manner (Yurgel and Kahn, 2004; Xu *et al.*, 2004). An NtrC response regulator with its histidine kinase partner NtrB are also found in *S. meliloti* as part of a nitrogen stress response cascade (Labes *et al.*, 1993; Yurgel and Kahn, 2008). CbrA, mentioned previously as a regulator of succinoglycan production, is a histidine kinase with an unknown response regulator partner. Another known but incompletely understood two-component system is ExoS/ChvI, the subject of this thesis.

1.4.3 ExoS/ChvI regulatory system

The ExoS sensor histidine kinase is a transmembrane protein that has an N-terminal periplasmic domain and a C-terminal cytoplasmic kinase domain. ChvI is its cognate response regulator, a cytoplasmic protein that is phosphorylated by ExoS (Cheng and Walker, 1998). From sequence similarity analysis, ChvI belongs to the OmpR family of response regulator proteins (Stock *et al.*, 2000).

Aside from overproducing succinoglycan, it has been demonstrated that *exoS96* mutants are non-motile, do not harbor flagella (Wei and Bauer, 1999; Yao *et al.*, 2004), and form loose biofilms (Fujishige *et al.*, 2006). However, the phenotypes that are associated with the *exoS96* mutant and indeed the N-terminal truncated ExoS, do not prevent bacteria from nodulating and invading alfalfa plants (Yao *et al.*, 2004). Recently, it has been shown that ExoR is a periplasmic protein that interacts with ExoS and inhibits its activity (Chen *et al.*, 2008). A K214T mutation that most likely results in an altered putative DNA-binding domain of ChvI can suppress the nodulation phenotype associated with *exoR*::Tn*5* (Wells *et al.*, 2007). The *exoS*::Tn*5* and *chvI(K214T)* mutations were also used to show the involvement of ExoS/ChvI in the regulation of *lpsS*, a sulfotransferase required under symbiotic conditions to modify lipopolysaccharides (Keating, 2007b). As previously mentioned, it is proposed that SyrA mediates transcriptional regulation of *lpsS* and *exo* genes through the ExoS/ChvI regulatory system.

1.4.4 ExoS/ChvI orthologous systems

S. meliloti exoS and *chvI* orthologs studied in other α-proteobacteria have been shown to play important roles in plant and animal pathogenesis. *A. tumefaciens* C58 *chvG* and *chvI*

genes were isolated by complementation of detergent sensitive mutants (Charles and Nester, 1993). Another important phenotype of these mutants is their inability to grow on complex media. The cause of this phenotype is still unknown even though genes have been identified as ChvG/I-regulated. The *A. tumefaciens* ChvG/ChvI two-component regulatory system is required for bacterial virulence and acts on the regulation of some acid-inducible genes: *aopB, katA, virB*, and *virE* (Li *et al.*, 2002). A microarray study found the expression of *A. tumefaciens chvG*, *chvI*, as well as genes involved in succinoglycan production to be induced under acidic conditions (Yuan *et al.*, 2008). This is in accordance with the inability of the mutant strains to grow in acidic media (Charles and Nester, 1993). The *A. tumefaciens* ChvG/ChvI regulatory system is recognized as a transduction system responding to low pH in the environment but the mechanism is still unknown. Is ChvG directly affected by a change in pH or does ChvG respond to a signal that is affected by pH? Does ChvI respond by a direct interaction with transcriptional machinery of *aopB, katA, virB* and *virE* genes or are these genes indirectly affected by ChvG/ChvI activation? These questions are still unanswered.

The orthologous two-component system in *Brucella abortus* is BvrR/BvrS (Guzmán-Verri *et al.*, 2002). Null mutants are impaired in virulence and show extensive alterations in cell envelope protein profile (Sola-Landa *et al.*, 1998). Proteomic studies using outer membrane fragments of mutant strains showed a general increase in levels of periplasmic proteins and 25 outer membrane proteins were identified as differentially produced (Lamontagne *et al.*, 2007). The BvrR/BvrS system also impacts on LPS by changing lipid A acylation (Manterola *et al.*, 2005). Similar to the *A. tumefaciens* ChvG/ChvI system, details about the molecular interactions of the BvrR/BvrS system, including the molecular activating signal and directly responsive genes, are still unknown.

1.5 This work

As one can notice in a rapid survey of the literature, trying to understand the role EPS plays in helping bacteria to invade their plant host has attracted lots of attention from the community of scientists studying *Rhizobia*. This level of interest is warranted as EPS have been shown to be essential for the invasion of the plant by bacteria and thus for the symbiosis to occur. While knowledge about *S. meliloti* EPS biosynthesis and its role in symbiosis has increased, a question remains unanswered. What role does the ExoS/ChvI transduction system play in this EPS biosynthesis schema? One reason that has limited the research on ExoS/ChvI has been the inability to isolate null mutants. Using genetic tools and hints from the work accomplished in *A. tumefaciens*, a *S. meliloti exoS* mutant strain was created in our laboratory and ignited this work. With the availability of an *exoS* mutant strain we undertook the task of analysing its phenotype in an attempt to understand the role of the ExoS/ChvI two-component regulatory system. This work also led to the construction of a *chvI* mutant strain, which allowed us to compare their phenotypes and confirm that these two genes are phenotypically related. ExoS and ChvI are both required for EPS biosynthesis and for symbiosis to occur but also for the bacteria to grow in a variety of conditions. No other known *exo* or *wg* mutants have such pleiotropy and it thus seems plausible that the ExoS/ChvI regulatory role is broader than originally expected and their direct control could be on pathways affecting the overall output of exopolysaccharides.

As an alternative to a genetic approach, we also developed a molecular technique using the purified ChvI protein to identify DNA sequences that bind with the protein. These results helped us to directly identify genes that are part of the ChvI regulon. Gene fusion assays confirmed that ChvI is involved in regulating the rhizobactin 1021, the *msbA2*, and

the SMc00262 operons. A number of other potential members of the regulon are parts of pathways for phospholipid, histidine and thiamine biosynthesis as well as transport and degradation of peptides, disaccharides, and methionine. Results from this work suggest that the ExoS/ChvI two-component regulatory system is a global regulator influencing numerous pathways and perhaps essential to maintenance of homeostasis during drastic environmental changes.

Chapter 2

Materials and Methods

Other methods can be found in the following Chapters or in Appendix A.

2.1 Bacterial strains, plasmids and growth conditions

Table 2.1 lists strains and plasmids used in this study. *Escherichia coli* strains were grown with Luria-Bertani (LB) medium at 37°C. *S. meliloti* strains were cultured at 30°C using modified TY or M9 media. TY contained 3 mM $CaCl_2$ instead of 6 mM as originally described (Beringer, 1974). M9-minimal medium was used with modifications described elsewhere (Finan *et al.*, 1986). A further modified M9-minimal medium, which we named MM9-minimal medium, was prepared by the addition of a micronutrient cocktail containing 500 μM K_2SO_4, 4 μM H_3BO_3, 0.4 μM $CuSO_4$, 2 μM $MnSO_4$, 0.8 μM $ZnSO_4$, 0.2 μM $CoSO_4$, and 0.2 μM Na_2MoO_4. 15 mM sodium succinate was added to M9 or MM9-minimal media as the carbon source unless indicated otherwise. To prepare solid media, 1.5% (w/v)

Bacto^{TM} Agar (BD) was added. Antibiotics were used at the following concentrations (μg ml^{-1}): gentamicin (Gm), 10 for *E. coli* and 20 for *S. meliloti*; neomycin (Nm), 200 or 400 for SmUW6; spectinomycin (Sp), 100; streptomycin (Sm), 200; tetracycline (Tc), 10 or 2 for *exoS* and *chvI* mutants; ampicillin (Amp) 100; kanamycin (Km) 20; and chloramphenicol (Cm), 20. Carbon sources were filter sterilized and used at a concentration of 0.1% (w/v) unless indicated otherwise. Detergent sensitivity was assessed as previously described (Charles and Nester, 1993). When *E. coli* MT609 (Thy^{-}) strains were used, thymine (60 μg/ml) was added in the media.

The autoinduction medium used for protein over-expression was made with 6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 20 g/L tryptone, 5 g/L yeast extract, 5 g/L $NaCl_2$. The pH of this solution was adjusted to 7.2 with NaOH and autoclaved. 0.2% lactose, 0.6% glycerol, and 0.05% glucose were added to the medium just prior to the inoculation using stock solutions of 8%, 60%, and 10% respectively.

Table 2.1: Bacterial strains used in this study.

Strain	Relevant characteristics	Reference or source
Sinorhizobium meliloti		
RCR2011	Same as SU47	(Meade *et al.*, 1982)
Rm1021	SU47 *str-21*, Sm^r, wild type	(Meade *et al.*, 1982)
Rm7055	Rm1021 *exoF55*::Tn*5*	(Leigh *et al.*, 1985)
Rm11026	RCR2011△*exoS-hprK*::*nptII* (*exoS192*)	This study
Rm11390	Rm1021 △*exoS-hprK*::*nptII* (*exoS192*)	This study
RmG182	Rm1021 Ω317::Tn*5*-233, $expR^+$ Gm^r Sp^r	(Miller-Williams *et al.*, 2006)
Rm11428	Rm1021 *ccmC*::Tn*5*	(Capstick, 2004)
Rm11506	RmG182 *ccmC*::Tn*5*, $expR^+$ Nm^r Gm^s Sp^s	This study
RmP110	Rm1021 $pstC^+$	(Yuan *et al.*, 2006)
SmUW1	Rm1021 $nolR^+$ $pstC^+$	This study
SmUW2	Rm1021 *ccmC*::Tn*5* $expR^+$ $nolR^+$ $pstC^+$	This study
SmUW3	Rm1021 $expR^+nolR^+$ $pstC^+$	This study
SmUW6	Rm1021 $expR^+$ Nm^s Gm^s Sp^s	This study
SmUW10	Rm1021 *chvI*::*nptII* (*chvI261*) pTC190	This study
SmUW24	SmUW6 *exoY*::Tn*5*	This study
SmUW37	Rm1021 △*exoS-hprK*::*nptII* (*exoS192*)	This study

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Strain	Relevant characteristics	Reference or source
SmUW38	Rm1021 *chvI*::*nptII* (*chvI261*)	This study
SmUW39	SmUW6 △*exoS-hprK*::*nptII* (*exoS192*)	This study
SmUW40	SmUW6 *chvI*::*nptII* (*chvI261*)	This study
SmFL112	RmP110 *msbA2*::pTH1522	(Cowie *et al.*, 2006)
SmFL4665	RmP110 SMb21189::pTH1522	(Cowie *et al.*, 2006)
SmFL5401	RmP110 SMb21190::pTH1522	(Cowie *et al.*, 2006)
SmFL918	RmP110 SMc00262::pTH1522	(Cowie *et al.*, 2006)
SmFL4392	RmP110 SMc00261::pTH1522	(Cowie *et al.*, 2006)
SmFL2950	RmP110 *rhbB*::pTH1522	(Cowie *et al.*, 2006)
SmFL5628	RmP110 *rhtX*::pTH1522	(Cowie *et al.*, 2006)
SmFL5755	RmP110 *rhbF*::pTH1522	(Cowie *et al.*, 2006)
SmUW43	Rm1021 *msbA2*::pTH1522	This study
SmUW58	Rm1021 SMb21189::pTH1522	This study
SmUW59	Rm1021 SMb21190::pTH1522	This study
SmUW46	Rm1021 SMc00262::pTH1522	This study
SmUW57	Rm1021 SMc00261::pTH1522	This study
SmUW55	Rm1021 *rhbB*::pTH1522	This study
SmUW62	Rm1021 *rhtX*::pTH1522	This study
SmUW63	Rm1021 *rhbF*::pTH1522	This study

Continued on next page

Strain	Relevant characteristics	Reference or source
SmUW133	SmUW38 *msbA2*::pTH1522	This study
SmUW148	SmUW38 SMb21189::pTH1522	This study
SmUW149	SmUW38 SMb21190::pTH1522	This study
SmUW136	SmUW38 SMc00262::pTH1522	This study
SmUW147	SmUW38 SMc00261::pTH1522	This study
SmUW145	SmUW38 *rhbB*::pTH1522	This study
SmUW152	SmUW38 *rhtX*::pTH1522	This study
SmUW153	SmUW38 *rhbF*::pTH1522	This study
Agrobacterium tumefaciens		
A6007	A348 Pho$^-$ Sm^r	(Cangelosi *et al.*, 1991)
A7678	A6007 *chvG*$_{678}$::Tn*phoA*	(Cangelosi *et al.*, 1991)
Escherichia coli		
DH5α	F$^-$, ϕ80d*lac*ZΔM15, *end*A1, *rec*A1, Δ(*lac*ZYA-*arg*F)U169, *hsd*R17(r$_K^-$ m$_K^+$,) *deo*R, thi-1, *sup*E44, λ^-, *gyr*A96, *rel*A1	(Hanahan, 1983)
MT616	MT607 pRK600, mobilizer	(Finan *et al.*, 1986)
BL21(DE3)pLysS	F$^-$, *omp*T, *hsd*S$_B$ (r$_B^-$, m$_B^-$), *dcm*, *gal*, λ(DE3), pLysS, Cm^r	(Studier and Moffatt, 1986)

Table 2.2: Plasmids used in this study.

Plasmid	Relevant characteristics	Reference or source
pKNG101	Suicide vector, $sacB^+$ mob_{RK2} ori_{R6K}, Sm^r	(Kaniga *et al.*, 1991)
pRK600	Helper plasmid for conjugal transfer, Cm^r	(Finan *et al.*, 1986)
pGEM®-T Easy	Cloning of PCR products, Amp^r	Promega (USA)
pJQ200SK	Suicide vector, $sacB^+$, Gm^r	(Quandt and Hynes, 1993)
pHP45Ω-Km	pBR322 derivative with ΩKm^r, Amp^r	(Fellay *et al.*, 1987)
pUC7	ColE1 cloning vector, Amp^r	(Vieira and Messing, 1982)
pUC18	Cloning vector, GenBank/EMBL accession L09136	(Yanisch-Perron *et al.*, 1985)
pUC19	Cloning vector, GenBank/EMBL accession L09137	(Yanisch-Perron *et al.*, 1985)
pGS220	pGS330Ω::Tn*5*, Km^r	(De Vos *et al.*, 1984)
pTC147	pRK7813::4kb-HindIII *chvI-chvG* from	
	A. tumefaciens, Tc^r	(Charles and Nester, 1993)
pTC147*chvI1*::Tn*5*	pTC147Ω1::Tn*5*	(Charles and Nester, 1993)
pTC147*chvG5*::Tn*5*	pTC147Ω5::Tn*5*	(Charles and Nester, 1993)
pTC147*chvI9*::Tn*5*	pTC147Ω9::Tn*5*	(Charles and Nester, 1993)
pTC147*chvG18*::Tn*5*	pTC147Ω18::Tn*5*	(Charles and Nester, 1993)
pTC147*chvG19*::Tn*5*	pTC147Ω19::Tn*5*	(Charles and Nester, 1993)
pTC147*pckA20*::Tn*5*	pTC147Ω20::Tn*5*	(Charles and Nester, 1993)

Continued on next page

Plasmid	Relevant characteristics	Reference or source
pTC147*hprK21*::Tn*5*	pTC147Ω21::Tn*5*	(Charles and Nester, 1993)
pTC190	pVK101::4kb-HindIII *chvI-chvG* from *A. tumefaciens*, Tc^r Km^s	(Charles and Nester, 1993)
pRML1	*S. meliloti* cosmid clone complementing A7678	This study
pSP329	Broad-host-range vector, Tc^r	(Zhan *et al.*, 1990)
pTC198	pUC19::5-kb KpnI fragment from pRML1	This study
pTC201	pSP329::5-kb KpnI fragment from pRML1	This study
pTC223	pTC201△2kb-XhoI *exoS-hprK* fragment	This study
pTC236	pTC223::2.35 kb-*nptII* containing XhoI fragment of Tn*5*	This study
pTC264	pKNG101::KpnI fragment of pTC236	This study
pLB001	pGEM®-T Easy::*chvI*	This study
pLB002	pUC7 containing EcoRI *chvI* fragment of pLB001	This study
pLB003	pLB002*chvI261*::*nptII* from pHP45Ω-Km	This study
pLB005	pJQ200SK::3.2-kb EcoRI fragment from pLB003	This study
pET30a-c(+)	His•Tag expression vectors	Merck (Novagen)
pLB010	pGEM®-T Easy::*chvI* (T7 orientation)	This study
pLB011	pGEM®-T Easy::*chvI* (SP6 orientation)	This study
pJF011	pET30a::*chvI* (NotI-fragment from pLB010)	This study

Continued on next page

Plasmid	Relevant characteristics	Reference or source
pJF012	pET30a::*chvI* (NotI-fragment from pLB011)	This study
pKD001	pTC190::pKNG101, Tc^r	This study

Table 2.3: Primers used in this study.

Name	Sequence (5'-3')	Description
LB1	cacgagtgatccgcaatc	R *chvI* mutant
LB2	atcggttgaatcgcagctt	F *chvI* mutant
LB5	atgcagaccatcgcgctt	F *chvI* for His-tag
LB6	acatcgtgatccaacaagg	R *chvI* for His-tag
LB7	tcggcttctttaagccgc	R *At chvI* for His-tag
LB21	gcgcatccctttacgctgat	F to confirm $\triangle$*exoS/hprK*
LB22	cggcagatttctcccgatct	R to confirm $\triangle$*exoS/hprK*
LB31	atgaatattacgttgctcg	F to confirm *expR*
LB32	tcaggagatcagtccca	R to confirm *expR*
LB61	gtaaaacgacggccagt	improved M13F

2.2 Molecular biology techniques

Routine molecular biology techniques were performed using standard methods (Sambrook and Russell, 2001). PCR was performed using primers (Table 2.3) made by MOBIX Lab (McMaster University, Hamilton) or Sigma-Aldrich®. Taq DNA polymerase was used for routine amplification while Pfu DNA polymerase or KOD Hot Start DNA polymerase were used for cloning when high fidelity was required. Restriction enzymes came from Fermentas, Inc. unless they were unavailable and then were from New England Biolabs. Sequencing was done by MOBIX Lab (McMaster University, Hamilton) or University of Waterloo sequencing service.

Isolation of genomic DNA from *S. meliloti* was done using the UltraClean^{TM} Microbial DNA Isolation kit (MO BIO Laboratories, Inc., USA) or following the standard procedure from Ausubel *et al.* (1992).

The DIG-High Prime DNA Labeling and Detection Starter kit (Roche Applied Science) was used for Southern blot following standard procedures and manufacturer's instructions.

2.3 Bacterial genetic methods

Phage ΦM12 was used for transductions following the usual procedure except for the use of TY media instead of LBmc media to prepare and dilute lysates (Charles and Finan, 1990).

Bacterial matings using *exoS* or *chvI* mutant strains were done with modifications to the routine method (Finan *et al.*, 1986). *E. coli* cultures of donor and mobilizing (MT607/(pRK600)) strains were grown to late-log-phase in LB media and 50 μl of each were mixed on top of previously MM9 plate-grown recipient strain. Spots were dried at room temperature for 2-3 hours and incubated at 30°C for 2 to 3 days. After mating, spots were streaked on the appropriate media. Transconjugants were streak-purified three times.

2.4 Exopolysaccharide assays

2.4.1 Calcofluor method

To detect the presence of EPS I (succinoglycan) when bacteria were grown on agar plates, we used Fluorescent Brightener 28 (Sigma-Aldrich), a fluorescent dye also known as Calcofluor white M2R (Leigh *et al.*, 1985). 0.02% (w/v) Fluorescent Brightener 28 was added to M9-minimal medium agar plates made with ultra-pure water (0.0555-0.1 μS/cm). Calcofluor-medium plates were each supplemented or not with different metal trace solutions. Concentrations and metals used: 0.4 μM $CuSO_4$, 2 μM $MnSO_4$, 0.8 μM $ZnSO_4$, 0.2 μM $CoSO_4$, and 0.2 μM Na_2MoO_4. Bacterial spots were made using a metal rod and plates were incubated at 30°C for 4-5 days and up to 25 days. Dry conditions increased

fluorescence. Pictures were taken using a handheld UV lamp (UVS 11, Mineralight) and a digital camera (Canon PowerShot A570 IS).

2.4.2 Sudan Black B method

The presence of total EPS was detected on agar plates using the Sudan Black B method (Liu *et al.*, 1998). Bacteria were inoculated on M9-minimal medium agar plates and incubated at 30°C for 14 days prior to the staining procedure.

2.5 Plant assays

Medicago sativa cv. *Iroquois* seeds were sterilized and germinated as described elsewhere (Wang *et al.*, 2007). Seedlings were transfered and grown in Leonard assemblies as previously described (Jacob *et al.*, 2008). Five seedlings were used per assembly and each strain was inoculated to three different assemblies (15 plants/bacterial strain). Inoculation was done by resuspending bacteria grown on M9-minimal media agar-plates in 5 ml sterile water and quickly pouring the resuspension on vermiculite. Plants were grown for 40 days before nodules were examined and counted. Shoots were separated from the roots, weighed and dried at 65°C until no further reduction in mass was noted.

Chapter 3

S. meliloti exopolysaccharide mutant strains

3.1 Introduction

S. meliloti produces two distinctly biosynthesized acidic exopolysaccharides (EPS). These excreted polymers have been known for a long time to be required for symbiosis (Leigh *et al.*, 1985) and have been extensively studied. We have a good knowledge about EPS molecular structures, genes required for their production, and the environmental conditions that increase or lower their production. However, details about the many mechanisms regulating the biosynthesis and export of these extracellular polymers are still scarce. Many genes are known to have a regulatory effect on EPS biosynthesis but only a few have been demonstrated as having a direct link to EPS biosynthesis genes. This chapter presents the construction and phenotypic analysis of *S. meliloti* Rm1021 derivative strains

affected in EPS production. These strains could then be used to discriminate if particular genes impact only on EPS I regulation or if their impact is wider and affects also EPS II regulation.

In culture, *S. meliloti* Rm1021, which is the commonly used wild-type strain, does not produce EPS II at detectable levels unless it is grown under very low phosphate conditions, and even then, the high molecular weight EPS II produced is symbiotically inactive (Mendrygal and González, 2000). However, unlike *S. meliloti* Rm1021, *S. meliloti* strains that do not have an IS*Rm*2011-1 element disrupting the *expR* gene (e.g. Rm8530, RmG182, SRmA363), are able to produce a large amount of low molecular weight EPS II, resulting in a highly mucoid phenotype (Glazebrook and Walker, 1989; Pellock *et al.*, 2002; Miller-Williams *et al.*, 2006). Using these EPS II-producing Rm1021 derivatives, it was shown that EPS II synthesis suppresses the symbiotic defects of EPS I-deficient strains on *Medicago sativa* (alfalfa). Therefore the low molecular weight EPS II is able to substitute for EPS I in nodulation (González *et al.*, 1996; Pellock *et al.*, 2000).

In this thesis, the broad interest is to understand how ExoS/ChvI TCRS regulates succinoglycan production. Because no genes have been identified yet as having their transcription directly regulated by ChvI, it is still uncertain if the change in succinoglycan production observed with *exoR*, *exoS*, and *chvI* mutant strains is due to a direct effect of ChvI on *exo/exs* genes or if it is due to an indirect regulation through another regulator, or perhaps due to regulation of pathways influencing metabolites required for succinoglycan production. One approach to determine the extent of ExoS/ChvI regulation is to introduce a null *exoS* or *chvI* mutation into a low-molecular-weight (LMW) galactoglucan producing strain. If ExoS and ChvI influence only *exo/exs* genes required for succinoglycan biosynthesis, the presence of LMW galactoglucan should suppress the nodulation defect caused by a lack of succinoglycan. To perform these experiments, a *S. meliloti* $expR^+$ strain is required.

As previously mentioned, *S. meliloti* Rm1021 has an insertion sequence in *expR* that inactivates it. This chapter describes how we constructed a *S. meliloti* Rm1021 $expR^+$ strain, which is named SmUW6. This strain was then used to construct a double mutant strain, Rm1021 $expR^+$ $exoY^-$, named SmUW24. We showed that the latter strain is unable to produce succinoglycan and is used in the work described in the next chapter as a control strain. Phenotypic analyses of these exopolysaccharide mutant strains is also presented in this chapter and demonstrates that the amount of galactoglucan produced depends upon the type of carbon source used. Phosphate and salts are known to influence EPS production, we present evidence that some metals, such as zinc, cobalt and copper, also influence succinoglycan production. These findings contribute to a better understanding of which are direct or indirect regulatory mechanisms acting on EPS biosynthesis.

3.2 Materials and Methods

Materials and methods used in this chapter are as described in Chapter 2.

3.3 Results

3.3.1 Construction of *expR* mutant strains

Production of exopolysaccharides by *S. meliloti* is an important step for a successful invasion of its host. In the case of the absence of succinoglycan production, biosynthesis of low-molecular-weight galactoglucan can rescue the symbiosis. However, *S. meliloti* Rm1021, the strain used as wild-type in our laboratory and many others, has an IS*Rm2011* element

disrupting *expR* gene and as a consequence, the Rm1021 strain does not produce low-molecular-weight galactoglucan. Thus Rm1021 is unable to establish a symbiosis when a mutation prevents its biosynthesis of succinoglycan. To study the extent of EPS regulation, a strain able to produce LMW galactoglucan is necessary. Because the only available $expR^+$ strain (RmG182) had a Tn*5*-233 insertion in *rpoH2* (Miller-Williams *et al.*, 2006), we designed a strategy using a series of transductions with ΦM12 to isolate a *S. meliloti* Rm1021 $expR^+$ strain without the Tn*5*-233 insertion in *rpoH2* (Figure 3.1). The transposon Tn*5*-233 carries resistance to gentamicin/kanamycin (Gm^r/Km^r) and streptomycin/spectinomycin (Sm^r/Sp^r) (De Vos *et al.*, 1986). The transposon Tn*5* however carries resistance to neomycin/kanamycin (Nm^r/Km^r) and streptomycin (Sm^r) (De Vos *et al.*, 1984).

In *S. meliloti* Rm1021, the *ccmC* gene is found 24 kb from the *rpoH2* gene. Rm11428, a *ccmC*::Tn*5* mutant strain unable to grow on defined media with hypoxanthine as the carbon source (Capstick, 2004) was therefore used to co-transduce into RmG182 the *rpoH2* gene that does not contain the Tn*5*-233 insertion. Rm11428 was first grown and used to prepare a lysate. From this lysate, the *ccmC*::Tn*5* transposon insertion was transduced into RmG182 with selection for Nm^r colonies, and the resulting transductants screened for Gm^s, Sp^s, and mucoidy. One such transductant was named Rm11506. A lysate made from Rm1021 culture was used to transduce hypoxanthine prototrophy to Rm11506. After screening for Nm^s and mucoidy, a colony was purified and named SmUW6. Southern blot

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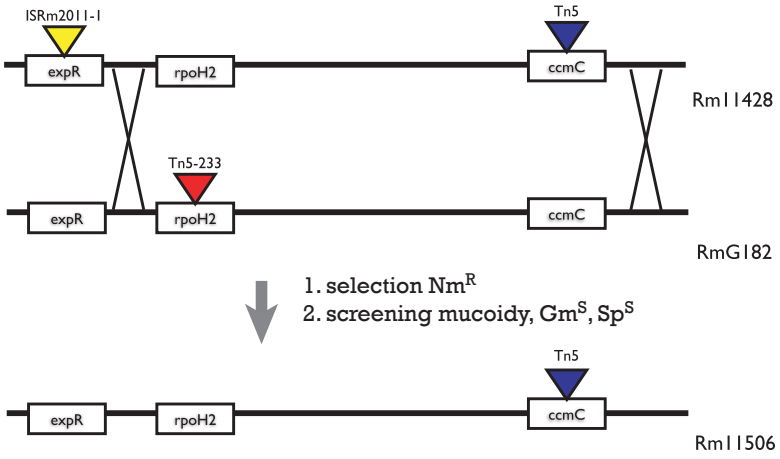
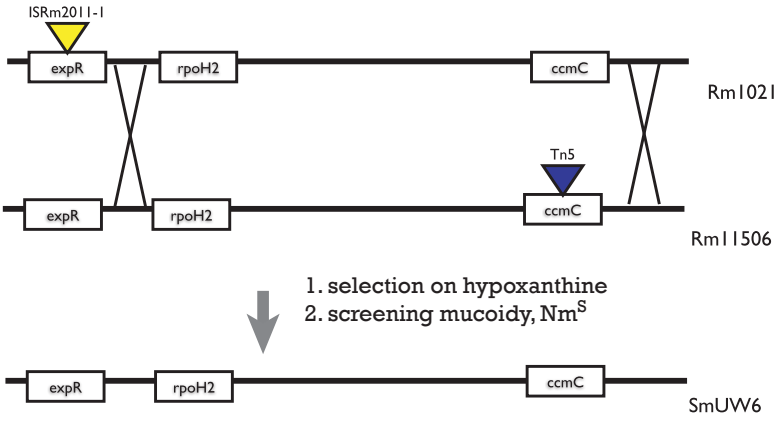
Figure 3.1 *(following page)*: Strategy to obtain *S. meliloti* SmUW6 strain. The transposon Tn*5*-233 carries resistance to gentamicin/kanamycin (Gm^r/Km^r) and streptomycin/spectinomycin (Sm^r/Sp^r) (De Vos *et al.*, 1986). The transposon Tn*5* carries resistance to neomycin/kanamycin (Nm^r/Km^r) and streptomycin (Sm^r) (De Vos *et al.*, 1984).

First transduction: Φ Rm11428 → RmG182



Second transduction: Φ Rm1021 → Rm11506



analysis was used to confirm the loss of any Tn*5*-derivative insertion (Figure 3.2) and PCR analysis was used to confirm the presence of an intact $expR^+$ gene.

To obtain a control strain, unable to produce succinoglycan but having a functional *expR* gene, the *exoY*::Tn*5* mutation from the Rm7055 strain was transduced into SmUW6 and selection was done accordingly with neomycin. One resulting transductant was named SmUW24 and used as the $expR^+exoY^-$ strain.

Construction of the SmUW3 strain (Rm1021 $expR^+nolR^+pstC^+$)

S. meliloti strain Rm1021 has two other genes, apart from *expR*, that are inactive: *nolR* and *pstC*. Strain SmUW1, available in our laboratory, has these two genes restored and functional. With the goal of having a new strain available in our collection that we could perhaps call truly wild-type, I did a series of transductions to obtain a SmUW1 $expR^+$ strain named SmUW3. A lysate was prepared with Rm11506 and used to co-transduce the $expR^+$ allele along with *ccmC*::Tn*5* into SmUW1 strain. Selection for neomycin resistance and mucoidy provided strain SmUW2. A lysate of SmUW1 culture was then used to transduce an active *ccmC* allele in SmUW2. Selection for hypoxanthine prototrophy and mucoidy allowed the isolation of SmUW3, a $nolR^+$, $pstC^+$, $expR^+$ strain. This strain was analyzed by Southern blot for the loss of any Tn*5*-derivative insertion (Figure 3.2).

3.3.2 A functional *expR* increases EPS II production and improves symbiosis.

The presence in *S. meliloti* Rm1021 of a functional *expR* gene increases considerably the production of galactoglucan (EPS II). The overproduction of exopolysaccharide due to an

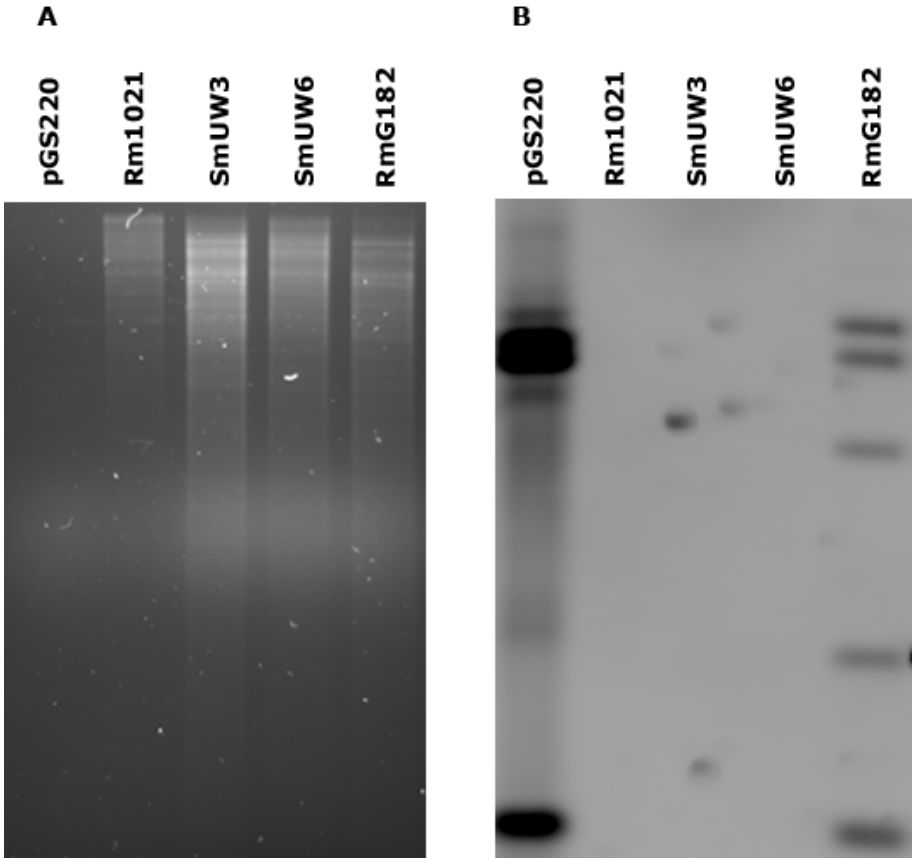


Figure 3.2: Agarose gel **A** and corresponding Southern blot analysis **B** confirming the absence of transposon Tn*5* insertion in *S. meliloti* Rm1021, SmUW3 and SmUW6. RmG182 and pGS220 DNA were used as positive controls while Rm1021 was used as a negative control. DNA samples were digested with HindIII and the membrane was hybridized with DIG-labelled pGS220.

active *expR* is noticeable on agar plates even in the absence of succinoglycan production caused by a mutation of the *exoY* gene (Rm7055 vs. SmUW24 strains) (Figure 3.3).

An important finding about the presence of a functional *expR* in *S. meliloti* Rm1021 strain is that it improves growth of plants under diazotrophic conditions. 40 days post-inoculation, plants that were inoculated with $expR^+$ strains presented higher shoot weights (Table 3.1).

3.3.3 Potassium and many carbon sources influence EPS II production.

As seen in Figure 3.3, *S. meliloti* Rm1021 with an active *expR* (SmUW6 strain) forms very mucoid colonies on agar plates. This mucoidy due to galactoglucan overproduction is influenced by media composition. SmUW6 grown on TY is much slimier than when grown on LB. Miller-Williams *et al.* (2006) have reported that increasing the concentration of Mg^{2+} or K^+ in media lowers mucoidy. We confirm that this is also true for our SmUW6 strain. Adding 350 mM KCl to LB or TY media decreased considerably the mucoidy of colonies. The same was found when using M9 minimal media; although in this condition the concentration of KCl added to the medium had to be no more than 175 mM for the strain to grow.

As we are interested in understanding what conditions trigger changes in exopolysaccharide production we streaked the SmUW6 strain on a large collection of MM9 minimal media plates, each containing a different and unique carbon source. Table 3.2 lists carbon sources on which the SmUW6 strain presented a mucoid phenotype or not. Growing SmUW6 on polyols or most pentoses, hexoses and oligosaccharides produced mucoid colonies while

nucleosides, dipeptides and most amino acids produced non-mucoid colonies.

3.3.4 Addition of Zinc, Copper or Cobalt influence succinoglycan production.

As we tested different compounds for their impact on mucoidy, we examined the effect on mucoidy of different metals usually added in RMM minimal medium, which is a medium different than M9 in composition and used to grow *Rhizobia*. Each metal was added separately to M9-succinate minimal medium agar plates and *S. meliloti* strains were streaked on these plates. Small differences in mucoidy of Rm1021 and SmUW6 were noted. However, when the same experiment was performed with added Calcofluor to test for succinoglycan production, fluorescence was greatly affected by the addition of the different metals (Figure 3.4). Addition of zinc considerably increased the fluorescent halos formed around both Rm1021 and SmUW6 strains. Cobalt, an essential metal to obtain an optimized growth of Rm1021 (Watson *et al.*, 2001), completely abolished the fluorescence. Copper, on the other hand, lowers the fluorescence when compared with results with no metal added but the fluorescence was not completely abolished. These results suggest an important effect of metals on succinoglycan detection. More experiments need to be done to understand if this effect is due to a change in the regulation of succinoglycan production or if these metals influence the calcofluor fluorescence.

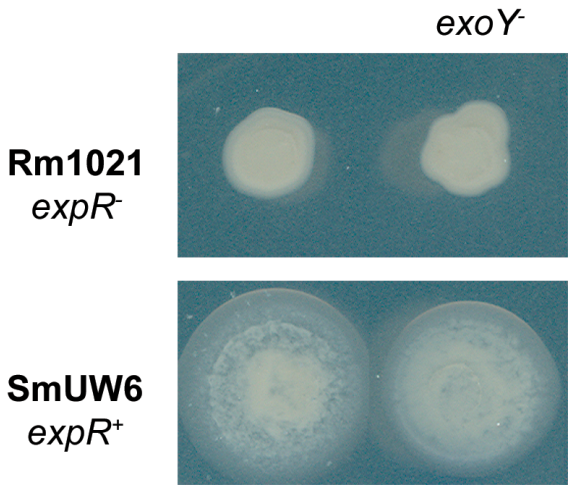


Figure 3.3: Mucoidy phenotype of *S. meliloti* strains grown on M9-succinate agar plates. First column: Rm1021 and SmUW6 strains ($exoY^+$); second column: Rm7055 and SmUW24 strains ($exoY^-$).

Table 3.1: Phenotypes of *Medicago sativa* cv *Iroquois* inoculated with *S. meliloti* mutant strains. Results represent the average for 15 plants and errors represent the standard deviation.

Strain	Genotype	Shoot weight				Number of nodule			
		Fresh		Dry		Pink		White	
		mg	±	mg	±		±		±
Rm1021		40	5.1	5.1	0.7	2	1	4	4
Rm7055	$exoY^-$	24	3	3.9	0.8	0	0	12	4
SmUW6	$expR^+$	59	12	7.8	0.7	3	1	1	1
SmUW24	$expR^+exoY^-$	51	4	6.7	0.6	4	1	2	2

Table 3.2: Mucoidy phenotype of *S. meliloti* SmUW6 strain grown on MM9 minimal media agar plates, each containing a unique carbon source.

Mucoid	**Mucoid** (cont.)	**Nonmucoid**
Carboxylic acids	**Polyols**	**Carboxylic acids**
D-3 hydroxybutyrate	D-mannitol	lactate
D-galactono lactone	D-maltitol	D(+) ribonic-γ-lactone
succinate	D-sorbitol	DL-malate
mono-methyl succinate	inositol	**Amino acids**
Amino acids	D-arabitol	L-alanine
L-lysine	L-arabitol	L-homoserine
L-glutamate	glycerol	L-asparagine
Pentoses	adonitol	L-serine
D-ribose	meso-erythritol	L-tyrosine
D-xylose	dulcitol	L-ornithine
methyl-β-D-xylopyranose	**Oligosaccharides**	L-proline
D-lyxose	D-melibiose	trans-4-hydroxy-L-proline
L-arabinose	D-trehalose	γ-aminobutyrate
Hexoses	cellobiose	aminovalerate
methyl-β-D-galactose	maltose	**Dipeptides**
N-acetyl D-glucosamine	maltotriose	Gly-Asp
D-fructose	lactulose	Ala-Gly
D-glucose	D-raffinose	Gly-Glu
D-mannose	sucrose	Gly-Pro
D-tagatose	D-(+)-turanose	**Pentoses**
6-deoxy-L-mannose	palatinose	D-arabinose
3-O-methyl-D-glucopyranose		**Hexoses**
L-fucose		D-galactose
		methyl-α-D-galactose
		N-acetyl D-galactosamine
		D-salicin
		Oligosaccharide
		D-(+)-lactose
		Nucleosides
		thymidine
		uridine

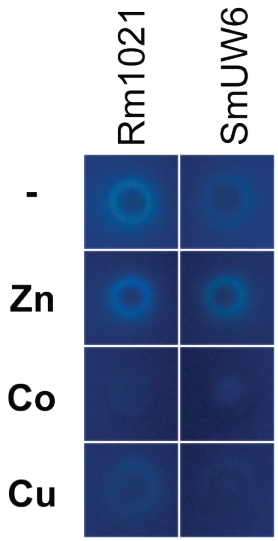


Figure 3.4: Effect of metals on succinoglycan phenotype of *S. meliloti* Rm1021 and SmUW6 ($expR^+$) strains grown on Calcofluor-medium agar plates. Picture was taken under UV light.

3.4 Discussion

In an effort to increase our knowledge about the symbiotically important acidic EPS of *S. meliloti*, we successfully constructed a Rm1021 $expR^+$ strain (SmUW6) and analyzed its associated phenotypes. As others have reported (Glazebrook and Walker, 1989) the presence of an active *expR* leads to the formation of very mucoid colonies. This mucoidy is due to an overproduction of galactoglucan (EPS II) that can function in place of succinoglycan (EPS I) in symbiosis. We confirmed that it was also true with our SmUW6 and SmUW6 $exoY^-$ strains, which validates these new strains.

The *S. meliloti* Rm1021 strain, although known to have some inactive genes, is the most common *S. meliloti* strain used for research. One of the reasons why Rm1021 is used rather than $expR^+$ strains is because it is technically difficult to work with a highly mucoid strain. The slimy phenotype on solid media makes it often extremely difficult to isolate colonies and the overproduction of EPS in liquid media alters the pelleting of cells. To obtain a better overview of conditions affecting the overproduction of EPS and perhaps find culture media more appropriate to the laboratory growth of *S. meliloti* $expR^+$ strains, we tested a number of different compounds for their effect on the overproduction of galactoglucan. SmUW6 showed a significantly lower production of galactoglucan when grown on solid-media in presence of KCl. This phenotype was also observed with SRmA363, another Rm1021 $expR^+$ strain (Miller-Williams *et al.*, 2006).

The mucoidy of SmUW6 could also be reduced by growth on a number of carbon sources. These carbon sources were mostly compounds containing nitrogen such as amino acids or nucleosides. It is tempting to speculate that the C:N ratio might influence the galactoglucan production. It has been previously reported that the concentration of ammonium in media influences the production of succinoglycan; lowering ammonia in the

medium increases the production of succinoglycan (Doherty *et al.*, 1988). Our results show the impact amino acids and nucleosides used as carbon sources have on the production of galactoglucan. More work needs to be done to understand how *S. meliloti* perhaps regulates EPS production based on C:N ratio available.

A recent microarray study has found that *exoK*, an endoglycanase acting on high-molecular-weight succinoglycan depolymerization, has an increased expression upon exposure to zinc (Rossbach *et al.*, 2008). Our study confirmed that these gene expression results are corroborated with phenotypic analyses. The presence of zinc in calcofluor media increased the intensity and size of fluorescent halos. Formation of halos on calcofluor-agar media is associated with a higher production of low-molecular-weight succinoglycan, which is believed to have a higher diffusion around colonies than high-molecular-weight succinoglycan (York and Walker, 1997).

A *bluB* mutant, deficient in cobalamin synthesis, has been shown to have a very-bright calcofluor phenotype (Campbell *et al.*, 2006; Taga *et al.*, 2007). The effect of added cobalt on succinoglycan production is then most likely related to a higher concentration of cobalamin in cells. *S. meliloti* has three cobalamin-dependent enzymes: BhbA, MetH, and NrdJ (Campbell *et al.*, 2006). BhbA (SMb20757) is a methylmalonyl-CoA mutase (EC 5.4.99.2) which could perhaps be linked to the calcofluor phenotype because it provides a source of succinyl-CoA necessary for succinoglycan succinylation (Charles and Aneja, 1999; Campbell *et al.*, 2006). MetH (SMc03112) is a methionine synthase (EC 2.1.1.13) required for L-methionine synthesis (Barra *et al.*, 2006). Although there is no obvious role of *metH* in succinoglycan production, methionine is most likely synthesized from homoserine via a O-succinyl-L-homoserine, and this reaction competes for succinyl-CoA. NrdJ (SMc01237) is a ribonucleoside-diphosphate reductase (EC 1.17.4.1) required for the *de novo* conversion of ribonucleoside diphosphates into deoxyribonucleoside diphosphates. Again there is

no direct link between this reaction and biosynthesis of succinoglycan but these pathways certainly compete for uridine triphosphates as UTP is required for the formation of UDP-glucose, a building block of succinoglycan. It appears plausible that the observed effect of cobalt on succinoglycan production might be due to a complex change in metabolism involving perhaps all three cobalamin-dependent enzymes. It would be interesting to study the calcofluor-phenotype of a *bhbA* mutant, which is available in our laboratory collection, in the presence and absence of cobalt.

A number of results remain obscure and need more investigation. For example, why is SmUW6 mucoid when grown with D-melibiose (α-galactoside) but not when grown with D-lactose (β-galactoside)? Both carbon sources have the exact same chemical composition ($C_{12}H_{22}O_{11}$) and are disaccharides composed of galactose and glucose. Their different configurations however, suggest that they are catabolized by different pathways. Indeed melibiose and lactose are transported and metabolized into β-D-glucose and β-D-galactose by different proteins (Gage and Long, 1998; Bringhurst *et al.*, 2001). Moreover, SmUW6 is also more mucoid when grown with D-glucose than when grown with D-galactose. How does the presence of D-lactose or D-galactose in the media interfere with the biosynthesis of galactoglucan, also a disaccharide made of galactose and glucose?

The construction of an *S. meliloti* Rm1021 $expR^+$ strain along with a *S. meliloti* Rm1021 $expR^+$ *exoY* strain provides us with a new genetic tool to study EPS production and regulation. The study of these strains and the different compounds that affect EPS production opens up new avenues of research to better understand pathways impacting on EPS biosynthesis and the role EPS plays in symbiosis.

Chapter 4

S. meliloti exoS and *chvI* mutant strains

4.1 Introduction

Difficulty isolating *S. meliloti exoS* and *chvI* null mutants has prompted the suggestion that these genes are essential for viability (Østerås *et al.*, 1995). We report here our success in isolating *S. meliloti exoS* and *chvI* null mutants using a merodiploid facilitated strategy. Phenotypic characterization of the mutants allows us to improve our understanding of the role ExoS/ChvI two-component regulatory system (TCRS) plays. *S. meliloti exoS* and *chvI* null mutants, like *A. tumefaciens chvG* and *chvI* mutants, do not grow on TY or LB media but are able to grow on defined media. Mutant strains have pleiotropic growth defects in laboratory conditions, do not produce EPS and are unable to establish an effective symbiosis with *Medicago sativa* cv. *Iroquois*. These phenotypes are restored to wild-type

by the presence of *A. tumefaciens* orthologs. *S. meliloti* ExoS/ChvI TCRS is required for symbiosis while the characterized orthologs *A. tumefaciens* ChvG/ChvI and *B. abortus* BvrR/BvrS are required for virulence. These findings are evidence that this TCRS is key to the regulation of host interaction and we propose to identify it as the Alpha-Proteobacteria Host Interaction (APHI) regulatory system.

4.2 Materials and Methods

Materials and methods used in this chapter are as described in Chapter 2.

4.3 Results

4.3.1 Null mutations in *exoS/hprK* and *chvI*.

The originally-described *exoS96* mutant (Doherty *et al.*, 1988) carries a Tn*5* insertion that results in a N-terminal deletion of *exoS*. This causes constitutive activation rather than loss of function (Cheng and Walker, 1998). Other researchers have reported unsuccessful attempts to create genomic disruptions of *exoS* and *chvI*, prompting their designation as essential genes (Østerås *et al.*, 1995). Our work on *S. meliloti exoS* and *chvI* was initiated in the context of a *S. meliloti* cosmid clone that was able to functionally complement *A. tumefaciens chvG* and *chvI* mutants (Charles and Nester, 1993). A clone that was able to complement the detergent sensitivity of *A. tumefaciens chvG*::Tn*phoA* strain (A7678; Table 2.1) was isolated from the *S. meliloti* pLAFR1 cosmid library (Friedman *et al.*, 1982). This plasmid, designated pRML1 (Table 2.2) was also able to complement the

atumorigenic phenotype of the *A. tumefaciens chvG* mutant strain. Having demonstrated the conservation of function between the *S. meliloti* and *A. tumefaciens* ChvG orthologs, we then investigated whether we could construct stable null mutations in the *S. meliloti* background.

A *chvI-exoS*-complementing 5-kb KpnI fragment from pRML1 was subcloned to pSP329 to make plasmid pTC201 (Figure 4.1). The restriction endonuclease cleavage pattern of this plasmid was consistent with the DNA sequence information, and was confirmed by sequence analysis. Cleavage of pTC201 with XhoI removed a 2013 bp fragment spanning from nucleotide 193 of the *exoS* ORF SMc04446 to 353 nucleotides past the end of the ORF sequence, into *hprK* ORF (SMc02752), and this fragment was replaced with the 2.35 kb *nptII*-containing XhoI fragment of Tn*5*. The resulting KpnI fragment from pTC236 was made blunt with mung bean nuclease (MBN) and then sub-cloned into the unique SmaI site of the homogenotization vector pKNG101, to make plasmid pTC264. Conjugation of pTC264 into the streptomycin sensitive (Sm^s) *S. meliloti* strain RCR2011 and selection for streptomycin resistance (Sm^r) resulted in cointegrate formation since the pKNG101 *oriV* is unable to function in *S. meliloti*. To minimize phenotypic effects that might preclude the isolation of double-crossover recombinants, the plasmid pTC190, which carries *A. tumefaciens chvI/chvG*, was introduced into the cointegrate strain. The meroploid cointegrate constructs were then subjected to selection on TY sucrose neomycin (Nm), and Sm^s isolates were retained as possible *exoS/hprK* (*exoS192*) deletion mutants. Transduction of Nm^r into strain RCR2011 gave rise to colonies with distinctive non-mucoid morphology and they did not grow on complex media (TY and LB). One of these transductants was designated Rm11026. The mutant phenotype could be complemented by the introduction of plasmid pTC147, carrying *A. tumefaciens chvI/chvG* (Charles and Nester, 1993).

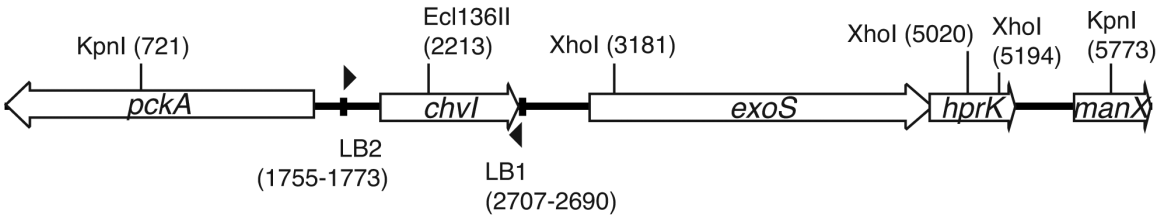


Figure 4.1: Genetic map of *chvI* (SMc02560), *exoS* (SMc04446), and surrounding genes. Positions between parentheses are relative to the last nucleotide coding for *pckA* (1) and the last nucleotide coding for *manX* (5967). Small arrowheads represent primers LB1 and LB2.

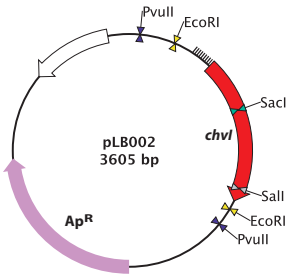
To transfer the mutation to the Rm1021 background, plasmid pTC190 was introduced into strain Rm11026, a ΦM12 lysate was prepared and used to transduce Nm^r to strain Rm1021, selecting on M9 glucose Nm, to make strain Rm11390. Plasmid pTC190 was introduced into strain Rm11390 and a lysate was made and subsequently used to transduce Nm^r into strain Rm1021 but this time the selection was done on M9 succinate Nm, resulting in strain SmUW37. Southern blot (Figure 4.3) and PCR analyses confirmed the nature of the *exoS192* deletion-insertion mutation. Using strain Rm11390 we discovered the inability of the bacteria to grow in liquid media. This phenotype and the inability to grow on complex media were restored by the introduction of plasmids containing *chvG* from *A. tumefaciens* (Table 4.1) or *S. meliloti exoS*. The liquid growth defect prompted us to adjust all methods to eliminate the growth in liquid media and rather use only bacteria grown from M9 or MM9-succinate agar plates.

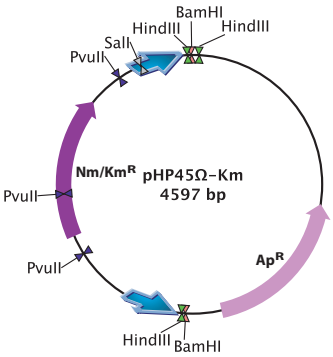
To obtain a *chvI*::*nptII* mutant strain, the *S. meliloti chvI* gene SMc02560 was amplified from Rm1021 genomic DNA using primers LB1 and LB2 (Table 2.3). The am-

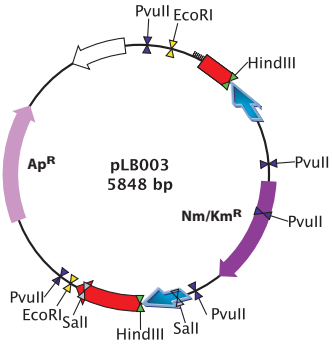
plification spanned from 197 nucleotides upstream to 29 nucleotides downstream of the coding sequence of Smc02560 (Figure 4.1). The 953-bp amplified fragment was cloned into pGEM®-T Easy vector and then subcloned into pUC7 using EcoRI restriction sites to create pLB002 (Figure 4.2). A 2.2-kb BamHI fragment carrying the Ω-kanamycin/neomycin resistance cassette from pHP45Ω-Km (Fellay *et al.*, 1987) was made blunt (MBN) and ligated into pLB002 previously cut with Ecl136II restriction enzyme. This subcloning step disrupted the *chvI* open reading frame (after nucleotide 261) and created pLB003 (Figure 4.2). The 3.2-kb EcoRI fragment from pLB003 was blunt-ended with MBN and subcloned into the SmaI restriction site of the *sacB* suicide vector pJQ200SK (Quandt and Hynes, 1993) to make plasmid pLB005 (Figure 4.2). Using a similar strategy to what we used to obtain the *exoS/hprK* null mutant, pLB005 plasmid was introduced by conjugation into the heterologous merodiploid strain *S. meliloti* Rm1021(pTC190). Transconjugants were selected for Nm^r and resulting meroploid cointegrate colonies were streak-purified three times on TY-NmSm tetracycline (Tc) before being subjected to selection on TY-NmSmTc supplemented with 5% sucrose. Fifteen isolates sensitive to gentamicin were identified as possible double-crossover *chvI* insertion mutants. Two of these were examined by Southern blot (Figure 4.3) and confirmed to have the desired *chvI261* insertion mutation. One was designated SmUW10 and used to prepare a ΦM12 lysate. This lysate was used for transduction of Nm^r into strain Rm1021 to obtain haploid strains. One chosen Rm1021 transductant was designated SmUW38 and confirmed by PCR analysis to have the expected insertion in *chvI* (data not shown). Similar to the *A. tumefaciens chvI* mutants, SmUW38 was not able to grow on complex media. The *S. meliloti chvI261* mutant strain was unable to grow in liquid media, like the *exoS192* mutant strain. Introduction of *chvI*-expressing plasmids resulted in restoration of the ability to grow in liquid and on TY agar (Table 4.1). *S. meliloti exoS192* and *chvI261* mutant strains were both unable to grow in the presence of 0.2 mg ml^{-1} sodium dodecyl sulfate (SDS), a condition that did not

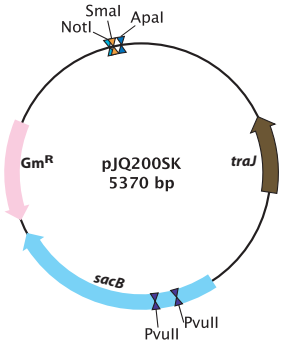
prevent the growth of the parental strain.

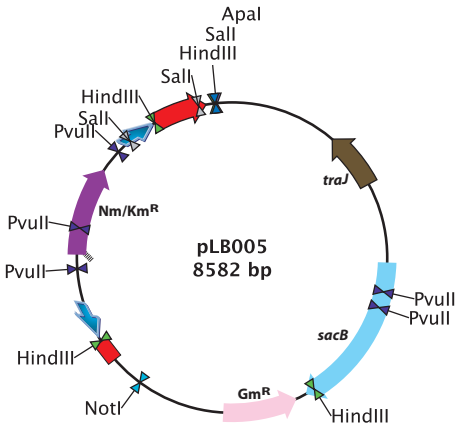
Figure 4.2 *(following page)*: Graphic representation of plasmids used to make *chvI* mutant strain.


PvuII
EcoRI
SacI
pLB002
3605 bp
chvI
SalI
EcoRI
PvuII
Ap^R


BamHI
HindIII
HindIII
SalI
PvuII
Nm/Km^R
pHP45Ω-Km
4597 bp
PvuII
PvuII
Ap^R
HindIII
BamHI


PvuII
EcoRI
HindIII
PvuII
Ap^R
pLB003
5848 bp
Nm/Km^R
PvuII
PvuII
EcoRI
SalI
HindIII
SalI
PvuII


SmaI
NotI
ApaI
Gm^R
pJQ200SK
5370 bp
traJ
sacB
PvuII
PvuII


ApaI
SalI
HindIII
SalI
HindIII
SalI
PvuII
traJ
Nm/Km^R
PvuII
pLB005
8582 bp
PvuII
PvuII
PvuII
sacB
HindIII
Gm^R
NotI
HindIII

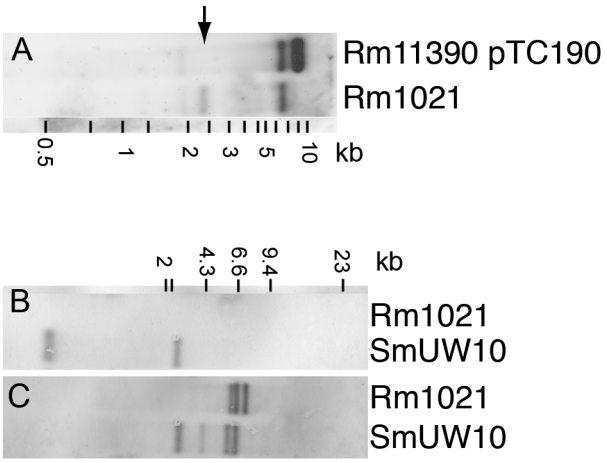


Figure 4.3: Southern blot analysis to confirm *exoS192* and *chvI261* mutations. *S. meliloti* Rm1021 was used as negative control. **A**: XhoI-digested DNA samples were transferred to membrane following agarose gel electrophoresis. Membrane was then hybridized with DIG-labelled pTC198. The arrow indicates the missing fragment corresponding to the deletion. **B** and **C**: DNA samples were digested with *Pst*I and the membrane was first hybridized with DIG-labelled pGS220 (**B**) then stripped and rehybridized with pTC198 (**C**).

Table 4.1: Heterologous complementation analysis of *S. meliloti exoS192* and *chvI261* mutant strains grown on TY medium.

Plasmid	SmUW37 *exoS192*	SmUW39 *exoS192 expR*$^+$	SmUW38 *chvI261*	SmUW40 *chvI261 expR*$^+$
no plasmid	-	-	-	-
pTC147	+	+	+	+
pTC147*chvI*1::Tn*5*	+	+	-	-
pTC147*chvG*5::Tn*5*	-	-	+	+
pTC147*chvI*9::Tn*5*	+	+	-	-
pTC147*chvG*18::Tn*5*	-	-	+	+
pTC147*chvG*19::Tn*5*	-	-	+	+
pTC147*pckA*20::Tn*5*	+	+	+	+
pTC147*hprK*21::Tn*5*	+	+	+	+

4.3.2 ExoS/ChvI TCRS is required for the production of EPS I.

Previous studies indicated the involvement of the ExoS/ChvI system as a regulator of succinoglycan (EPS I) production (Cheng and Walker, 1998; Yao *et al.*, 2004; Wells *et al.*, 2007), but since these studies were done using the *exoS96* or *chvI*(K214T) mutants, which are not null mutants, it was not confirmed whether the system was essential for succinoglycan synthesis. The N-terminal deleted ExoS96 is believed to stay in an active form that leads to the overproduction of EPS I and therefore, ExoS was originally described as an activator of EPS I synthesis. The *exoS192* and *chvI261* null mutant strains gave rise to colonies with distinctive non-mucoid morphology. The inability to grow these strains in liquid culture precluded quantitative measurements of EPS, however a qualitative method was used and deemed sufficient to determine the presence or absence of EPS I on solid-media. To confirm that ExoS and ChvI are required for the production of EPS I, we added Calcofluor white M2R (Fluorescent Brightener 28), which is known to fluoresce under UV light once bound to EPS I, to M9 minimal media containing succinate as the carbon source. *S. meliloti* mutant strains SmUW37 (*exoS192*), SmUW38 (*chvI261*), and Rm7055 (*exoY*) were dim under UV light whereas Rm1021 (WT) was bright (Figure 4.4). The Rm7055 strain was used as a negative control because this strain, originally described as a *exoF55*::Tn*5* mutant but more recently analyzed to be an *exoY* mutant (Miller-Williams et al., 2006), does not produce EPS I. The introduction of the *A. tumefaciens chvG* and *chvI* expressing plasmid pTC147 into mutant strains gave rise to bright colonies under UV light (Figure 4.4). Derivatives of pTC147 plasmid carrying Tn*5* insertions were also used to confirm mutations by complementation (data not shown). Fluorescence was also visible when mutant strains were complemented with the pTC201 plasmid, confirming that the calcofluor-dark phenotype of *exoS192* and *chvI261* strains is due to the mutation of these genes and not secondary mutation (data not shown).

While trying to optimize growth conditions for mutant strains we discovered that to obtain bright colonies using M9-succinate minimal media with Bacto-agar and Calcofluor white M2R we had to use ultra-pure water (0.0555-0.1 μS/cm). Our mutant strains exhibited superior growth with the use of ultra-pure water and the addition of a metal cocktail (Zn, Co, Cu, Mn, and Mo). This metal cocktail however prevents the calcofluor fluorescence of the wild-type strain. To optimize the growth of all strains while keeping the maximum level of brightness on calcofluor plates the addition of 0.8 μM $ZnSO_4$ was found to yield best results (chapter 3). The addition of Zn also increased halo formation around bright colonies of Rm1021, indicating an increased production of low molecular weight succinoglycan. As mentioned in the previous chapter, the influence of Zn on EPS I corroborates the recent finding that *exoK* is up-regulated during exposure to Zn (Rossbach *et al.*, 2008).

4.3.3 ExoS/ChvI TCRS is required for production of EPS II.

Production of exopolysaccharides by *S. meliloti* is an important step for a successful invasion of its host. In case of an absence of EPS I, biosynthesis of EPS II can rescue the symbiosis. Having shown that *exoS* and *chvI* are required for the production of EPS I, we wondered whether production of EPS II in an $expR^+$ background would result in restored symbiotic ability.

We used SmUW6 as the recipient strain for introduction of *exoS192* and *chvI261* mutations by transduction resulting in strains SmUW39 and SmUW40 respectively. Non-mucoid

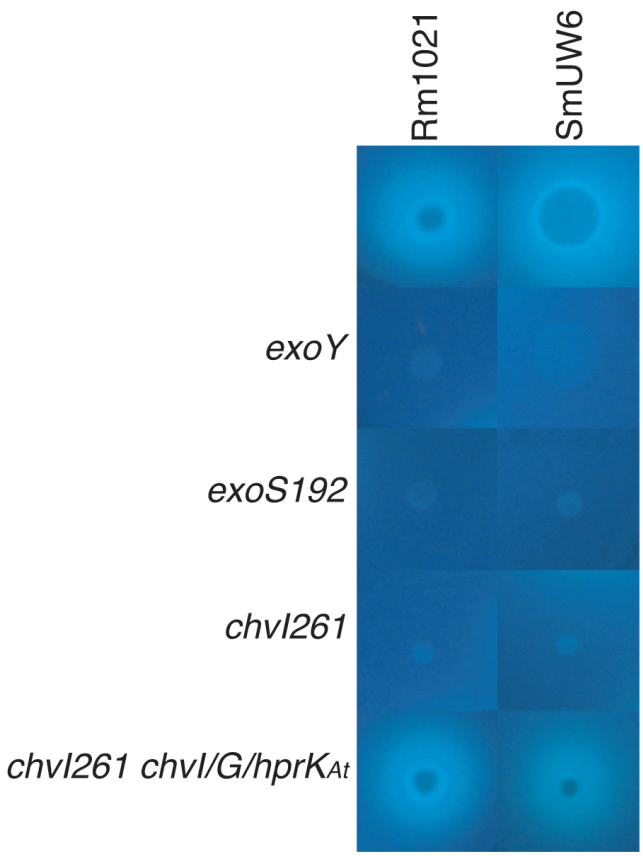


Figure 4.4: Succinoglycan phenotype of *S. meliloti* strains grown on Calcofluor-medium plates. Picture was taken under UV light. Bacterial strains used are: first column; Rm1021, Rm7055, SmUW37, SmUW38, SmUW38(pTC147), second column; SmUW6, SmUW24, SmUW39, SmUW40, SmUW40(pTC147).

colony morphologies of these strains different from SmUW6 ($expR^+$) and SmUW24 ($exoY$ $expR^+$) suggested the involvement of ExoS and ChvI in regulation of EPS II production (Figure 4.4). We also tested for the presence of exopolysaccharides by using the Sudan Black B staining method that has been used to identify *S. meliloti* strains unable to produce EPS I and EPS II (Liu *et al.*, 1998). The Sudan Black B staining method showed a lack of EPS from *exoS192* and *chvI261* grown on M9-succinate Bacto-agar plates. After staining, the large colonies of SmUW6 were mostly white with fuzzy light-blue staining (data not shown). Strain SmUW24, containing the $exoY$ mutation in the $expR^+$ background, also showed weak light-blue staining by Sudan Black B, which confirms that $exoY$ is not required for EPS II production. In contrast, the *exoS192* $expR^+$ and *chvI261* $expR^+$ strains exhibited a Sudan Black B dark coloration of colonies that indicates a complete lack of exopolysaccharide.

4.3.4 Carbon source utilization

Mutation of *A. tumefaciens chvG* or *chvI* prevents growth of the bacteria in complex media (Charles and Nester, 1993), and we have demonstrated here that this phenotype also characterizes *S. meliloti* that have null mutation of *exoS* or *chvI*. Because we could not grow mutant strains using any media that contained tryptone or yeast extract, we used M9 minimal media. We first used M9 media with glucose as the carbon source because it was uncertain if *pckA* was under the control of the genetically adjacent *exoS/chvI* but soon realized that succinate was a better carbon source; the colony growth of mutant strains was faster using succinate, although still lagged by 2 to 3 days compared to the wild-type strain. This apparent difference in carbon source utilization led us to search for carbon

sources that could either optimize or were unable to support growth of *exoS* and *chvI* mutant strains. We streaked mutant strains along with parental controls on MM9 minimal media Bacto-agar plates containing each of 64 different carbon sources that support the growth of *S. meliloti* Rm1021.

The *exoS192* mutant strain (SmUW37) only grew on 16 carbon sources out of 64 tested (Table 4.2). For those carbon sources that supported growth, the *exoS192* mutant strain in general grew poorly. SmUW37 grew on succinate and mono-methyl succinate but no other carboxylic acids. It did not grow on any amino acids, polyols or nucleosides tested. Weak growth was exhibited on the dipeptide Gly-Glu, the pentose D-arabinose, many hexoses and oligosaccharides.

The *chvI261* mutant strain (SmUW38) was able to grow on 51 carbon sources, considerably more than the *exoS192* mutant (Table 4.2). Also, as was observed for the *exoS192* mutant strain, carbon sources that allowed the growth of the *chvI* mutant strain did not, in general, result in growth as extensive as the parental strains. SmUW38 grew on all dipeptides and oligosaccharides tested. It grew on most carboxylic acids but not on aminovalerate or malate. It could not grow on most amino acids except for L-ornithine, L-proline, and showed only poor growth on L-lysine. Weak growth of SmUW38 was found on most pentoses tested but not on D-ribose or L-arabinose. Growth of SmUW38 on hexoses varied, from no growth on D-galactose, D-mannose, and D-tagatose to full growth on N-acetyl D-galactosamine. Only three polyols (L-arabitol, glycerol, dulcitol) supported the growth of SmUW38. Uridine but not thymidine was able to sustain the growth of SmUW38.

We confirmed that one of the better carbon sources to grow *exoS192* and *chvI261* mutant strains is succinate (Table 4.2). These growth phenotypes were restored to a wild-type (Rm1021) comparable growth after conjugation of pTC147 derivatives into SmUW37 and SmUW38. Transposon insertion in pTC147 allowed inactivation of *chvI*, *chvG*, or *hprK* and enabled us to precisely determine the *A. tumefaciens* complementing gene (Charles and Nester, 1993).

Using the same agar plate growth assay, we also tested if the ExpR regulator could affect growth of mutant strains. We streaked *exoS192* $expR^+$ and *chvI261* $expR^+$ mutant strains along with *exoS192*, *chvI261*, wild-type and $expR^+$ strains. The *exoY* and *exoY* $expR^+$ mutant strains were also used as controls to determine if the lack of EPS I production could be the reason for changes in the ability of the bacteria to use certain carbon sources. We observed no differences in growth between the wild-type (Rm1021) and *exoY* (Rm7055) mutant, or between $expR^+$ (SmUW6) and *exoY* $expR^+$ (SmUW24) mutant strains, suggesting that growth phenotypes are not linked to the lack of EPS I biosynthesis. By comparing growth of SmUW37 (*exoS192*) and SmUW38 (*chvI261*) to SmUW39 (*exoS192* $expR^+$) and SmUW40 (*chvI261* $expR^+$), it became apparent that on most saccharides and polyols, the presence of an intact *expR* allowed improved growth.

A difference between Rm1021 and SmUW6 growth is the slimy character of SmUW6 colonies due to a high level of EPS II. This is true on many carbon sources used in this assay but not all. SmUW6 mucoidy is mostly associated with growth on saccharides; however, the use of a few saccharides such as D-(+)-lactose, D-galactose, and D-arabinose did not lead to mucoidy (Table 4.2). Strains SmUW39 and SmUW40 were always dry on plates no matter which carbon source was used, another indication of a defective production or

export of EPS.

Table 4.2: Growth of *exoS192* or *chvI261* mutant strains on MM9 minimal media with various sole carbon source. Growth was recorded after 14 days relative to the growth of corresponding parental strains Rm1021 or SmUW6 ($expR^+$). [+++] represents growth equivalent to wild-type and [-] represents no growth. [*] SmUW6 and its derivative strains were not mucoid on these carbon sources.

Carbon source	SmUW37 *exoS192*	SmUW39 *exoS192 expR*$^+$	SmUW38 *chvI261*	SmUW40 *chvI261 expR*$^+$
Carboxylic acids				
D-3 hydroxybutyrate	-	-	+++	+++
lactate*	-	-	+	++
D(+) ribonic-γ-lactone*	-	-	++	++
D-galactono lactone	-	-	+	+
succinate	+++	+++	+++	+++
mono-methyl succinate	++	++	++	++
DL-malate*	-	+	-	+
Amino acids				
L-lysine	-	-	+	+
L-glutamate	-	-	++	++
L-alanine*	-	-	-	-
L-homoserine*	-	-	-	-
L-asparagine*	-	-	-	-
L-serine*	-	-	-	-

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Carbon source	SmUW37 *exoS192*	SmUW39 *exoS192 expR*$^+$	SmUW38 *chvI261*	SmUW40 *chvI261 expR*$^+$
L-tyrosine*	-	-	-	-
L-ornithine*	-	-	++	++
L-proline*	-	++	++	+++
trans-4-hydroxy-L-proline*	-	+	-	+
γ-aminobutyrate*	-	-	++	++
aminovalerate*	-	-	-	-
Dipeptides				
Gly-Asp*	-	-	++	++
Ala-Gly*	-	-	+++	+++
Gly-Glu*	+	+	+++	+++
Gly-Pro*	-	-	-	++
Pentoses				
D-ribose	-	+	-	+
D-xylose	-	+	+	+
methyl-β-D-xylopyranose	-	+	+	+++
D-lyxose	-	+	+	+
L-arabinose	-	+	-	-
D-arabinose*	+	+	++	++
Hexoses				
D-galactose*	-	+	-	+
methyl-α-D-galactose*	+	++	+	++
methyl-β-D-galactose	-	+	+	++

Continued on next page

Carbon source	SmUW37 *exoS192*	SmUW39 *exoS192 expR*$^+$	SmUW38 *chvI261*	SmUW40 *chvI261 expR*$^+$
N-acetyl D-galactosamine*	+	+	+++	+++
N-acetyl D-glucosamine	++	+	++	++
D-fructose	++	++	++	++
D-glucose	+	+	+	+
D-mannose	-	+	-	++
D-tagatose	-	+	-	+
6-deoxy-L-mannose	++	++	++	++
3-O-methyl-D-glucopyranose	-	+	+	++
L-fucose	-	++	++	++
D-salicin*	-	++	+	++
Oligosaccharides				
D-(+)-lactose*	+	+	+++	+++
D-melibiose	+	++	++	+++
D-trehalose	-	++	++	++
cellobiose	+	+++	++	+++
maltose	+	+++	++	+++
maltotriose	+	++	++	++
lactulose	-	+	+	++
D-raffinose	-	++	+	++
sucrose	-	++	+	++
D-(+)-turanose	-	++	+	+
palatinose	++	++	++	++

Continued on next page

Carbon source	SmUW37 *exoS192*	SmUW39 *exoS192 expR*$^+$	SmUW38 *chvI261*	SmUW40 *chvI261 expR*$^+$
Polyols				
D-mannitol	-	-	-	-
D-maltitol	-	-	-	-
D-sorbitol	-	+	-	+
inositol	-	+	-	++
D-arabitol	-	++	-	++
L-arabitol	-	++	++	++
glycerol	-	+	++	++
adonitol	-	++	-	++
meso-erythritol	-	+	-	+
dulcitol	-	-	+++	++
Nucleosides				
thymidine*	-	-	-	-
uridine*	-	-	++	++

4.3.5 ExoS/ChvI TCRS is required to establish an effective *S. meliloti*-alfalfa symbiosis.

To establish an effective symbiosis between *S. meliloti* and *M. sativa*, exopolysaccharides are required. Without production of EPS by *S. meliloti*, the bacterial invasion of the plant root hair is blocked in the infection thread and nodules are then devoid of bacteroids. Having demonstrated the lack of EPS I and EPS II production in strains carrying null mutations of *exoS* and *chvI*, we examined the ability of these mutants to establish an effective

symbiosis. Since *exoS192* and *chvI261* mutations are pleiotropic and also affect growth in many conditions we grew the plants for a full 40 days after inoculation (Table 4.3). Uninoculated control plants did not form nodules, while plants inoculated with Rm1021 or SmUW6 formed pink nitrogen-fixing nodules. No plant inoculated with *exoS192* or *chvI261* mutant strains presented pink nodules, whether in Rm1021 or SmUW6 background. White non-fixing nodules were however present. Shoots were stunted and with yellow leaves, another indicator of defective symbiosis. Plants inoculated with complemented *exoS192* and *chvI261* mutant strains were green and healthy, with pink nodules. These plant assays confirmed that a null mutation of *exoS* or *chvI* cannot be suppressed by an active *expR* in the same way as for a null mutation in *exoY*. The *exoS192* and *chvI261* mutant strains were able to trigger the formation of nodules by *M. sativa* but were not able to establish an effective symbiosis.

Table 4.3: Phenotypes of *Medicago sativa* cv *Iroquois* inoculated with *S. meliloti* strains complemented or not with heterologous *chvI/G/hprK* from *A. tumefaciens*. Results represent the average for 15 plants and errors represent the standard deviation.

Genotype	Shoot weight				Number of nodule			
	Fresh		Dry		Pink		White	
	mg	±	mg	±		±		±
wild type	40	5.1	5.1	0.7	2	1	4	4
exoY	24	3	3.9	0.8	0	0	12	4
exoS192	26	2	4.9	0.4	0	0	4	3
exoS192 pTC147*chvI*1::Tn*5*	40	12	5.6	1.4	1	1	6	6
exoS192 pTC147*hprK*21::Tn*5*	38	10	6.2	1.1	2	1	4	4
chvI261	27	7	4.9	0.9	0	0	3	3
chvI261 pTC147*chvG*18::Tn*5*	36	7	4.9	1.2	4	3	3	2
chvI261 pTC147*hprK*21::Tn*5*	39.0	0.4	5.6	0.4	3	3	5	5
expR$^+$	59	12	7.8	0.7	3	1	1	1
expR$^+$ *exoY*	51	4	6.7	0.6	4	1	2	2
expR$^+$ *exoS192*	26	2	4.4	0.4	0	0	4	4
expR$^+$ *exoS192* pTC147*chvI*1::Tn*5*	37	3	4.9	0.3	4	2	4	3
expR$^+$ *exoS192* pTC147*hprK*21::Tn*5*	31	3	5	0.2	2	2	10	6
expR$^+$ *chvI261*	26	2	4	0.8	0	0	6	6
expR$^+$ *chvI261* pTC147*chvG*18::Tn*5*	51	13	6.9	1.7	5	4	5	4
expR$^+$ *chvI261* pTC147*hprK*21::Tn*5*	49	16	7	2.5	6	3	4	3

4.4 Discussion

The previously reported description of *S. meliloti exoS* and *chvI* genes as essential for viability was based on the inability to obtain null mutants of this regulatory system (Østerås *et al.*, 1995). We have successfully constructed such mutants, and have presented evidence that this system is not required for viability, but is essential for adaptation to particular laboratory conditions. While growth of our *S. meliloti exoS* and *chvI* mutants is seriously compromised, these strains are able to divide and form colonies when cultivated on solid defined media. The ability to culture these strains allowed us to perform phenotypic analyses to refine our understanding of the role of the ExoS/ChvI TCRS.

It was previously shown that *exoS* and *chvI* are able to complement *A. tumefaciens chvG* and *chvI* mutants respectively (Østerås *et al.*, 1995). We have confirmed that this complementation works in the reverse direction as well, as the *A. tumefaciens chvG/I* fully complements the growth and symbiotic deficiencies of *S. meliloti exoS* and *chvI* mutants. Orthologs of *exoS* and *chvI* are found in many host-associated alpha-proteobacteria, and in all cases where it has been investigated the system has been shown to be involved in interaction with the eukaryal host (Sola-Landa *et al.*, 1998; Charles and Nester, 1993). We propose that this TCRS be designated as the Alpha-Proteobacteria Host Interaction (APHI) regulatory system.

Consistent with the results of other investigators studying the constitutively activated *exoS* mutant (Cheng and Walker, 1998) and the *chvI* missense mutant (Wells *et al.*, 2007), we have determined the involvement of these genes in regulating EPS I production. Since direct targets for ChvI in the genes for EPS I synthesis have not been identified (Cheng

and Walker, 1998; Cheng and Yao, 2004; Wells *et al.*, 2007), and our findings indicate that the ExoS/ChvI system also positively regulates EPS II production, we suggest that the regulatory role of ExoS/ChvI might not be on specific EPS genes, but rather on genes required for both EPS I and EPS II production. EPS I and EPS II polymers are biosynthesized through two distinct pathways, and genes coding for these pathways are found within different DNA clusters, but they do share some intermediates. The *exoB*-encoded UDP-glucose 4-epimerase (EC 5.1.3.2), catalyzing the interconversion of UDP-glucose and UDP-galactose, is thus required for both EPS I and EPS II synthesis (Buendia *et al.*, 1991). However, because recent findings suggest that ExoR acts as a regulator by inhibiting ExoS (Chen *et al.*, 2008) and a previous study indicated that an *exoR* mutation did not increase the levels of *exoB* transcript but did increase the transcripts of *exoAMNP*, *exoYFQX*, and *exoHK* (Reed *et al.*, 1991), *exoB* does not appear to be a good ChvI-target candidate.

UDP-glucose is produced from UTP and alpha-D-glucose 1-phosphate by a UTP-glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) such as the enzyme predicted to be encoded by the EPS I cluster-located *exoN* (SMb20960), and a distantly located *exoN2* (SMc04023) (Doherty *et al.*, 1988; Pobigaylo *et al.*, 2008). The suggested redundancy of this enzyme perhaps reflects the importance of maintenance of a steady level of UDP-glucose which argues against ChvI-mediated control. Given the observation that uridine, but not thymidine or ribose, as sole carbon source sustains growth of the *chvI* mutant on minimal media, we hypothesize, instead, that the lack of ExoS/ChvI regulation results in UMP limitation. This could occur through the depletion of 5-phosphoribosyl 1-pyrophosphate (PRPP), which is the intermediate between the pentose phosphate pathway and biosynthesis pathways for tryptophan, histidine, NAD, thiamine, purine and pyrimidine nucleotides. A depletion of PRPP would drain out D-ribose, preventing it from entering the Entner-

Doudoroff and glycolysis pathways. Generally, substrates further away from D-ribose-5P and PRPP were found to better sustain growth of the mutants, for example D-xylose and D-lyxose are better carbon sources than D-ribose. Others have also demonstrated a phenotypic link between succinoglycan and UMP *de novo* synthesis (Noel *et al.*, 1988; Nogales *et al.*, 2006). ExoS/ChvI transcriptional control of a pathway that feeds on PRPP could explain the lack of metabolic balance in *exoS* and *chvI* mutant strains that is reflected by the EPS phenotype as well as the growth defect on so many different carbon sources.

A recent finding indicates that EPS I and EPS II, in addition to pathway substrate sharing, also share an outer membrane protein that is required for export (Cosme *et al.*, 2008). Periplasmic and extracellular proteins required for the transport or the depolymerization of EPS need to be translocated across membranes. This can be done by using Sec-dependent or independent systems. Mutation of *tolC* (SMc02082), an outer membrane protein part of a Sec-independent type I secretion system, prevents the secretion of EPS I and EPS II (Cosme *et al.*, 2008). TolC is required for the secretion of ExsH, WgeA (ExpE1), and a putative hemolysin-type calcium-binding protein (SMc04171). ExsH is known as a EPS I depolymerase (York and Walker, 1997), and WgeA as a calcium-binding protein whose function is not clearly defined but suspected to be required for EPS II secretion (Becker *et al.*, 1997). It is perhaps noteworthy that the three genes encoding the TolC-dependent secreted proteins are all expressed at a higher level in the presence of a functional ExpR (Hoang *et al.*, 2004). However, strains deficient in ExoR or producing a constitutively active ExoS exhibit lower expression of *wgeA* in contrast to the overexpression of most *exo* genes in these genetic backgrounds (Wells *et al.*, 2007). Regulation of *wgeA* expression by the ExoR/ExoS/ChvI system might explain the EPS phenotype of the mutants if an overexpression of *wgeA* could somehow inactivate TolC. Also important to consider is the

observation that disruption of *tolC* causes SDS ultrasensitivity, a phenotype shared with our *exoS* and *chvI* mutants. Such detergent sensitivity is associated with membrane defect and not with EPS alteration (Ferguson *et al.*, 2002). This suggests a broader role for ExoS/ChvI than as solely a regulator of EPS biosynthesis.

Similar to *A. tumefaciens chvG* and *chvI* mutants, *S. meliloti exoS* and *chvI* mutants do not grow on complex media (Charles and Nester, 1993). A recently reported broad screen identified fifty-nine *S. meliloti* genes as being essential for growth in complex medium, but it is not known if these genes are also essential for growth in minimal media (Cowie *et al.*, 2006). Three of these genes, *secA*, *tolA* and *tolB*, encode parts of secretory pathways, and SecA, TolB and TolC have been detected in cultured bacteria but not in nodules suggesting that they might be down-regulated after the release of the bacteria from the infection thread in an expression pattern mirroring that of EPS I biosynthesis genes (Djordjevic, 2004). In contrast, SecB was found in nodules where it likely acts as a chaperone only. The *E. coli* SecB/A secretory system is affected by the lipid composition of the membrane and stabilized by zinc (Rietveld *et al.*, 1995; Fekkes *et al.*, 1999), and *secB* is required for growth on complex media, but not on minimal media (Kumamoto and Beckwith, 1985). Comparatively little is known about the *S. meliloti* translocase systems. Our results indicate that zinc increases the production of calcofluor fluorescent colony halos, an indication of low-molecular weight EPS I (York and Walker, 1997). This could result from increased expression of glycanase encoding *exoK* (Rossbach *et al.*, 2008), but also perhaps by stabilizing proteins of the Sec system required for the translocation of ExoK across the membrane. Transcription of some of the genes affecting the activity of these proteins might be controlled by ExoS/ChvI TCRS, thus providing a link between the EPS and the complex media phenotypes.

Investigation of mutants of the *Brucella abortus* BvrR/BvrS APHI system has shown considerable alterations in the patterns of membrane and periplasmic proteins (Lamontagne *et al.*, 2007). Similarly, microarray analysis performed using an *exoR* or constitutively active *exoS* mutant strain showed differential expression of several genes encoding components of ABC transport systems (Wells *et al.*, 2007). Our carbon source utilization results might therefore be associated with reduced expression of particular transport systems. For example, both *exoS* and *chvI* mutant strains do not grow on most polyols, similar to the inability of the *smoS* (SMc01500) mutant strains to grow on the same polyols (mannitol, maltitol, sorbitol, myo-inositol, meso-erythritol) (Jacob *et al.*, 2008). The *smoS* gene encodes a probable sorbitol dehydrogenase that is up-regulated in ExoR deficient and ExoS constitutively activated strains (Wells *et al.*, 2007). Moreover, the *smoS* gene is part of an operon that also contains genes for an ABC transporter induced by and used for the uptake of C6 polyols (Mauchline *et al.*, 2006); these genes are all consistently up-regulated in *exoR* knockout and *exoS* constitutively active mutant strains (Wells *et al.*, 2007).

The *exoS* and *chvI* genes lie between *pckA* and *hprK* on the chromosomal DNA (Galibert *et al.*, 2001). *S. meliloti* HprK is part of the phosphotransferase system (PTS), which has been demonstrated to regulate succinate-mediated catabolite repression (Pinedo and Gage, 2009). Phosphoenolpyruvate carboxykinase, encoded by *pckA*, catalyses the formation of phosphoenolpyruvate (PEP) from oxaloacetate. PEP is used by PTS as a phosphoryl donor and may thus be viewed as an important link between the TCA cycle and PTS. Most proteobacteria that contain *hprK* are missing homologues of enzyme IIB and enzyme IIC, which are required for the transport and phosphorylation of incoming sugar. It has been suggested that in the alpha-proteobacteria, PTS does not in fact facilitate sugar uptake, but instead controls transcriptional regulators via a phosphorylation cas-

cade (Boël *et al.*, 2003). Comparison of the phenotypes of our *exoS192* ($\triangle$*exoS-hprK*) and *chvI261* mutant strains with the phenotype of a *hprK* mutant strain from another study (Pinedo and Gage, 2009) suggests that PTS does not activate the phosphorylation of ExoS/ChvI. Because ExoS and ChvI are both required for EPS production and growth on complex media, phosphorylated ChvI seems to be the active state. In this case, if PTS was to act as a phospho donor or activator of phosphorylation, mutations in some of the PTS components should present similar phenotypes and this has been shown not to be the case (Pinedo *et al.*, 2008; Pinedo and Gage, 2009). A *S. meliloti* Rm1021 $\triangle$*hprK* mutant demonstrated better growth in complex (TY) than minimal (M9) media and the deletion of *hprK* increases the production of EPS I (Pinedo and Gage, 2009). These phenotypes are contrary to those of our *exoS192* ($\triangle$*exoS-hprK*) and *chvI261* mutant strains and suggest that if there is a relationship between ExoS/ChvI TCRS and PTS it involves a deactivation of ExoS/ChvI or is in the other direction with ExoS/ChvI controlling the transcription of PTS components or a combination of both. In this latter hypothesis, ExoS/ChvI would activate the transcription of PTS components, perhaps *manX/hpr* since *hprK* is most likely co-transcribed with *exoS*, and this could lead ultimately to dephosphorylation of ExoS and ChvI.

The close metabolic relationship between PTS and PckA and their genetic proximity to the ExoS/ChvI TCRS suggest that perhaps the transcriptional regulation of PTS components and *pckA* are tightly linked with ExoS/ChvI. It has been shown that *A. tumefaciens pckA* acid-induced transcription is ChvG/I dependent (Liu *et al.*, 2005). We are investigating if this is true for *S. meliloti*. While our *exoS* and *chvI* mutants grow better on minimal media with succinate than with glucose, it is possible that the *ppdK* gene (SMc00025), encoding pyruvate orthophosphate dikinase (Østerås *et al.*, 1997), is overexpressed under these conditions and compensates for a lower *pckA* expression. Analysis of *pckA* transcrip-

tion in *exoS* and *chvI* mutant strains grown under different conditions, for example low pH, is required to verify if ExoS/ChvI TCRS could be an activator or a repressor of *pckA* or PTS components. This future work should give us a better understanding of possible relationships between, PTS, PckA and ExoS/ChvI.

There is an increasing number of studies reporting an inverse relationship between the presence of EPS and motility of *S. meliloti* and *A. tumefaciens* cells. In *A. tumefaciens*, a lack of phosphatidylcholine (PC) in membranes causes a reduction in motility and an increased biofilm formation (Klüsener *et al.*, 2009). Mutation of *S. meliloti cbrA*, another regulatory histidine protein kinase, causes a decrease in motility and an increase in expression of *exo* genes (Gibson *et al.*, 2007). A downward shift in extracellular pH also causes a down-regulation of flagellar and motility genes, along with up-regulation of *exo* genes, in both *S. meliloti* Rm1021 and *A. tumefaciens* (Hellweg *et al.*, 2009; Yuan *et al.*, 2008). Very similar changes in *S. meliloti* gene expression are also observed in response to osmotic upshift (Domínguez-Ferreras *et al.*, 2006), iron limitation (Chao *et al.*, 2005), or when cells are grown in minimal versus complex media (Barnett *et al.*, 2004). These gene expression patterns are similarly associated with *exoR* and constitutively-activated *exoS* mutations (Cheng and Walker, 1998). As more high-throughput analyses are published, the evidence mounts for a general stress response that includes an increase in *exo* gene expression and a decrease in expression of genes required for flagella and motility. Our results suggest that the ExoS/ChvI system is central to this response, and in fact is required to cope with some of these situations. Genetic and phenotypic analyses lead us to hypothesize that ExoS/ChvI does not solely regulate EPS I, but also substantially affects carbon metabolism and cell envelope functions. The considerable pleiotropy that we observed suggests that ExoS/ChvI may have a wide regulon, and thus finding direct targets for ChvI becomes an essential task in the quest to understand the fundamental

roles of APHI regulons in symbiosis and pathogenicity.

Chapter 5

Genes directly regulated by the ChvI response regulator

5.1 Introduction

Efficient genetic tools, such as transduction, conjugation and transposon mutagenesis, were developed early in the study of the symbiotic model organism *S. meliloti* (Finan *et al.*, 1984, 1986; Meade *et al.*, 1982). These tools have been proven very successful in, for example, identifying genes required for symbiosis or determining gene location (Charles *et al.*, 1991; Charles and Finan, 1990). They can also be very useful in determining biochemical and regulatory linkages between pathways and in helping uncover new gene functions (Oresnik *et al.*, 1994). The previous chapter has shown that using genetic tools and phenotypic analyses to understand the role of regulatory proteins can be informative but it also has its limitations. In this chapter we present our combination of the use of gel electrophoretic

mobility shift assay (EMSA) and the use of our mutant strains to better understand ChvI role in gene regulation.

EMSA was developed based on the observation that a DNA fragment has its mobility in a gel retarded when it complexes with a DNA-binding protein (Garner and Revzin, 1981). By comparing the gel electrophoretic mobility of a DNA fragment in the presence or absence of a protein, one can determine if a DNA fragment interacts with the protein of interest. Following the development of the assay, a large number of methods have been elaborated or adapted for different purposes (Hellman and Fried, 2007). The most common methods use a labeled-PCR amplified fragment and a purified protein or a crude cell extract. These methods are designed to determine whether a particular DNA fragment contains a sequence of interest. Although these methods are of importance for the confirmation of hypotheses raised using non-direct techniques, they cannot be used in screens to identify protein-binding DNA fragments from a large set of fragments. Other methods have been elaborated for this purpose. For example, an EMSA using phage DNA followed by a second dimension gel electrophoresis at higher temperature was used to identify *E. coli* integration host factor binding sites in phage Lambda and Mu DNA (Boffini and Prentki, 1991). This method was also used with genomic DNA (Boffini and Prentki, 1993). Another similar method uses *E. coli* digested genomic DNA to perform EMSA followed by detection of complexes with the labeled protein (Subrahmanyam and Cronan, 1999). These two methods use EMSA to isolate DNA fragments with affinity for a protein and to identify these DNA fragments by PCR amplification using linkers followed by cloning and sequencing.

In this chapter, I present how I adapted and modified these latest methods to identify DNA fragments binding to ChvI. In this method I used digested genomic DNA, a sodium boric acid buffer and extended electrophoresis to separate bound from unbound fragments. Analyses of these fragments suggest functional relationships between some of them but

also hint at a diverse ChvI regulon. Reporter gene fusion libraries were used to confirm transcriptional influence of ChvI on three operons identified using the genomic DNA EMSA method. Results from these experiments suggest that ChvI has a dual role of activator and repressor. We present evidence that ChvI activates the expression of the SMb21189-SMb21190-*msbA2* operon. ChvI also represses transcription of SMc00262-SMc00261 genes and the *rhtXrhbABCDEF* operon. This chapter presents successful identification of members of the ChvI regulon and based on this new understanding we present a new model for the regulatory role of ExoS/ChvI.

5.2 Materials and Methods

5.2.1 Cloning of *chvI* for His•Tag-ChvI expression and purification

S. meliloti Rm1021 *chvI* was amplified using primers LB5 and LB6 (Table 2.3). The 800-bp PCR fragment was then gel-purified and cloned in pGEM®-T Easy vector. Plasmids pLB010 (*chvI* in T7 orientation) and pLB011 (*chvI* in SP6 orientation) were isolated and sequenced to confirm *chvI* sequence. NotI *chvI*-containing fragments were then cut out of pLB010 and pLB011 and ligated in NotI-digested pET-30a, pET-30b, and pET-30c. Orientation of inserts in pET-30 plasmids were determined by PCR. To be in the correct reading frame, a gene from pGEM®-T Easy vector and cut with NotI needs to be cloned in pET30a vector if it is in the T7 orientation in pGEM®-T Easy and in pET30b vector if it is in the SP6 orientation. One plasmid for each orientation in each pET-30 vector were then picked and transformed in *E. coli* BL21(DE3)pLysS to test for His•Tag-ChvI expression. *E. coli* clones were grown overnight using an autoinduction medium (Chapter

2) with kanamycin (Km) and chloramphenicol (Cm). Cells were harvested, mixed with 6X SDS-PAGE sample buffer, boiled for 10 minutes and loaded for electrophoresis on a 10% SDS-polyacrylamide gel (SDS-PAGE). Over-expression of the predicted 34-kDa His•Tag-ChvI was evident after staining of the gel with standard Coomassie blue staining. Western blot was also performed using a His•Tag monoclonal antibody from mouse (Novagen) and Alexa Fluor 488 goat anti-mouse IgG (H+L) (Invitrogen, Molecular Probes) as the secondary antibody. Imaging of Alexa Fluor 488 fluorescence on membrane was done with a Typhoon 9400. Plasmid pJF011 was confirmed to overproduce the His•Tag-ChvI protein and the bacterial strain was then sent to the laboratory of Professor Bi-Cheng Wang at University of Georgia (USA) where the His•Tag-ChvI purification using nickel-affinity chromatography was performed.

5.2.2 Electrophoretic mobility shift assay using genomic DNA (GD.EMSA) or plasmid DNA (PD.EMSA)

A modified method was used to perform an electrophoretic motility shift assay using Bsp143I-digested genomic DNA.

Sample preparation for GD.EMSA or PD.EMSA

S. meliloti Rm1021 genomic DNA or pTC198 plasmid DNA was digested overnight with Bsp143I restriction enzyme (Sau3AI isoschizomer, Fermentas Life Sciences) and the reaction was then heat-inactivated. Purified His•Tag-ChvI protein was mixed with digested DNA in a solution of 9% glycerol, 3 mM acetyl phosphate, 0.8 mM Tris-acetate, 0.25 mM

magnesium acetate, 1.65 mM potassium acetate, 2.5 μg/ml BSA. Reactions were incubated for 30 minutes at room temperature and loaded directly on gel without dye.

Preparation, running and staining of nondenaturing polyacrylamide gel

A sodium boric acid buffer (SB buffer, Appendix A.2) was made following recommendations from Brody and Kern (2004) and used in place of TBE buffer to perform electrophoresis. 5% nondenaturing polyacrylamide gels (14 cm x 16 cm) were cast following the standard procedure for resolution of small DNA fragments (Ausubel *et al.*, 1992) but using SB buffer instead of TBE buffer. Gels were run in 1X SB buffer between 25 to 40 mAmps for 3-6 hours. Gels were then stained for 1 hour in a 3X GelRed^{TM} staining solution containing 0.1 M NaCl and following manufacturer's recommendation for post gel staining (Biotium, USA, CA) prior to visualization on a UV transilluminator. Shifted DNA bands in the highest part of the gel were then excised and stored in 2-ml plastic tubes at -20°C.

Fragment recovery, cloning and sequencing

The method from Ausubel *et al.* (1992) was used to recover DNA fragments from polyacrylamide gel. The elution buffer used contained 0.5 M ammonium acetate, 1 mM EDTA, 0.1% SDS and final pH 8. 200 μl elution buffer was added to each tube containing a piece of gel. The gel was then crushed in smaller pieces using a pipet tip. Tubes were incubated overnight at 37°C with shaking. Gel pieces were then pelleted in a microcentrifuge at room temperature for 10 minutes at 10,000 rpm. Supernatant was removed and transfered to a clean 2.0 ml tube. 500 μl ethanol was added to precipitate the DNA and tubes were placed at -20°C overnight. DNA was pelleted at 13,000 rpm for 10 minutes. Supernatant was

removed and DNA solubilized in 100 μl 10 mM Tris pH 8 and 15 μl 5 M sodium chloride was added. DNA was then precipitated a second time with 2 volumes of ethanol and kept at -20°C overnight. DNA was pelleted at 13,000 rpm for 15 minutes, supernatant was removed and DNA was dried. Final resuspension of DNA was done with 10 μl 10 mM Tris pH 8.

After recovery from gel, DNA fragments were cloned in pUC18 at the BamHI restriction site. Prior to ligation, BamHI-digested pUC18 was dephosphorylated using shrimp alkaline phosphatase (Fermentas Inc.) and the reaction stopped by heat-inactivation. Ligation was done overnight at room temperature with T4 DNA ligase (Fermentas Inc.). Transformation of calcium chloride competent *E. coli* DH5α cells was done following standard procedure (Sambrook and Russell, 2001). Over 40 transformant colonies were streaked from each of the genomic and pTC198 DNA experiments. A number of them were then used for plasmid preparation and tested for the presence of an insert using restriction digest with EcoRI and PstI. Fragments cloned in pUC18 were sequenced using primers M13F provided by the sequencing facility (University of Waterloo) or LB61 (Table 2.3).

Sequences were first analyzed by searching for Sau3AI (Bsp143I) restriction sites to determine the limits of each fragment. Each fragment sequence was then searched against *S. meliloti* Rm1021 genomic sequence using Blast tool from Toulouse annotation website (http://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi). Fragments identified in each plasmid are listed in Appendix B.1.

Genes in closest proximity to identified sequences and potentially regulated by ChvI were searched against STRING 8.1 databases (June 28, 2009) for functional relations (Jensen *et al.*, 2009). The search was directed from the Toulouse annotation website previously mentioned.

5.2.3 Reporter gene fusion strains

Transcriptional fusion strains were obtained by transduction from the reporter gene fusion library made by Cowie *et al.* (2006). Table A.1 in Appendix A lists the 23 strains used and their derivatives. All 23 SmFL strains were used to prepare transduction lysates to transfer the gene fusions from the original *S. meliloti* RmP110 background into the Rm1021 background. Selection of transductants was done on LB Gm60. The same lysates were also used to transduce gene fusions into SmUW37 (pKD001) and SmUW38 (pKD001) with selection on LB Gm60 Nm200. Four transductants per transduction were picked and streaked on LB Gm60 Nm200. Transductants were then cured of pKD001 by streaking them on MM9-succinate Gm20 and 2.5% sucrose and incubated at 30°C for four days. pDK001-cured strains were finally streaked on MM9-succinate Gm20.

We found that high yield of transductants required the use of Bacto^{TM}-Agar, Bacto^{TM}-Tryptone, and Bacto^{TM}-Yeast extract (BD). The transduction method was modified to use lower solution volume and 2-ml plastic tubes. Lysates were diluted in 1.2 ml of TY. 0.5 ml of diluted lysate was mixed with 0.5 ml of cell suspension and incubated at room temperature for 30 minutes. Cells were then spun down at 5,000 rpm for 10 minutes and washed twice with 2 ml of saline. Final resuspension was done with 400 μl saline and then spread on two agar plates. Plates were incubated at 30°C for four days.

5.2.4 β-Glucuronidase Assay

To measure transcription from reporter gene fusion strains, the β-glucuronidase assay described in Cowie *et al.* (2006) was adapted. Strains were grown in MM9-succinate plus 0.1% proline, 0.1% uracil, and Gm until log phase (OD between 0.2 and 0.8). These cells

were then used directly for the assay in microplates as described in Cowie *et al.* (2006). More details about the protocol can be found in Appendix A.4.

5.3 Results

To better understand the role ChvI plays as a response regulator, it is necessary to identify genes that have their transcription directly influenced by the presence of ChvI. To identify specific DNA sequences from the genomic DNA for which ChvI might have binding affinity, we developed a new method using the electrophoretic mobility shift principle. This method uses a purified DNA-binding protein incubated with Bsp143I-digested genomic DNA and then DNA samples with or without protein are run on a native polyacrylamide gel using SB buffer. The use of SB buffer and a long gel (14 cm) as well as running the gel for a long period of time, 3-6 hours, allowed unbound DNA fragments to migrate far from the top of the gel while bound fragments remained higher in the gel at about 1-2 cm from top. These higher bands were then excised from the gel and purified. The DNA fragments were then cloned, sequenced and analyzed. Cloned fragments could also be used to make transcriptional fusions to study the activator or repressor role of the DNA-binding protein. Presented in this section are important results obtained while developing this method and confirmation of the ChvI regulatory role by the use of transcriptional gene fusion assays.

5.3.1 EMSA using plasmid DNA (PD.EMSA)

As a test control, the Bsp143I-digested pTC198 plasmid DNA was used to perform an EMSA. This pUC19 plasmid contains a 5-kb KpnI-fragment from *S. meliloti* Rm1021

spanning across the entire *chvI-hprK* genomic sequence including the intergenic sequence between *pckA* and *chvI* (Figure 4.1). This plasmid allowed testing of the method with a lower number of fragments than with genomic DNA, thus providing a better resolution on the gel but also increasing the chances of binding to areas surrounding *chvI* and *exoS* to test for possible autoregulation of ExoS/ChvI system. Regulation of the adjacent gene *pckA* by the APHI system has been shown for *A. tumefaciens* although not by direct binding but using fusion assays and this experiment was also aimed at testing if *S. meliloti* ChvI could bind the upstream sequence of *pckA*.

When comparing gel images of the same experiment run on different gel sizes (6 cm vs 14 cm) (Figure 5.1), it became apparent that this assay requires the use of the larger 14-cm gel to allow the excision from gel of distinctly shifted bands. Although it is easier to visualize the individual shifted bands on the smaller gel, the separation between shifted and non-shifted bands is too small to effectively excise them. 14-cm gels were then used in all experiments that required the excision of bands for further analyses.

Following the excision of bands, DNA fragments were cloned and sequenced. Out of nine inserts sequenced, three represent a 176 bp fragment coding for the region upstream SMc02753, including its start codon (Tables 5.1 and B.1). Another plasmid contained a region spanning the upstream sequence of *chvI* and past the translational start site. The five other plasmids contained different fragments from pUC19. These results suggest that ChvI might autoregulate its transcription but most importantly, it shows a direct binding affinity between the APHI system and the upstream sequence of *manXhpr* operon part of the PTS system.

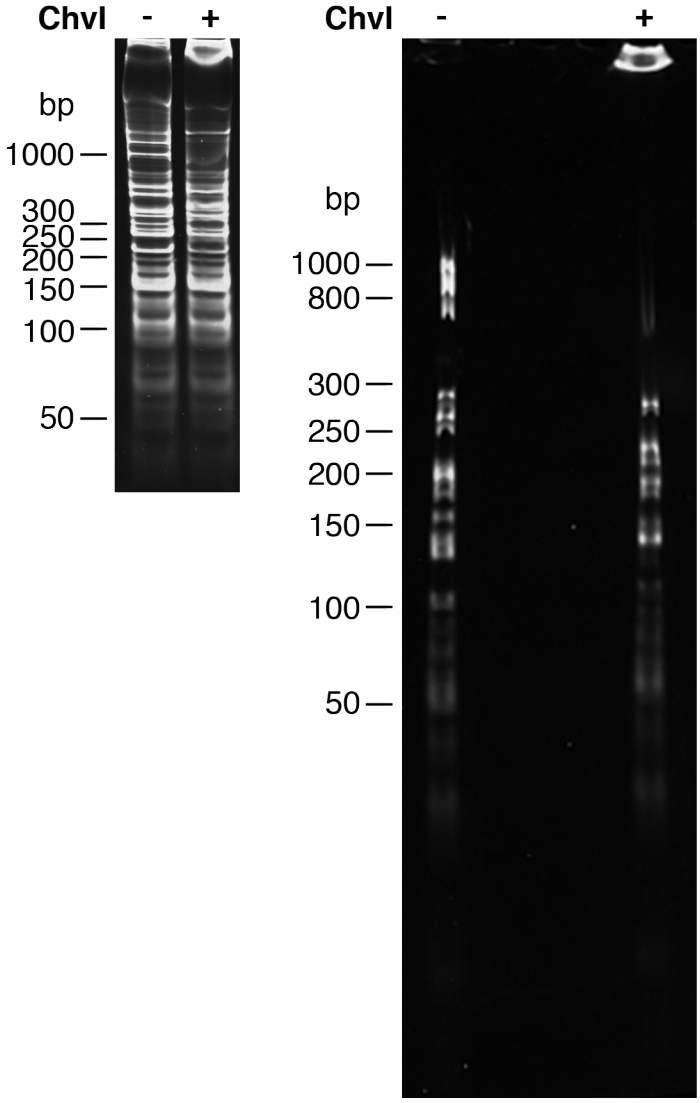


Figure 5.1: PD.EMSA to compare DNA shifts on 6-cm versus 14-cm 5% nondenaturing polyacrylamide gel and using SB buffer. Prior to the electrophoresis, the Bsp143I restricted pTC198 plasmid was incubated or not with the His•Tag-ChvI protein.

Table 5.1: *S. meliloti* DNA fragments recovered from PD.EMSA

Position		**Size**	**From**	**Gene**	**Gene**	**Gene**
Begin	End	(bp)	Up/In		**forward**	**reverse**
51887	52281	395	Up/In	SMc02560 (*chvI*)		SMc02562 (*pckA*)
48523	48699	176	Up/In	SMc02753	SMc02754	

5.3.2 EDTA inhibits His•Tag-ChvI binding to DNA and confirms the importance of magnesium.

UniProt annotation of ChvI sequence (P50350) suggests potential magnesium binding sites, a feature that might be essential for protein activity and thus is important to consider. To test if His•Tag-ChvI binding to DNA required Mg^{2+}, a 5% nondenaturing polyacrylamide gel was made using Tris-borate (TB) buffer. This buffer was made using TBE recipe but omitting EDTA as the presence of this strong chelator would be inappropriate to test for the requirement of Mg^{2+}. The assay confirmed that His•Tag-ChvI binds the DNA fragment from digested pLB102 (Figure 5.2). This assay also presented evidence that ChvI-DNA binding depends on the presence of EDTA. Adding the chelator in the reaction mix lowered the amount of DNA fragments shifted (Figure 5.2) and suggests that Mg^{2+} availability in the reaction is important.

Response regulators have been shown to autophosphorylate in the presence of acetylphosphate and ChvI is no exception (Cheng and Walker, 1998). To determine if acetylphosphate, and by extrapolation the phosphorylated state of ChvI, is important for its binding to DNA, the assay was performed in presence and absence of acetylphosphate. Figure 5.2 shows that no changes in the DNA shifted are observed due to the absence of acetylphos-

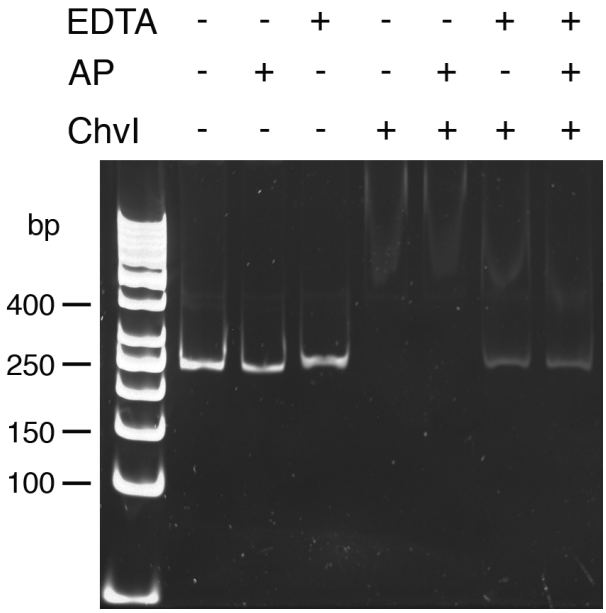


Figure 5.2: PD.EMSA to compare specifity in presence of EDTA or acetylphosphate. A 5% nondenaturing polyacrylamide gel made with TB buffer was used for the electrophoresis of the EcoRI-PstI double restricted pLB102 plasmid. The plasmid DNA was incubated or not with His•Tag-ChvI protein in presence or not of EDTA and in presence or not of acetylphosphate (AP) prior to the electrophoresis.

phate. These results suggest that the His•Tag-ChvI binding to DNA does not require the phosphorylated form of the protein. However, it does not rule out the possibility that the phosphorylation could be necessary for the activity affecting transcription.

5.3.3 EMSA using genomic DNA (GD.EMSA)

The use of plasmid DNA demonstrated that an electrophoretic mobility shift assay can be successfully performed to isolate shifted DNA from a large set of DNA fragments. The

next challenge was to use the developed method to try to isolate DNA shifted-fragments from restricted *S. meliloti* genomic DNA. In this latter situation, the use of 14-cm gel was also successful in giving a large separation between shifted and non-shifted fragments. This allowed an easy excision of lower mobility bands containing a large number of various sizes of shifted-DNA fragments. Table 5.2 presents a subset of fragments from excised bands that were purified, cloned and sequenced. The use of genomic DNA resulted in the identification of a large number of potential targets for ChvI regulation. Surprisingly, the majority of fragments identified are fragments found in coding sequences and not intergenic sequences. Moreover, ChvI-binding fragments are widely distributed across the genome and are not confined to a particular metabolic pathway. Although no one fragment was isolated more than once, two non-contigous fragments that are part of the same gene (*rhtX*) were independently cloned and sequenced.

Genes potentially regulated by ChvI are of diverse functions (Table 5.3). Because DNA fragments binding ChvI are often found within a coding sequence and not in intergenic areas it is hard to predict if ChvI acts as an activator of the following gene or a repressor of the gene it binds to. In many cases, such as the rhizobactin gene cluster and the *msbA2* gene cluster, the ChvI-binding fragment is found in the first gene of what is predicted to be an operon. Table 5.3 lists genes found closest to a ChvI-binding DNA fragment but it is possible in many instances that genes further downstream could be part of the same transcript and also be ChvI-regulated. It is also important to note that this is a subset of cloned-fragments and perhaps other ChvI targets exist.

Table 5.2: Fragments recovered from GD.EMSA. All these genes have been found in close proximity to a potential ChvI binding site. "Up" means that the identified fragment is found upstream from the following gene. "In" means that the identified fragment is found in the following gene.

Position		**Size**	**From gene**	**Gene**		**Gene**		**Gene**	
Begin	End	bp	Up/In			**forward**		**reverse**	
Chromosome									
1085493	1086087	595	In	SMc00051	*phaA2*	SMc00052	*phaB2*	SMc00117	*msrB1*
1830765	1831000	236	In	SMc00262		SMc00261		-	
1220301	1220439	139	In	SMc00550		SMc00551	*psd*	-	
1260626	1260767	142	In	SMc00589		-		SMc00588	*gal*
1639710	1639854	145	In	SMc02076	*cls*	SMc02074		SMc02077	*xthA2*
654156	654459	304	In	SMc02281		SMc02282		-	
3303566	3303853	288	In	SMc02491		-		SMc02489	*xerC*
60821	60949	129	In	SMc02574	*hisB*	SMc02573		SMc02575	*hslV*
1183131	1183282	152	In	SMc02637		SMc02635	*recO*	SMc02639	
2587012	2587177	166	In	SMc02733		SMc02732		-	
3117150	3117277	128	In	SMc03159	*metN*	SMc03158	*metI*	-	
3383057	3383240	184	Up	SMc03267		SMc03268		-	

Continued on next page

Position		**Size**	**From gene**	**Gene**		**Gene**		**Gene**	
Begin	End	bp	Up/In			**forward**		**reverse**	
3412878	3413020	143	Up	SMc03297		-		-	
2991422	2991677	256	In	SMc03993		SMc03992		-	
pSymB									
43628	43775	148	Up	SMb20032		SMb20031		SMb20034	
132970	133119	150	Up	SMb20119		SMb20118		-	
221089	221185	97	In	SMb20213		SMb20214		SMb20211	
492546	492612	67	In	SMb20478		SMb20479		-	
1588216	1588436	221	In	SMb20574		-		SMb20573	
1634582	1634726	145	In	SMb20615	*thiC*	SMb20616	*thiO*	SMb20614	
938297	938439	143	In	SMb21188		SMb21189		-	
1091213	1091353	141	Up	SMb21552	*aacC4*	SMb21554		-	
1266	1449	184	Up & In	SMb21653	*lacF*	SMb21654	*lacG*	-	
pSymA									
1186198	1186417	220	In	SMa2103		-		SMa2105	
1278398	1278609	212	In	SMa2295		SMa2297		-	
1305222	1305315	94	In	SMa2337	*rhtX*	SMa2400	*rhbA*	SMa2333	*kdpA*
1305714	1305861	148	In	SMa2337	*rhtX*	SMa2400	*rhbA*	SMa2333	*kdpA*

Table 5.3: Functions of genes potentially regulated by ChvI. All these genes have been found in close proximity to a potential ChvI binding site. "Up" means that a fragment from upstream of this gene has been found binding ChvI. "In" means that a fragment inside this gene has been found binding ChvI.

Closest ORFs	**Up or In**	**Function**
SMc00051 (*phaA2*)	In	Probable Na^+/H^+-antiporter
R SMc00117 (*msrB1*)	Up	Probable peptide methionine sulfoxide reductase
SMc00262	In	Putative 3-ketoacyl-CoA thiolase
F SMc00261	Up	Putative fatty-acid-CoA ligase
SMc00550	In	Probable ABC transporter ATP-binding transmembrane protein
F SMc00551 (*psd*)	Up	Probable phosphatidylserine decarboxylase
SMc00589	In	Conserved hypothetical protein
R SMc00588 (*gal*)	Up	Putative D-galactose 1-dehydrogenase
SMc02076 (*cls*)	In	Putative cardiolipin synthetase transmembrane protein
SMc02281	In	Conserved hypothetical protein
F SMc02282	Up	Putative copper-containing oxidoreductase
SMc02491	In	Hypothetical protein
SMc02560 (*chvI*)	Up & In	Transcriptional regulatory protein
SMc02574 (*hisB*)	In	Probable imidazoleglycerol-phosphate dehydratase (histidine biosynthesis)

Continued on next page

Closest ORFs	Up or In	Function
SMc02637	In	Hypothetical protein
SMc02733	In	Hypothetical protein
SMc02753	Up	Putative IIA component of PTS system
SMc03159 (*metN*)	In	Methionine import ATP-binding protein
SMc03267	Up	Putative dipeptidase
F SMc03268	Up	Peptide transport ATP-binding protein
SMc03297	Up	Hypothetical/unknown protein
SMc03993	In	Hypothetical transmembrane protein
SMb20032	Up	Hypothetical protein
SMb20119	Up	Putative site-specific recombinase
SMb20213	In	Conserved hypothetical protein
F SMb20214	Up	Probable oxidoreductase
SMb20478	In	Putative dipeptide ABC transporter permease and ATP-binding protein
SMb20574	In	(1→4)-alpha-D-glucan 1-alpha-D-glucosylmutase
SMb20615 (*thiC*)	In	Putative thiamine biosynthesis protein
SMb21188	In	Putative acyltransferase
F SMb21189	Up	Putative glycosyltransferase
SMb21653 (*lacF*)	Up & In	Lactose ABC transporter, permease component
SMb21552 (*aacC4*)	Up	Putative aminoglycoside 6'-N-acetyltransferase

Continued on next page

Closest ORFs	**Up or In**	**Function**
SMa2103	In	Oxidoreductase
SMa2295	In	Penicillin-binding protein
SMa2337 (*rhtX*)	In	RhtX rhizobactin transporter

5.3.4 Functional interactions between probable ChvI-regulated genes.

Using the list of potentially ChvI-regulated genes obtained from GD.EMSA, I searched databases for functional relationships between targets: MetaCyc (Caspi *et al.*, 2007), KEGG (Kanehisa *et al.*, 2007) and STRING 8.1 (Jensen *et al.*, 2009). A number of links can be made between some potential ChvI targets.

Two fragments are linked to lactose catabolism. One is found in front of the *lacFGZ1K* gene cluster and the second one is found in SMc00589, about 300 bp upstream of *gal*. The *lacFGZ1K* gene cluster encodes genes for lactose ABC-transporter and a β-galactosidase (E.C. 3.2.1.23). β-D-galactose is degraded through the De Ley-Doudoroff pathway in *S. meliloti* (Arias and Cerveñansky, 1986) and *gal* codes for the galactose dehydrogenase (EC 1.1.1.48). STRING 8.1 also found the link between *lacZ1* and *gal* (Figure 5.3).

Two other fragments suggest that ChvI is involved in regulating phospholipid biosynthesis. One fragment is found in SMc02076 (*cls*) and another one is found in SMc00550, about 300 bp upstream of *psd* and followed by *pssA*. Cardiolipin is produced in *S. meliloti* and the only gene coding for a cardiolipin synthetase is *cls* (López-Lara *et al.*, 2003). Proteins encoded by *psd* and *pssA* are responsible for the biosynthesis of phosphatidyl-ethanolamine and phosphatidylserine respectively, both of these phospholipids are also intermediates for phosphatidylcholine biosynthesis (Vences-Guzmán *et al.*, 2008). Aside from phospholipids synthesis, another link was found between SMc00550 and *msbA2* using STRING (Figure 5.4). These two genes are homologs and might have similar functions.

The involvement of ChvI in lipid metabolism is also suggested by the fragment found in SMc00262, a putative 3-ketoacyl-CoA thiolase, followed by SMc00261, a putative fatty-

acid-CoA ligase. These genes are putatively involved in fatty acid β-oxidation.

A fragment found in *thiC* and one found in *hisB* do not present a directly evident link between the two pathways they are respectively involved in but there is an indirect metabolic link that can be followed in MetaCyc or KEGG and in STRING. ThiC catalyzes the reaction between 5-aminoimidazole ribonucleotide (AIR) and hydroxymethylpyrimidine phosphate in the thiamine biosynthesis pathway (Figure 5.5). AIR is biosynthesized from 5-phosphoribosyl 1-pyrophosphate (PRPP). PRPP is also required for the synthesis of histidine. In STRING this link is made through *pur* genes (Figure 5.6 and 5.7), which code for enzymes involved in purine synthesis. Pyrimidine, purine and pyridine nucleotide synthesis are all dependent on the availability of PRPP.

ChvI was also found to bind fragments from genes involved in peptide and methionine transport. A fragment belongs to the upstream sequence of a putative dipeptidase (SMc03267) followed by a dipeptide ABC-transporter. Another fragment is from SMb20478, part of a gene cluster coding for another dipeptide ABC-transporter. MetN involved in importing methionine also has a fragment of its gene having affinity for ChvI.

Following these analyses, I could not find a direct link between these potentially ChvI-regulated genes and the exopolysaccharide biosynthesis pathway, one of the most important phenotypes of the *chvI* mutant strain. However, an indirect link is suggested from the regulation of thiamine and histidine biosynthesis. These pathways are inter-related with the synthesis of pyrimidine and consequently the availability of UTP required for the synthesis of UDP-glucose. Perhaps the imbalance caused by deregulating thiamine and histidine synthesis affects UDP-glucose synthesis and therefore polysaccharide production. To test this hypothesis, I added 0.1% uracil to the MM9-succinate minimal media and was able to improve significantly the growth of the *chvI* mutant strain, although still not to a

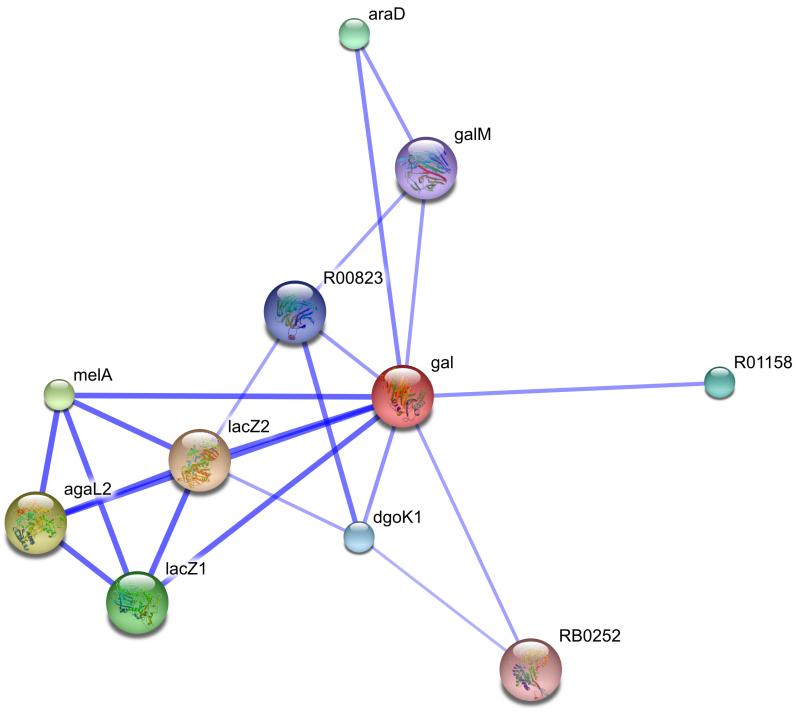


Figure 5.3: Functional interaction between *gal* and *lacZ1*. Link colour intensity represents the confidence level from STRING 8.1.

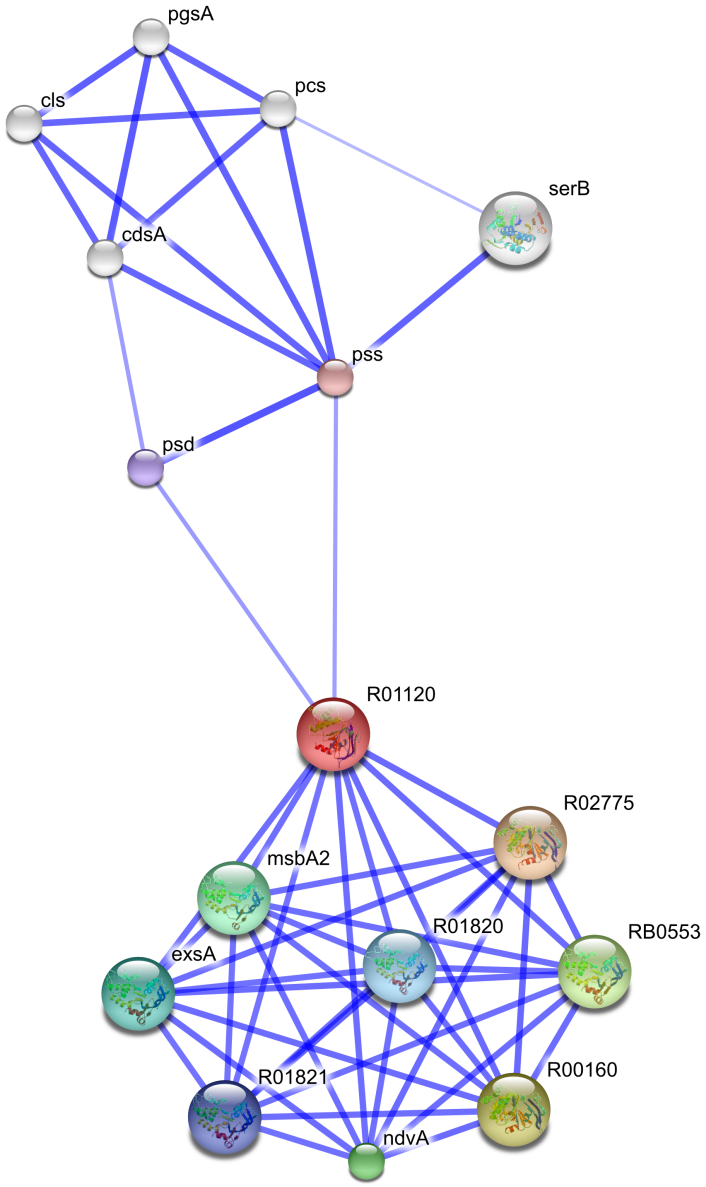
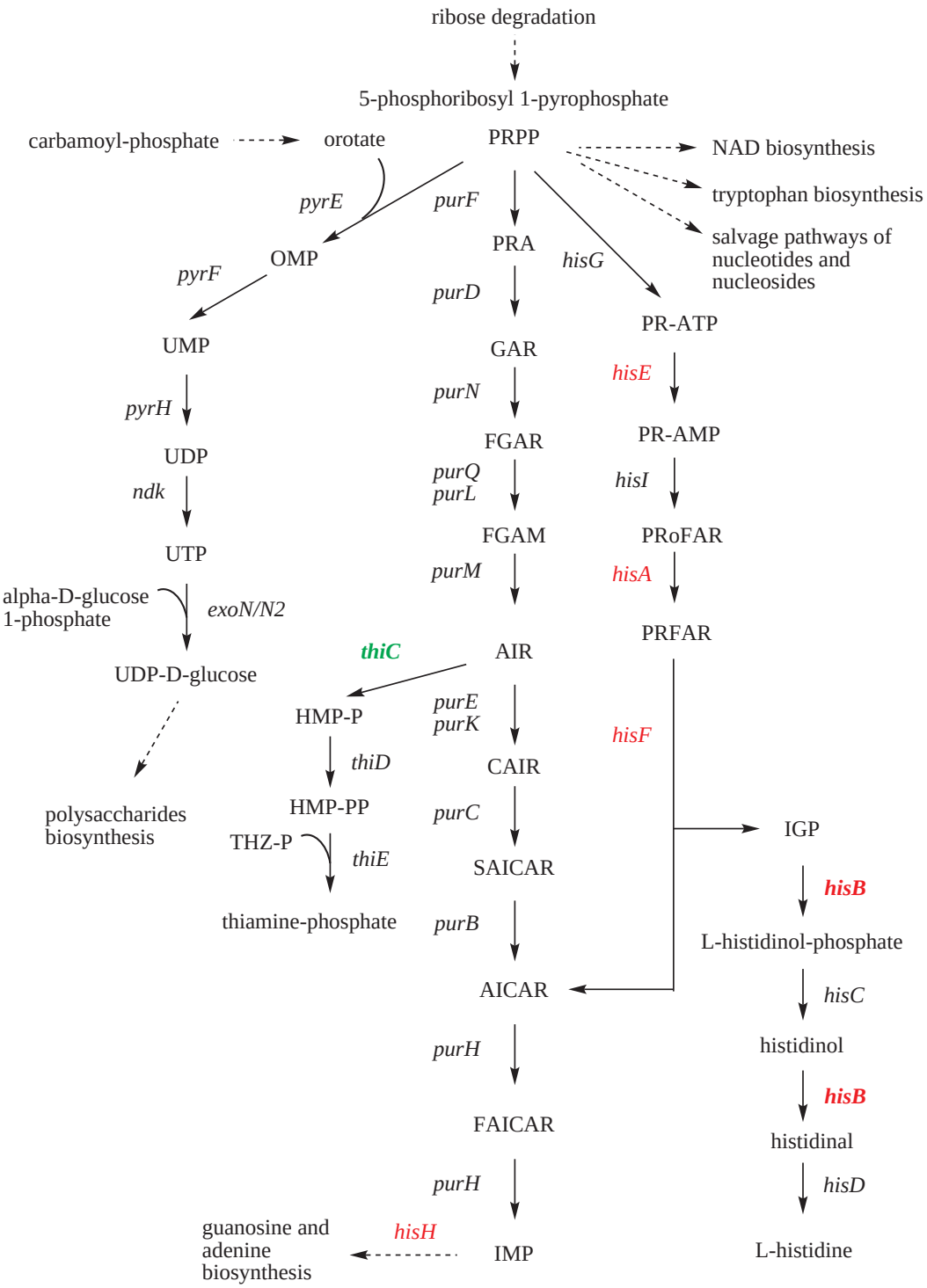


Figure 5.4: Functional interactions between SMc00550 (R01120), *msbA2*, *psd*, and *cls*. Link colour intensity represents the confidence level from STRING 8.1.

level comparable to wild-type (Table 5.4). Addition of uracil allows SmUW38 to grow in liquid media.

From carbon source utilization analyses performed in the previous chapter, we showed that proline or ornithine are good carbon sources for the *chvI* mutant strains, therefore 0.1% proline was added to M9-succinate media supplemented also with 0.1% uracil. This improved even more the growth of the mutant strain (Table 5.4). Addition of uracil improved growth but adding proline is even better. Interestingly, adding uracil to the Rm1021 strain reduces its growth while it improves SmUW38 growth.

Figure 5.5 *(following page)*: PRPP metabolic pathway. Genes in red and green are potentially regulated by ChvI. 5-phosphoribosyl 1-pyrophosphate (PRPP), orotidine-5'-phosphate (OMP), uridine-5'-phosphate (UMP), uridine-5'-diphosphate (UDP), uridine-5'-triphosphate (UTP), hydroxymethylpyrimidine phosphate (HMP-P), 4-amino-5-hydroxymethyl-2-methylpyrimidine-pyrophosphate (HMP-PP), 4-methyl-5-(β-hydroxyethyl)thiazole phosphate (THZ-P), 5-phospho-β-D-ribosyl-amine (PRA), 5-phospho-ribosyl-glycineamide (GAR), 5'-phosphoribosyl-N-formylglycineamide (FGAR), 5-phosphoribosyl-N-formylglycineamidine (FGAM), 5-aminoimidazole ribonucleotide (AIR), 4-carboxyaminoimidazole ribonucleotide (CAIR), 5'-phosphoribosyl-4-(N-succinocarboxamide)-5-aminoimidazole (SAICAR), aminoimidazole carboxamide ribonucleotide (AICAR), phosphoribosyl-formamido-carboxamide (FAICAR), inosine-5'-phosphate (IMP), phosphoribosyl-ATP (PR-ATP), phosphoribosyl-AMP (PR-AMP), phosphoribosylformiminoAICAR-P (PRoFAR), phosphoribulosylformimino-AICAR-P (PRFAR), D-erythro-imidazole-glycerol-phosphate (IGP)


ribose degradation
5-phosphoribosyl 1-pyrophosphate
PRPP
carbamoyl-phosphate
orotate
NAD biosynthesis
tryptophan biosynthesis
salvage pathways of nucleotides and nucleosides
pyrE
purF
OMP
PRA
hisG
pyrF
purD
PR-ATP
UMP
GAR
hisE
pyrH
purN
PR-AMP
UDP
FGAR
purQ
purL
hisI
ndk
FGAM
PRoFAR
UTP
purM
hisA
alpha-D-glucose 1-phosphate
exoN/N2
PRFAR
thiC
AIR
UDP-D-glucose
HMP-P
purE
purK
hisF
thiD
CAIR
polysaccharides biosynthesis
HMP-PP
purC
IGP
THZ-P
thiE
SAICAR
hisB
thiamine-phosphate
purB
L-histidinol-phosphate
AICAR
hisC
purH
histidinol
hisB
FAICAR
histidinal
purH
hisD
guanosine and adenine biosynthesis
hisH
IMP
L-histidine

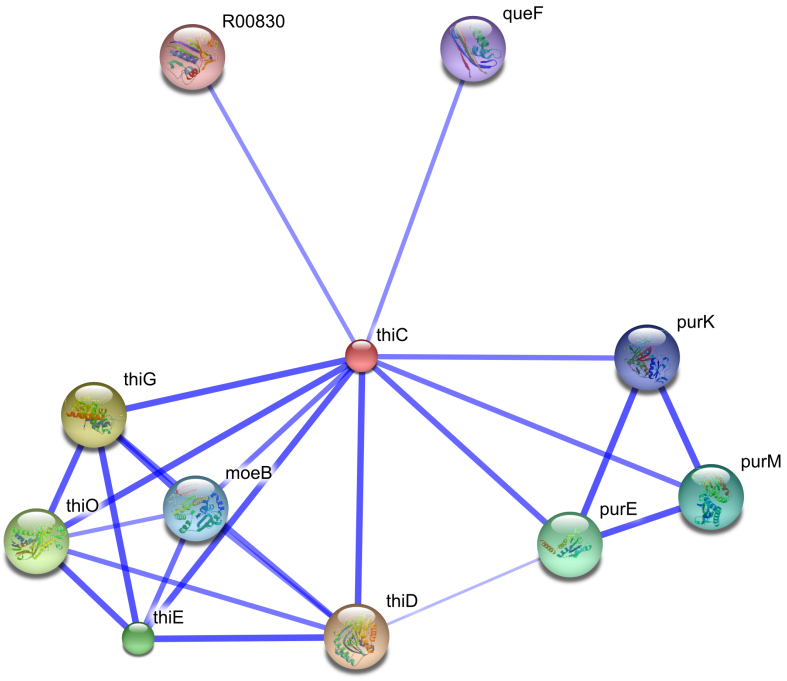


Figure 5.6: Functional interactions between *thi* genes and *pur* genes. Link colour intensity represents the confidence level from STRING 8.1.

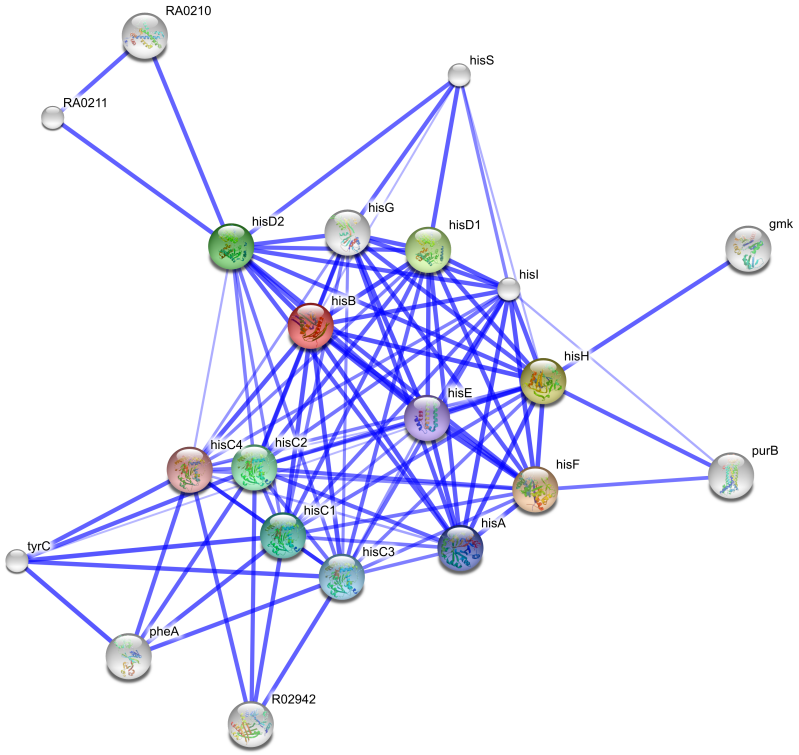


Figure 5.7: Functional interactions between *his* genes and *purB*. Link colour intensity represents the confidence level from STRING 8.1.

Table 5.4: Impact of adding 0.1% proline and uracil on growth rate constant of SmUW38 and Rm1021 growing in MM9-succinate liquid media. Growth rate constants were calculated for duplicates and the average is presented in the table followed by the standard deviation.

Addition	Rm1021 wild-type	SmUW38 *chvI261*
none	0.182 ± 0.004	0.043 ± 0.003
proline	0.201 ± 0.014	0.159 ± 0.025
uracil	0.167 ± 0.006	0.144 ± 0.004
proline & uracil	0.192 ± 0.003	0.161 ± 0.002

5.3.5 Transcriptional fusion to genes identified as ChvI-targeted.

Having identified genes that might be regulated by ChvI, the next step was to confirm the regulation by transcriptional gene fusion assays. To perform these assays, we used strains from a *S. meliloti* fusion library (Cowie *et al.*, 2006). The library is made using a vector that contains the reporter genes *lacZ* or *gusA* depending on the orientation of the insert. Because of the possible involvement of ChvI in regulating the *lac* operon, we selected fusion strains to measure transcriptional activity using *gusA* and the β-glucuronidase assay. Gene fusions were transduced into *chvI* mutant background strain and in the Rm1021 wild-type strain. Gene fusion strains in each background were assayed for β-glucuronidase activity and compared. These assays confirmed the regulation of three operons by ChvI.

A ChvI-binding DNA fragment was identified in SMb21188 and therefore we tested three gene fusions in the three following genes: SMb21189, SMb21190, and *msbA2* (Figure

5.8). These fusions had a higher expression level in wild-type than in *chvI* mutant background. In fact, the activities in *chvI* mutants were null for the the first two fusions and extremely low for the third one. These results suggest that ChvI is responsible for activation of the co-transcription of SMb21189, SMb21190, and *msbA2* genes. Using a neural network promoter prediction tool (http://www.fruitfly.org/seq_tools/promoter.html), I identified a putative transcriptional start site (P2) adjacent to the area containing a ChvI binding site (B). Another putative transcriptional start site (P1) further upstream from SMb21188 suggest that transcription might be directed from two differently regulated promoters and could include (or not) the SMb21188 gene.

The same assay and promoter prediction were done with fusions in genes SMc00262 and SMc00261 (Figure 5.9). In this case, a promoter was predicted right before the ChvI binding area in SMc00262 and accordingly the fusions further in SMc00262 and in SMc00261 presented higher expression levels in *chvI* mutant strains than in wild-type. These results suggest that ChvI acts by repressing the transcription of SMc00261 and also perhaps SMc00262.

S. meliloti produces an iron-siderophore, rhizobactin 1021, when in conditions of iron-depletion (Lynch *et al.*, 2001). Genes for the synthesis and transport of rhizobactin are found together in an operon. The rhizobactin transporter (*rhtX*) was found to contain two DNA fragments binding ChvI (Figure 5.10). Fortunately, we were able to test a fusion following the first binding site (B1) and two other fusions further in *rhbB* and in *rhbF*. The promoter prediction suggests the presence of a promoter before *rhtX* and another one before *rhbA*. The β-glucuronidase assays presented a significantly higher expression in *chvI* background for all three fusions. This suggests that ChvI represses the expression of genes required for the synthesis and transport of rhizobactin 1021. Both binding areas seem to be important in repressing the transcription as shown by a higher expression in the fusion

found before the second binding. Rhizobactin fusion mutants were spotted on calcofluor plates to test for a possible effect on succinoglycan production and no differences were found between wild-type and rhizobactin mutants. These results rule out the possibility that rhizobactin production causes a change in succinoglycan production.

More gene fusion strains are available and additional ones can be constructed to confirm the activator or repressor role of ChvI in operons identified in previous sections.

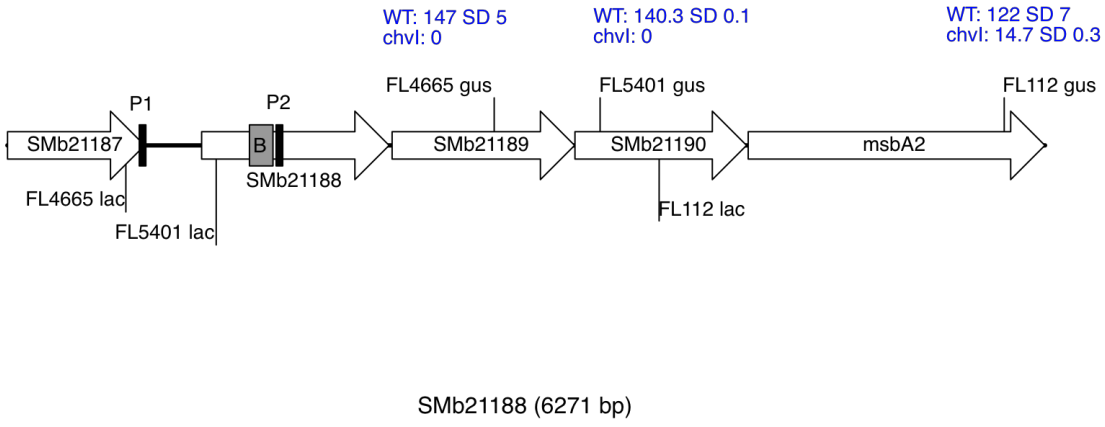


Figure 5.8: The *msbA2* operon. The grey box represents the sequence of the 143 bp fragment selected with GD.EMSA. Ps represent predicted promoters. FL# gus or FL# lac represent insertion of fusion and *gus* or *lac* indicate the orientation of the insertion. Numbers in blue are the β-glucuronidase activity (Miller Units) for wild-type or *chvI* mutant strains carrying the fusion indicated below. SD is the standard deviation.

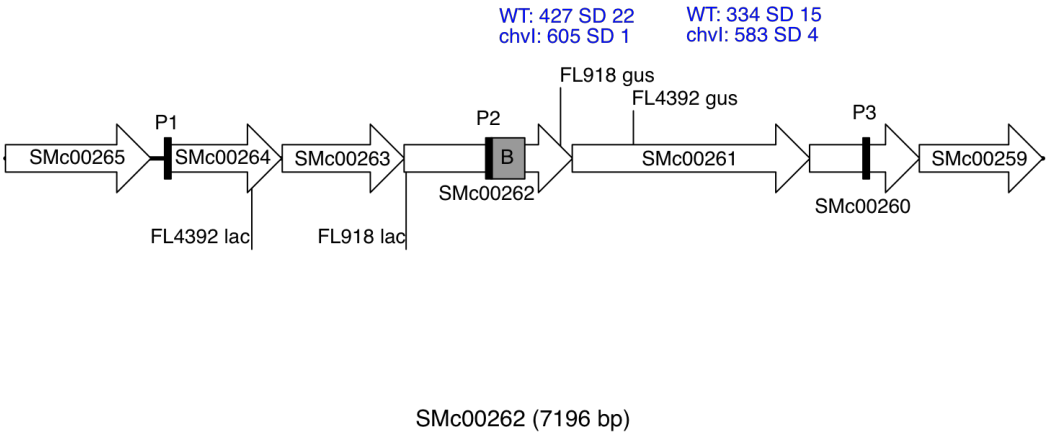


Figure 5.9: The SMc00261 operon. The grey box represents the sequence of the 236 bp fragment selected with GD.EMSA. Ps represent predicted promoters. FL# gus or FL# lac represent insertion of fusion and *gus* or *lac* indicate the orientation of the insertion. Numbers in blue are the β-glucuronidase activity (Miller Units) for wild-type or *chvI* mutant strains carrying the fusion indicated below. SD is the standard deviation.

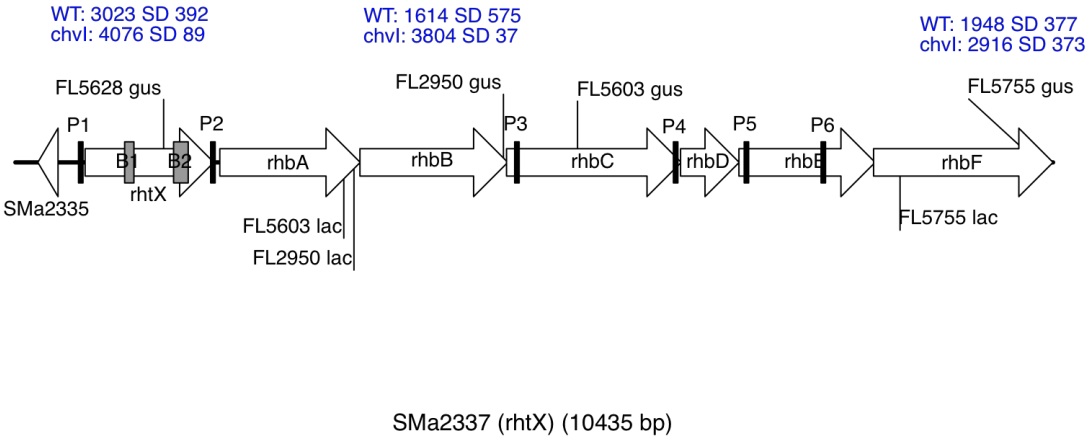


Figure 5.10: The rhizobactin 1021 operon. The grey boxes represent sequences of the 94 bp fragment (B1) and the 148 bp fragment (B2) selected with GD.EMSA. Ps represent predicted promoters. FL# gus or FL# lac represent insertion of fusion and *gus* or *lac* indicate the orientation of the insertion. Numbers in blue are the β-glucuronidase activity (Miller Units) for wild-type or *chvI* mutant strains carrying the fusion indicated below. SD is the standard deviation.

5.4 Discussion

S. meliloti ChvI, identified as a response regulator homolog of OmpR, has been shown to autophosphorylate in presence of acetylphosphate *in vitro* (Cheng and Walker, 1998; Wells *et al.*, 2007). ExoS, the histidine kinase partner, can also provide a phosphoryl group for the *in vitro* phosphorylation of ChvI. We report for the first time the *in vitro* DNA-binding activity of ChvI.

The addition of acetylphosphate to the binding reaction did not influence the shift in the mobility assay. These results suggest that the protein phosphorylation is not required for the protein to bind DNA but perhaps acts on changing the transcriptional activity through changing the protein-protein interactions as it has been demonstrated for other response regulators (Sinha *et al.*, 2008). *E. coli* PhoB is an example where phosphorylation by acetylphosphate changes the affinity of the protein for the DNA-binding sequence but it does not change the binding specificity (McCleary, 1996). The latest model suggests that PhoB phosphorylation enhances DNA-binding by promoting dimerization, which gives PhoB the ability to regulate transcription (Mack *et al.*, 2009). The presence of magnesium was necessary for ChvI DNA-binding activity and this is important because most EMSA are performed with EDTA in their buffer. EDTA is not required in gel electrophoresis buffers and we recommend to avoid its usage (Brody and Kern, 2004).

An adaptation of methods to perform gel electrophoresis mobility shift assays allowed us to identify DNA fragments with higher affinity for ChvI. Analyses of these results forces us to revise our perception from phenotypic analyses of ExoS/ChvI as mainly a regulatory system for exopolysaccharide production. Our results suggest that the ChvI regulon includes genes from diverse pathways. Moreover, ChvI appears to have a dual regulatory role, activating and repressing different operons.

While we have identified 29 DNA fragments with higher affinity for ChvI (Tables 5.1 and 5.2), this discussion is focused on four of them. These four fragments were tested and confirmed by gene fusion assay to be important for ChvI-dependent regulation. The other fragments have not been tested yet by gene fusion assays and are still considered to contain potential ChvI-binding sites.

ChvI binds in SMa2337 sequence to repress *rhtXrhbABCDEF* gene transcription (Figure 5.10). This operon is known to be upregulated by *rhrA* in iron-depleted conditions (Lynch *et al.*, 2001) and downregulated by *rirA* in iron-repleted conditions (Viguier *et al.*, 2005). Our fusion assay results confirmed that the rhizobactin operon is highly expressed in M9-minimal media (iron-depleted conditions). However, this expression is even higher in strains with mutated *chvI* gene. This could suggest that following the sensing of a signal other than the presence of iron, ExoS/ChvI represses genes for rhizobactin 1021 production. Iron is an important micronutrient found in many cofactors required for cytochrome and nitrogenase activity. Its acquisition however is difficult for two main reasons, its solubility at pH 7 is low and a high concentration of iron can cause the generation of hydroxy radicals. Bacteria produce siderophores to scavenge iron and therefore control iron availability. A tight control over the production of siderophore is thus important. Mutation of *rirA* derepresses rhizobactin production and as a result causes a growth defect of the strain relative to the presence of iron (Chao *et al.*, 2005). The reduced viability due to oxidative stress of the *rirA* mutant suggested that perhaps this strain would also be affected in its symbiotic properties but it was not the case (Chao *et al.*, 2005). This study suggested that *in planta* another unknown regulatory system might control the production of rhizobactin. From our findings, it is tempting to speculate that ExoS/ChvI might be this system.

Another important finding is that ChvI is important for activating the expression of SMb21189, SMb21190, and *msbA2*. These genes have only been described recently in

the literature although *msbA2* in particular seems to play an important but incompletely defined role in symbiosis (Griffitts *et al.*, 2008; Beck *et al.*, 2008). SMb21189 and SMb21190 encode glycosyltransferases and *msbA2* is part of an ABC-transporter family involved in macromolecule export. The above mentioned recent studies proposed that the operon including SMb21188, a putative acyltransferase, is involved in the production and export of an unknown polysaccharide which uses intermediates from the succinoglycan production pathway. The regulation of this operon by ExoS/ChvI is therefore the closest link to the succinoglycan phenotype of *exoS* and *chvI* mutant strains. Although this ChvI-regulated operon is not required for succinoglycan production it seems to be functionally related to succinoglycan production.

The third operon that we confirmed differentially regulated by ChvI encodes proteins putatively involved in fatty acid β-oxidation. SMc00262 putatively produces a 3-ketoacyl-CoA and SMc00261, a fatty-acid-CoA ligase. These genes are also followed by SMc00260 coding for a putative short-chain dehydrogenase and SMc00259 coding for a hypothetical protein. Upstream of these genes lie genes for a transcriptional regulator of the IclR family (SMc00263) and another short-chain dehydrogenase (SMc00264). A tripartite ATP-independent periplasmic (TRAP) transporter system upregulated by the presence of acetoacetate and 3-methyl oxovaleric acid is encoded by genes in the same orientation upstream of SMc00264. All these genes are organized in the same orientation and close enough to each other to be part of the same transcript. However, our finding of a ChvI binding site in SMc00262 after the gene encoding a IclR regulator suggest a complex regulation of these genes. In fact, a N-Acyl homoserine lactone (AHL) also impacts on them (Chen *et al.*, 2003). The fatty-acid-CoA ligase (SMc00261) has been found differentially accumulated in early log phase cultures of *S. meliloti* Rm1021 treated for 2h with 3-oxo-$C_{16:1}$-HL while the periplasmic binding protein (SMc00265) accumulated in stationary phase cultures in-

dependently of the presence of AHLs. Perhaps under conditions that activate ChvI, the first part of the gene cluster is upregulated to allow the import of an organic acid but the second part responsible for its degradation and entry in the TCA cycle is downregulated. This hypothesis would suggest the use of this organic acid, under certain conditions, as a readily available building block rather than an energy source.

An important finding from this work is that proline or uracil improved the growth of the *chvI* mutant strain. This finding now allows us to grow the mutant strain in liquid media, greatly facilitating experimental analysis. Binding of ChvI in *thiC* (SMb20615) and in *hisB* (SMc02574), perhaps to repress the thiamine and histidine biosynthesis operons, made us hypothesize that a derepression of these operons in *exoS* or *chvI* mutants could lead to a deficiency in UTP formation and could explain the pleiotropy of these mutants. Rhizobial purine and pyrimidine auxotrophic mutants have been found affected in polysaccharides synthesis and plant invasion (Noel *et al.*, 1988; Clover *et al.*, 1989; Newman *et al.*, 1994; Djordjevic *et al.*, 1988). Further work needs to be done to confirm that *chvI* mutant auxotrophy is truly caused by a derepression of operons for thiamine and histidine biosynthesis.

We showed that ChvI may act as a repressor or activator of gene expression, and surprisingly ChvI seems to often bind within coding sequences. This finding is intriguing because it has not often been reported, however the Fur regulator in *Helicobacter pylori* does have targets within polycistronic operons and does act as a repressor and an activator of gene expression (Danielli *et al.*, 2006). The tendency to search for transcriptional regulatory elements in intergenic areas rather than considering equally an ORF upstream region whether this area is intragenic or not may need to be revisited. The use of GD.EMSA or Chromatin-Immunoprecipitation (ChIP) techniques are examples of techniques that do not have a bias towards intergenic or intragenic areas and their usage might eventually

increase our knowledge about transcriptional regulators.

In an attempt to find a sequence logo specific for ChvI transcriptional regulator, I used bioinformatic tools to search for a motif within fragments identified during this study. The outcome was an overwhelming discrepancy between results obtained from different computer programs used and none of the logos found seemed to have a convincing specificity. An experimental approach in this case is probably more appropriate. While we paved the way to define the ChvI regulon, a number of potentially ChvI-regulated genes still need to be confirmed if they are activated or repressed. These findings combined with footprinting analyses should also help determine a specific ChvI binding-site.

Chapter 6

General Discussion

This thesis presents evidence that the ExoS/ChvI system is essential for effective symbiosis and therefore understanding the role this two-component regulatory system plays is important. We demonstrated that null mutations of *exoS* and *chvI* are pleiotropic and thus it is difficult to predict which genes are directly regulated by this transduction system based solely on phenotypic analyses. Orthologs of *exoS* and *chvI* genes in *Brucella* and *Agrobacterium* are also important for their respective interaction with their eukaryal hosts (Sola-Landa *et al.*, 1998; Charles and Nester, 1993). Although genes have been previously determined to be differentially regulated by the ExoS/ChvI orthologs it is unknown if the regulation is direct or not. Because mutation of *exoS* or *chvI* produces strains difficult to grow on a number of media, we decided to take a direct approach to search for DNA fragments having a higher affinity for ChvI and this proved to be successful. This thesis is the first report to identify genes directly regulated by ChvI. In turn, finding genes that are part of the ChvI regulon, directed us towards a better understanding of the biological function of ExoS/ChvI and helped us find better conditions to grow mutant strains.

This chapter is a discussion of how phenotypic analyses combined with the identification of genes that are directly regulated by ChvI are revealing a previously obscured side of the ExoS/ChvI system. I will present in this chapter how I came full circle in my analysis of results presented in previous chapters and how these results provide evidence to support a suggestion that ExoS/ChvI is central to bacterial homeostasis.

6.1 How does the ExoS/ChvI system regulate exopolysaccharide production?

It has been known for a long time that ExoS regulates succinoglycan production but how this happens is unknown (Doherty *et al.*, 1988). Our mutational analyses showed that aside from regulating succinoglycan, ExoS and ChvI are also important for galactoglucan production and perhaps other membrane components because the mutants are also more sensitive to detergents; detergent sensitivity is often correlated to cell envelope defects (Ferguson *et al.*, 2002). The lack of EPS production in ExoS and ChvI mutants might explain their inability to form symbiotically active nodules but it does not explain any of the other phenotypes. Slow growth of mutant strains or in many cases their inability to grow on many carbon sources suggests that the lack of EPS might be a consequence of a metabolic imbalance. A number of genes *a priori* unrelated to EPS production are known to affect EPS synthesis. For example mutation of *bluB*, a gene required for cobalamin biosynthesis, results in an increased fluorescence on calcofluor (Campbell *et al.*, 2006). It was suggested that the succinoglycan succinylation is affected by methylmalonyl-CoA mutase deficiency, a cobalamin-dependent enzyme catalyzing the formation of succinyl-CoA. Pyruvyl substitution is also important for succinoglycan production and mutating

exoV, the gene responsible for this modification of the polymer, creates a strain with slower growth (Glucksmann *et al.*, 1993). Perhaps limiting the availability of phosphoenolpyruvate could have a similar effect. Phenotypes associated with the mutation of *exoS* or *chvI* point towards a role for ExoS and ChvI other than in regulating *exo* genes or this system does regulate *exo* genes but in concert with a number of other metabolic pathways.

Phenotypic analyses provide a number of new hypotheses about the regulatory role of ExoS/ChvI but the inability to grow mutant strains in liquid media and their slow growth on agar-plates make it extremely difficult to perform experiments to confirm these hypotheses. Because of this burden, we took a different approach and developed a method to identify DNA fragments from genomic DNA that bind to ChvI and have their mobility in gel retarded. These results confirm that the ExoS/ChvI system has a regulatory role on genes related to succinoglycan biosynthesis but perhaps not directly on *exo* genes.

ChvI activates the transcription of the *msbA2* operon containing genes related to succinoglycan production. A mutation that inactivates *msbA2* alters the polysaccharide profile and blocks bacteria in the infection thread (Beck *et al.*, 2008). At this point more work has to be done to understand the role of the *msbA2* transporter and glycosyltransferases (family 2) upstream of it (Cantarel *et al.*, 2009). Are these genes involved in producing an unknown polysaccharide as suggested by Griffitts *et al.* (2008)? Alternatively, could these genes be important for succinoglycan production but in a manner that does not affect the fluorescence on LB-calcofluor, perhaps by altering the high to low molecular-weight ratio or by helping to increase the exopolysaccharide production at a critical point of invasion? A number of questions regarding the role of this operon and its link to succinoglycan production need to be resolved to assess the importance of its regulation by ExoS/ChvI.

Analysis of potentially ChvI-regulated genes has unveiled a new avenue to explain the

exopolysaccharide phenotypes, and this is worth exploring. Polysaccharides are built from nucleotide sugars and UDP-D-glucose is essential for succinoglycan production. Figure 5.5 presents the synthesis pathway for UDP-D-glucose. This diagram highlights the metabolic relationship between pyrimidines, purines, thiamine, and histidine. In Chapter 4, I discussed how carbon source utilization phenotypes suggested that UMP levels might be limiting. The finding that fragments from *thiC* and *hisB* were isolated in the GD.EMSA experiment supports this hypothesis. Moreover, adding uracil or uridine to M9-succinate permits the growth of the *chvI* mutant strain in liquid medium. This suggests that the *chvI* mutant is a uracil auxotroph and growth seen on media-agar is due to its presence as an impurity in commercial agar. Others have reported that agar can be permissible to purine or pyrimidine auxotrophs (Diebold and Noel, 1989). Adding proline also restored growth of the *chvI* mutant in liquid and this could be explained by an increased production of carbamoyl-phosphate and orotate thus shifting the flux towards UMP biosynthesis (Jochimsen *et al.*, 1985).

The role of ExoS/ChvI in regulating the thiamine and histidine operons is extremely interesting from many points of view and should be addressed in detail. 5-Phosphoribosyl 1-pyrophosphate (PRPP) is central to these pathways and it is also a substrate for anthranilate phosphoribosyltransferase (E.C. 2.4.2.18, *trpD*). This enzyme catalyzes the formation of N-(5'-phosphoribosyl)-anthranilate, an intermediate in the tryptophan biosynthesis pathway. Mutants with lower 2-dehydro-3-deoxyphosphoheptonate aldolase (DAHP synthase, E.C. 2.5.1.54) activity, the first enzyme in the tryptophan biosynthesis pathway, do not grow on complex media and are defective in symbiosis (Jelesko *et al.*, 1993). All these pathways would have a requirement for coordinated regulation and ExoS/ChvI could play an important role in balancing the metabolic flux through histidine and thiamine biosynthesis pathways.

6.2 Above and beyond exopolysaccharide regulation.

The ExoS/ChvI two-component regulatory system regulates the production of exopolysaccharides but it also has a number of other regulatory roles. It represses the transcription of genes producing the rhizobactin 1021 iron-siderophore and putative fatty acid β-oxidation genes. The ExoS/ChvI-repression of genes producing rhizobactin 1021, a hydroxamate siderophore, could be necessary during infection. Genes producing rhizobactin 1021 are activated by RhrA during iron-depleted conditions and repressed by RirA during iron-repleted conditions (Lynch *et al.*, 2001; Viguier *et al.*, 2005). The mutation of any of these genes do not change the outcome of symbiosis and perhaps ExoS/ChvI could be responsible for the down-regulation of the siderophore production during the invasion because ExoS and ChvI are found during symbiosis (Ampe *et al.*, 2003; Djordjevic *et al.*, 2003). The role for SMc00261 and SMc00262 genes is associated with fatty acid β-oxidation by sequence similarity but nothing is known about which compounds these genes could be involved in degrading.

A long list of genes still need to be confirmed by a second method and their activation or repression by ChvI need to be determined, however an overall picture may still be drawn from these results. Regulation of genes for methionine import, cardiolipin, phosphatidylserine and phosphatidylethanolamine synthesis strongly suggests an impact of ExoS/ChvI on phospholipid homeostasis. Regulation of the *lac* operon and *gal* gene by ExoS/ChvI would also affect disaccharide catabolism. Our results also point towards ChvI autoregulation and its regulation of the *manXhpr* operon involved in succinate-mediated catabolite repression (PTS).

The involvement of ExoS/ChvI in exopolysaccharide production and its genomic position between *pckA* and genes for the PTS system have directed us towards the hypothesis

that ExoS/ChvI might play a central role in carbon metabolism. The large number of growth defects of mutant strains on different carbon sources highlighted this hypothesis. However, results from GD.EMSA and the discovery that the *chvI* mutant is a uracil and proline auxotroph changes the perspective on the regulatory role of ExoS/ChvI. The work done in this thesis points to the ExoS/ChvI two-component regulatory system as a global regulator required to maintain homeostasis during the drastic change from free-living to eukaryal-associated conditions. This change perhaps requires maintenance of a tight control on amino acid, purine and pyrimidine biosynthesis to maintain steady growth while the cell envelope adapts to the new conditions.

Brucella abortus is a mammalian pathogen for which membrane homeostasis is important for its virulence. Accordingly, the BvrR/BvrS two-component regulatory system has been found to affect lipid A acylation and the cell envelope protein profile (Manterola *et al.*, 2005; Lamontagne *et al.*, 2007). *Agrobacterium tumefaciens* is a plant pathogen that has to counteract acidic pH at the wound site of the plant. Activation of virulence genes depends on this acidification and the ChvG/ChvI two-component sytem has been found to be key to this process (Li *et al.*, 2002). *S. meliloti* ExoS/ChvI is required for EPS production and without EPS the symbiosis is blocked at an early stage. In all cases the invasion of the host depends upon the presence of this important two-component regulatory system. Although phenotypes of mutants have directed research toward specific host-interaction factors, this study could open new avenues for researching commonalities between these orthologous systems. The regulation of the histidine biosynthesis operon is one interesting avenue because it is found close to the two-component regulatory system and also because these genes are important for virulence (Joseph *et al.*, 2007).

Appendices

Appendix A

Additional Materials and Methods

A.1 Acetylene reduction assay

Plants were harvested and shoots were cut, weighed, and dried. Root systems were kept for acetylene reduction assays and placed in 24-ml glass vials with a rubber stopper. Each vial containing five alfalfa root systems, was injected with 0.2 ml acetylene at room temperature, normal pressure, and incubated for two hours at room temperature on a rotor. A 1-ml sample from each vial was analysed throught a HP-AL/M column (J&W, 30 m, I.D. 0.53 nm, Film 15 μm) using a Shimadzu GC-17A gas chromatograph and a flame ionization detector. Gasses were carried by helium with a flow rate set at 6 ml/min. and 36 kPa total pressure. Under these conditions, if the oven is set for isothermal 100°C runs, the injector set at 120°C and detector set at 150°C, ethylene elutes after 1.9 min. and acetylene after 3.0 min. The amount of ethylene produced per plant was standardized by making an ethylene standard curve. A total of 15 alfalfa plants for each bacterial strain inoculum were analysed.

A.2 Sodium boric acid (SB) buffer

20X SB buffer

200 mM NaOH

Adjust pH to 8 using boric acid

To make 500 ml:

4 g NaOH in about 400 ml of distilled water

pH is adjusted to 8 with about 20 g of boric acid

Add water to have a final volume of 500 ml

To make 1 Liter of 1X SB buffer:

50 ml 20X SB buffer

950 ml distilled water

pH should be 8.5

A.3 Preparation of a 5% polyacrylamide gel for DNA separation or EMSA

To make 10 ml:

500 μl 20X SB buffer

7.7 ml distilled water

1.7 ml 29:1 acrylamide/bisacrylamide (40%)

125 μl 10% APS

12.5 μl TEMED

A.4 β-Glucuronidase Assay

This assay is used to determine the expression of *gusA* under control of a fused gene. This protocol is adapted from Cowie *et al.* (2006).

Reagents and Solutions:

1 M Na_2CO_3

17.6 mg/ml 4-nitrophenyl beta-D-glucuronide *(Dissolve in PE buffer and stored frozen in 250 μl aliquot.)*

0.1% SDS

500 mM DTT *(Dissolved in PE buffer and stored frozen in 1 ml aliquot.)*

Phosphate-EDTA (PE) Buffer

To prepare 500 ml:

- *8.05 g $Na_2HPO_4 \cdot 7H_2O$ (60 mM final)*
- *2.75 g $NaH_2PO_4 \cdot H_2O$ (40 mM final)*
- *2 ml 250 mM EDTA (1 mM final)*

GUS buffer *(prepared fresh)*

To prepare 10 ml (enough for a 96 well microplate):

- *Add to 7.5 ml PE buffer*
- *1.25 ml of 0.1%SDS*
- *1.0 ml of 500 mM DTT*
- *250 µl of 17.6 mg/ml 4-nitrophenyl beta-D-glucuronide*

Procedure:

1. Transfer 200 µl of each culture in a 96 well microplate and measure culture turbidity (OD_{595}).
2. Use the 200 µl of each culture to aliquot 3 times 20 µl/well (triplicates).
3. Add 80 µl of GUS buffer and start the incubation at room temperature. Make sure to note starting time.
4. Incubate for 40 minutes or until most cultures turned yellow.
5. Stop the reaction by adding 100 µl of 1M Na_2CO_3.
6. Measure the activity at 405 nm and 550 nm.
7. Calculate units of specific activity with the following equation:

$$U(Miller) = \frac{1000 \times (OD_{405} - (1.75 \times OD_{550}))}{t \times v \times OD_{595}}$$

t = time of reaction (min)

v = volume of culture used in assay (ml)

OD_{595} = cell density at the start of the assay

OD_{405} = combination of absorbance by p-nitrophenol and light scattering by cell debris

OD_{550} = light scattering by cell debris

A.5 GusA activity on agar plates

Make a stock solution: 20 mg/ml X-GlcA in DMF.

Aliquot 200 μl per eppendorf tube and keep frozen at -20°C.

To prepare agar plate use one aliquot per 200 ml medium.
The final concentration in agar should be 20 μg/ml.

Table A.1: Transcriptional fusion strains used or made in this study.

RmP110	Rm1021	SmUW37	SmUW38	Fusion to	Annotation
SmFL91	SmUW42	-	-	SMb20615	*thiC*
SmFL112	SmUW43	SmUW155	SmUW133	SMb21191	*msbA2*
SmFL309	SmUW44	SmUW156	SmUW134	SMc02282	
SmFL430	SmUW45	SmUW157	SmUW135	SMa2295	
SmFL918	SmUW46	SmUW158	SmUW136	SMc00262	
SmFL1212	SmUW47	SmUW159	SmUW137	SMb20615	*thiC*
SmFL1784	SmUW48	SmUW160	SmUW138	SMb20213	
SmFL1886	SmUW49	SmUW161	SmUW139	SMb20213	
SmFL2130	SmUW50	SmUW162	SmUW140	SMb20478	
SmFL2629	SmUW51	SmUW163	SmUW141	SMa2294	*mrcA2*
SmFL2754	SmUW52	SmUW164	SmUW142	SMb20574	
SmFL2775	SmUW53	SmUW165	SmUW143	SMc03992	
SmFL2934	SmUW54	SmUW166	SmUW144	SMc02491	
SmFL2950	SmUW55	SmUW167	SmUW145	SMa2402	*rhbB*
SmFL3369	SmUW56	SmUW168	SmUW146	SMc02281	
SmFL4392	SmUW57	SmUW169	SmUW147	SMc00261	
SmFL4665	SmUW58	SmUW170	SmUW148	SMb21189	
SmFL5401	SmUW59	SmUW171	SmUW149	SMb21189	
SmFL5603	SmUW60	SmUW172	SmUW150	SMa2404	*rhbC*
SmFL5622	SmUW61	SmUW173	SmUW151	SMc02076	*cls*
SmFL5628	SmUW62	SmUW174	SmUW152	SMa2337	*rhtX*
SmFL5755	SmUW63	SmUW175	SmUW153	SMa2410	*rhbF*
SmFL6054	SmUW64	SmUW176	SmUW154	SMa2294	*mrcA2*

Appendix B

Data

B.1 Electrophoretic Mobility Shift Assay

Table B.1: Fragments recovered from EMSA and cloned in pUC18. Fragments were recovered from PD. EMSA (P) or GD.EMSA (G).

Closest ORF	From ORF	Position		Size	Found in	Exp.
	Up/In	Begin	End	(bp)		
SMc02753 (*manX*)	Up	48523	48699	176	pLB102	P
SMc02753 (*manX*)	Up	48523	48699	176	pLB110	P
SMc02753 (*manX*)	Up	48523	48699	176	pLB114	P
SMc02560 (*chvI*)	Up & In	51887	52281	395	pLB116	P
SMc02574 (*hisB*)	In	60821	60949	129	pLB126	G
SMc02281	In	654156	654459	304	pLB104	G
SMc03267	Up	3383057	3383240	184	pLB106	G
SMc03297	Up	3412878	3413020	143	pLB124	G
SMc03159 (*metN*)	In	3117150	3117277	128	pLB105	G
SMc02076 (*cls*)	In	1639710	1639854	145	pLB105	G
SMc02733	In	2587012	2587177	166	pLB129	G
SMc00051 (*phaA2*)	In	1085493	1086087	595	pLB123	G
SMc00262	In	1830765	1831000	236	pLB118	G
SMc00550	In	1220301	1220439	139	pLB129	G
SMc00589	In	1260626	1260767	142	pLB121	G
SMc03993	In	2991422	2991677	256	pLB127	G
SMc02637	In	1183131	1183282	152	pLB124	G
SMc02491	In	3303566	3303853	288	pLB121	G
SMb21188	In	938297	938439	143	pLB107	G
SMb20213	In	221089	221185	97	pLB129	G
SMb20478	In	492546	492612	67	pLB129	G
SMb21653 (*lacF*)	Up & In	1266	1449	184	pLB129	G
SMb21552 (*aacC4*)	Up	1091213	1091353	141	pLB128	G
SMb20574	In	1588216	1588436	221	pLB125	G
SMb20032	Up	43628	43775	148	pLB123	G
SMb20119	Up	132970	133119	150	pLB122	G
SMb20615 (*thiC*)	In	1634582	1634726	145	pLB119	G
SMa2295	In	1278398	1278609	212	pLB106	G
SMa2103	In	1186198	1186417	220	pLB103	G
SMa2337 (*rhtX*)	In	1305222	1305315	94	pLB107	G
SMa2337 (*rhtX*)	In	1305714	1305861	148	pLB129	G

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