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Characterization of Putative ExbB and ExbD Leads to the Identification of a Potential Tol-Pal System in Rhizobium leguminosarum ATCC 14479

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by

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ABSTRACT

Characterization of Putative ExbB and ExbD Leads to the Identification of a Potential Tol-Pal System in *Rhizobium leguminosarum* ATCC 14479

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Rhizobium leguminosarum is a Gram negative nitrogen-fixing soil bacterium. Due to the limited bioavailability of iron, bacteria utilize siderophores that scavenge and bind available iron. The transport of iron-siderophore complexes is achieved by the TonB-ExbB-ExbD complex. We have previously shown that a functional TonB protein is necessary for iron transport by creating Δ*tonB* mutants and assessing their growth and ^{55}Fe-siderophore transport ability. We attempted to identify and characterize the roles of putative *exbB* and *exbD* genes using a similar approach. Growth curves and sequence analyses suggest putative *exbB* and *exbD* may be the *tolpal*-associated genes *tolQ* and *tolR.* Phenotypic and sensitivity assays showed mutants do not exhibit the characteristic *tol* phenotype and are not sensitive to detergents or changes in ionic strength of the growth medium. We also expressed and purified the 120 amino acid fragment of the TonB C-terminus for further physical and chemical characterization.

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INTRODUCTION

Iron and Its Importance

Iron is an essential nutrient for not only bacteria, but virtually all living organisms. All metal ions, including iron, serve important biological functions and are often cofactors for various proteins and transcription factors (Porcheron et al., 2013). In microorganisms, the role of iron ranges from nitrogen fixation to metabolism. Deficiencies may cause decreased DNA or RNA biosynthesis and affect pathways such as the electron transport chain and citric acid cycle (Messenger et al., 2010). Although a plethora of iron can be found in nature, the amount of biologically usable iron is rather low (Miethke et al., 2007). Iron exists as either the reduced ferrous iron (Fe^{2+}) or the oxidized ferric iron (Fe^{3+}). At neutral pH ferrous iron is soluble and can readily be taken up by cells. However, in the presence of oxygen it spontaneously oxidizes to form ferric iron. Although microorganisms have receptors for ferrous and ferric iron, neither is freely available in required concentrations since ferric iron forms insoluble ferric hydroxide polymers in the presence of oxygen (Miethke et al., 2007). Inside the host, ferric iron is sequestered into proteins such as lactoferrin, transferrin, and ferritin (Krewulak et al., 2007). For pathogenic bacteria, this poses a dilemma since the concentration of free iron is greatly reduced. Ferrous iron, albeit soluble, does not assuage bacteria's burden of acquiring iron due to its potential toxicity when confronted with oxygen. By undergoing the Fenton and Haber-Weiss reactions, ferrous iron and oxygen react to form pernicious reactive oxygen species (ROS) which may harm DNA, proteins, and membrane lipids (Caza et al., 2013).

Iron Availability

Due to insoluble ferric hydroxides that form from ferric iron, the amount of available usable iron in the environment is approximately 10^{-9} to 10^{-18} M (Miethke et al., 2007). Inside a mammalian host, the iron is stored in proteins such as hemoglobin or ferritin resulting in an even lower free iron concentration of 10^{-24} M (Miethke et al., 2007). This certainly limits pathogenic microorganisms to a very restricted supply of iron. However, non-pathogenic bacteria are also devoid of free iron since the iron threshold for most bacteria is about 10^{-6} M (Miethke et al., 2007). In order for microorganisms to grow, the iron concentration must be maintained. As a result, bacteria have evolved intricate iron acquisition systems to capture this vital metal.

Iron Acquisition Systems

Through the course of evolution, some microbes developed alternative mechanisms of acquiring iron including direct and indirect iron acquisition systems. Those that evolved such mechanisms were able to gain a foothold over the deluge of competing microorganisms in the environment. Many bacteria possess both direct and indirect iron acquisition systems (Krewulak et al., 2007). Iron acquisition by direct mechanism involves the direct contact of a bacterium to iron or iron-containing source. Pathogenic bacteria are able to target iron-containing sources, such as hemoglobin, by secretion of hemolysins that lyse red blood cells, thus releasing hemoglobin. The heme from hemoglobin can further be extracted by proteases that specifically target and degrade hemoglobin (Caza et al., 2013). The heme released from hemoglobin can then be taken up through direct contact of bacterial cell surface receptors with the heme. In Gram negative bacteria, outer membrane receptors recognize and bind heme or hemoglobin, or both, depending on the organism (Wandersman et al., 2004). For the bacterium *Yersinia enterocolitica*,

the receptor HemR is able to bind both heme and hemoglobin, whereas Hmbr of *Neisseria meningitidis*, for example, only binds hemoglobin (Wandersman et al., 2004). Heme acquired intact or released from hemoglobin is transported across the periplasm and through the inner membrane by periplasmic permeases. The fate of heme once inside the cytoplasm has not been resolved. It may be that heme oxygenase-like enzymes, such as those in *Neisseriae* species, are present in the cytoplasm that further extract the iron from heme (Wandersman et al., 2000). Gram positive bacteria utilize iron from heme and other iron sources in a similar matter, i.e. receptors on the peptidoglycan surface that recognize and bind iron or iron-containing sources. In *Staphylococcus aureus*, iron-regulated surface determinant B (IsdB), a protein encoded by the Isd locus that also encodes for nine other proteins including surface proteins, a transporter, a transpeptidase, and heme-degrading monooxygenases, is capable of binding hemoglobin. *isdB* mutants show reduced hemoglobin binding and pathogenicity (Skaar, 2006). The disadvantage of direct iron uptake, however, is that a specific receptor is required for each iron source (Miethke et al., 2007).

Contrary to direct iron acquisition systems in which the iron binding occurs at the cell surface, indirect iron acquisition involves the release of compounds into the extracellular medium to capture iron. Iron acquisition through indirect methods is achieved by either hemophores or siderophores – chelators of heme and ferric iron, respectively. Indirect iron acquisition is present in Gram positive and Gram negative bacteria and offers certain advantages not provided by direct mechanisms. For microorganisms inhabiting environments devoid of heme iron, a direct mechanism of iron uptake would be futile. Therefore, an indirect mechanism of iron acquisition through the secretion of siderophores is more beneficial.

Siderophores

Siderophores are low molecular weight compounds (<1 kDa) produced by many Gram positive and Gram negative bacteria in response to iron-starvation conditions. Activation of genes for the synthesis of siderophores is initiated when intracellular iron concentrations drop below bacterium's threshold, usually 10^{-6} M (Miethke et al., 2007). Ferric uptake regulator (Fur), the global iron regulator in many Gram negative bacteria, controls the transcription of genes responsible for siderophore synthesis (Miethke et al., 2007). Using Fe^{2+} or Mn^{2+} as a corepressor, Fur recognizes and binds specific DNA-binding sequences, called the Fur box, and blocks transcription of iron uptake genes. When intracellular Fe^{2+} concentrations fall below a certain limit, the siderophore transcription is initiated (Troxell et al., 2013). In addition to regulation of siderophore synthesis, Fur regulates the transcription of more than 90 other genes, including those required for the transcription of proteins involved in the tricarboxylic acid (TCA) cycle and Fe-dependent superoxide dismutase (SodB) (Hantke, 1987; Dubrac and Touati, 2000). In certain Gram positive bacteria, iron regulation is controlled by the DtxR family of proteins first identified in *Corynebacterium diphtheria.* However, not all Gram negative organisms use Fur for the maintenance of iron homeostasis. In the nitrogen-fixing *Rhizobium leguminosarum*, Fur is replaced by the rhizobial iron regulator RirA. There is no sequence similarity between RirA and the Fur family of proteins, and RirA belongs to the Rrf2 family of transcription regulators (Rudolph et al., 2006). Homologs of RirA are only found in other alphaproteobacteria, a class to which *R. leguminosarum* belongs (Ngam et al., 2009). The regulator acts as a repressor of iron-responsive genes (Ngam et al., 2009) and its transcription is down-regulated by iron-rich conditions. The synthesis, uptake, and regulation of *R. leguminosarum*'s vicibactin siderophore is under the control of RirA (Rudolph et al., 2006).

Types of Siderophores

After secretion into the environment, siderophores are then brought inside the cell. Due to their size and polarity, siderophores must be transported across the cytoplasmic membrane by transport proteins (Furrer et al., 2002). With a high affinity for ferric iron, siderophores can bind and strip iron from the source with a lower affinity. Siderophores can be divided into one of three groups: hydroxamates, catecholates, or carboxylates, depending on the moiety donating the oxygen ligand for Fe^{3+} coordination (Miethke et al., 2007). A fourth group classified as mixed-type has been established for the increasing number of identified siderophores that contain more than one functional group used as the Fe^{3+} ligand (Miethke et al., 2007). In acidic conditions, carboxylates are more efficient in iron mobilization, and thus often preferred by microbes inhabiting such environments whereas catecholates are the more predominant and stronger siderophores of microbes found at physiological pH. This is due to protonation of donor atoms which plays a role in determining the effectiveness of a siderophore's affinity (Miethke et al., 2007).

Vicibactin

Belonging to the hydroxamate group of siderophores and produced by the nitrogen-fixing *Rhizobium leguminosarum*, vicibactin is a cyclic siderophore containing three hydroxamate functional groups that bind Fe^{3+}. Its synthesis is controlled by the gene clusters *vbsGSO, vbsADL, vbsC,* and *vbsP* arranged in four operons. Except for *vbsP*, the transcription of the operons is initiated in low-iron conditions (Carter et al., 2002). RpoI, a putative RNA polymerase σ factor of extracytoplasmic function (ECF) family, is required for the transcription of *vbsGSO* and *vbsADL* operons (Yeoman et al., 2003). Mutants defective in *rpoI* do not produce vicibactin,

and strains with cloned *rpoI* are shown to overexpress the siderophore. The regulator, however, does not control expression of *vbsC* and *vbsP* (Carter et al., 2002). RpoI most likely controls the transcription of *vbsGSO* and *vbsADL* operons by interacting with the promoter. The closely resembled PvdS σ factor of *Pseudomonas* is known to bind to the promoters of genes responsible for the biosynthesis of the pyoverdine siderophore (Carter et al., 2002). Through a mechanism proposed by Carter et al (2002), the vicibactin siderophore is synthesized as a monomer by the *vbs* genes and, in its final step, converted to a cyclic trimer, yielding the completed form of vicibactin. The siderophore then exits through the inner and outer membranes and into the environment where it binds Fe^{3+}, forming a siderophore-Fe complex. The complex is brought inside the cell with the aid of outer and inner membrane transporters, periplasmic binding proteins, and the TonB-ExbB-ExbD complex which presumably supplies the energy for the outer membrane transporters.

Outer Membrane Transporters

Porins located on the outer membrane of Gram negative bacteria serve as channels that allow the passage of charged molecules smaller than approximately 600 Daltons into and out of the cell via passive transport (Schirmer et al., 1998). Many siderophores are small enough that they may pass through porins. However, once bound to iron, the Fe-siderophore complex becomes too large for such passage. As a result, the complexes must be actively transported by siderophore-specific outer membrane transporters. These transporters are often referred to as TonB-dependent transporters (TBDTs) since their function is dependent upon the energy transducing TonB complex and, in addition to siderophores, are also responsible for the transport of vitamin B12 (Udho et al., 2012), nickel chelates, and carbohydrates (Noinaj et al., 2010).

Even though the sequence similarity among the receptors may be low, all outer membrane receptors share a similar structure. They are composed of a 22 β-stranded transmembrane barrel and an amino-terminal globular domain located inside the barrel often referred to as the "plug" (Krewulak et al., 2007). The "plug" prevents molecules from freely entering or exiting the cell. At the N-terminus of the "plug" is the TonB box – a stretch of amino acids with conserved motifs that interact with TonB to presumably signal and transduce energy to the transporter (Noinaj et al., 2010).

The β-barrel is made up of 10 periplasmic loops, 11 extracellular loops, and 22 β strands of the β-barrel (Krewulak et al., 2007). The β-barrels of outer membrane transporters such as BtuB, FecA, FepA, FptA, and FpvA are similar in structure when the Cα backbones of the barrel are overlayed. Though the lengths and/or widths of the barrel may differ among the transporters, in all cases the β-barrel extends above the lipid bilayer and contains a conserved phenylalanine residue necessary for proper folding and insertion of the transporter (Krewulak et al., 2007).

Occluding the β-barrel is an amino-terminal globular domain. Much speculation still exists as to how the siderophore-iron complexes or other ligands pass through the transporter. One suggested mechanism is that complete dissociation of the "plug" from the β-barrel occurs, as observed by Ma et al. (2007). Li Ma and colleagues engineered and fluorescein maleimide-modified 25 cysteine substitution mutations in the outer membrane transporter FepA of *Escherichia coli*. A cysteine residue buried within the N-terminal and labeled with fluorescein maleimide was observed in the periplasm during transport which suggested the plug exited from the β-barrel (Ma et al., 2007). A second proposed mechanism is that the plug does not leave the β-barrel, but instead undergoes a conformational change that creates a pore within the β-barrel (Noinaj et al., 2010). Using FepA and colicin B, Smallwood et al. detected no structural changes

in the plug domain during its interaction with colicin B, contradicting the idea that dissociation of the plug from the β-barrel occurs (Smallwood et al., 2009). Similar results were obtained by Chakraborty et al. in FhuA and FepA mutants using ^{55}Ferrichrome (Chakraborty, 2007). Further research is necessary to elucidate the exact mechanisms of TBDTs.

At the N-terminus of the plug domain is a conserved region of about seven amino acids called the TonB box (Schalk et al., 2012). Its interaction with the TonB protein is essential for the transport of substrates across the outer membrane transporter. Albeit conserved among other TBDTs, single amino acid substitutions in the TonB box show little to no reduced transport ability (Gudmundsdottir et al., 1989). The TonB box is thought to exist in a folded conformation within the β-barrel when not interacting with the TonB protein. Upon binding of the substrate, a conformational change occurs at the periplasmic side of the N-terminal domain and a reversible association of the TonB protein with the TonB box results in transduction of energy to the transporter (Kim et al., 2007).

TonB-ExbB-ExbD

In order for any transport via outer membrane transporters to occur, there must be an energy source to drive the transporters. In Gram negative bacteria, energy is produced at the inner membrane and is supplied to the energy-devoid outer membrane. To transport the Fe-siderophore complexes inside the cell, the outer membrane transporter must carry the complex across a concentration gradient since the concentration of iron is higher inside the cell (10^{-6} M) than outside the cell (10^{-18} M). Therefore, for active transport to occur, the proton motive force generated at the cytoplasmic membrane must be supplied to these transporters. A complex of three proteins – TonB, ExbB, and ExbD – located at the cytoplasmic membrane provide the pmf

to the Fe-siderophore transporters, hence the name TonB-dependent transporters (TBDTs) (Noinaj, 2010).

Much of the research on the TonB complex has been focused in *Escherichia coli*, though little is still known about its mechanism of transduction. In *E. coli*, TonB is a 26 kDa single transmembrane protein with three functional domains: a cytoplasmic N-terminal domain (residues 2-65), a periplasmic C-terminal domain (residues 103-239) and a proline-rich spacer (resides 66-102) separating the N- and C-terminal domains (Postle et al., 2007).

The N-terminal region contains the signal sequence for Sec-dependent export into the cytoplasmic membrane (Postle et al., 2007) and consists of a 32-residue transmembrane helix (Krewulak et al., 2007). The transmembrane helix not only anchors the protein into the CM, but also serves as a site of interaction with the other two proteins in the complex, ExbB and ExbD, whose function is vital for energy transduction (Krewulak et al., 2007).

The C-terminal domain of TonB resides in the periplasm and interacts with the N-terminal TonB box of the outer membrane transporter. Solution structures of the *E. coli* TonB C-terminal domain solved by Peacock et al (2005) reveal a monomeric protein with unstructured and structured residues 103-151 and 152-239, respectively. The structured region is made up of two α helices packed against a four-stranded antiparallel β sheet (Peacock et al., 2005).

The proline-rich spacer is located in the periplasm and contains a series of proline and glutamine residues and several proline-lysine repeats. More than one in every three residues is a proline (Kohler et al., 2010) which presumably provides rigidity and allows for the extension of the protein across the periplasm (Krewulak et al., 2007). Although energy transduction cannot be achieved without the interaction of the TonB C-terminal with the outer membrane transporter,

much of the residues of the proline-rich region can be deleted with no effect on transport (Larsen et al., 1993). Larsen and colleagues constructed an *E. coli tonB*Δ66-100 mutant devoid of the proline-rich region and observed no effect on transport of ϕ80 except when the bacterial cell was subjected to osmotic swelling, suggesting it plays no role in energy transduction but rather in extension across the periplasmic space (Larsen et al., 1993).

In complex with TonB are ExbB and ExbD, cytoplasmic membrane proteins of approximately 26 and 17 kDa, respectively. Inside the cell, the ratio of ExbB:ExbD:TonB is 2:7:1 (Ollis et al., 2012), though it is unclear if this ratio is retained while the complex is in an energy transducing state (Bulathsinghala et al., 2013). In *E. coli*, *exbB* and *exbD* genes are part of the *exb* operon whose transcription is initiated at the *exbB* promoter, and separating the open reading frames of *exbB* and *exbD* are only 9 base pairs (Ahmer et al., 1995). Furthermore, because both genes are co-transcribed as a single mRNA, ExbB and ExbD do not function independently in energy transduction (Held et al., 2002). However, distance between the open reading frames varies from organism to organism, and the genes may not be in an operon in other Gram negative organisms.

Traversing the cytoplasmic membrane three times, the majority of ExbB is located in the cytoplasm where, in conjunction with the neighboring ExbD, it harnesses and transduces the pmf to TonB (Ahmer et al., 1995). Though the mechanism of transduction has not been elucidated thus far, ExbB seems to play a role in stabilizing TonB and ExbD and may serve as a scaffold on which the aforementioned proteins assemble. It is the only protein in the complex that is stable when expressed independently (Bulathsinghala et al., 2013), and its absence results in a proteolytically unstable TonB and ExbD (Baker et al., 2013). As mentioned earlier, ExbB consists of three transmembrane domains (TMDs), and a significant portion of the protein lies in

the cytoplasm. It also has the reverse topology of TonB and ExbD – a periplasmic amino terminal and a cytoplasmic carboxy terminal. With much of the protein in the cytoplasm, it has been postulated that the cytoplasmic loops of ExbB function in communication between the periplasm and cytoplasm (Jana et al., 2011; Bulathsinghala et al., 2013). Earlier studies identified four cytoplasmic residues (N196, D211, A228, and G244) important for pmf-dependent formaldehyde crosslinking between ExbD and TonB, suggesting signal transduction occurs from the cytoplasmic residues to the periplasmic domains of ExbD and TonB (Jana et al. 2011). A more recent study using 10-residue-deletion scanning mutagenesis showed that the loop residues were necessary for all TonB-dependent interactions. Furthermore, expression of eight out of nine deletion mutants resulted in immediate, yet reversible, growth arrest independent of pmf (Bulathsinghala et al., 2013). The cytoplasmic loop of ExbB occurs between the first two of three TMDs. Due to ExbB and ExbD's homology to the flagellar motor proteins MotA and MotB, which also harness cytoplasmic pmf, several proton pathways through the ExbB TMDs have been proposed (Baker et al., 2013). Baker and Postle (2013) showed that when half of each TMDs is substituted as a block with alanines, ExbB is inactivated, suggesting all TMDs are necessary for ExbB function (Baker et al., 2013). Moreover, there were no individual TMD residues identified that were essential for signal transduction or that participated in proton translocation (Baker et al., 2013).

The third and smallest protein in the complex, ExbD, shares identical topology to TonB. It is a single transmembrane protein with a periplasmic C-terminus and a cytoplasmic N-terminus. Like TonB, majority of ExbD occupies the periplasm (Ollis et al., 2009). The periplasmic domains of both ExbD and TonB have been shown to interact with each other in the presence of proton motive force. Using formaldehyde crosslinking, Ollis et al. (2009) treated *E.*

coli cells with protonophores DNP and CCCP before and during the crosslinking. The presence of protonophores prevented crosslinking of TonB and ExbD, indicating the need for pmf for *in vivo* interaction of the periplasmic domains (Ollis et al., 2009). This interaction is disrupted when residue D25 in the transmembrane domain is substituted with asparagine or alanine (Ollis et al., 2009) or by the substitution of leucine 132 with glutamine in the periplasmic domain (Ollis et al., 2012). Likewise in the TonB transmembrane domain, an H20A substitution disrupts this TonB-ExbD periplasmic interaction (Ollis et al., 2009). A model for the early stages of energization of TonB proposed by Ollis and Postle (2012) corroborates the importance of residues H20 and L132. Based on ExbD mutants and using spheroplasts as an *in vivo* model, a three stage energization model shows TonB stymied in the first stage when its periplasmic domain fails to interact with the periplasmic domain of ExbD, due to either an H20A or L132Q mutation preventing their proper assembly (Ollis et al., 2012). Stage II results when the periplasmic domains of both proteins interact with each other with the aid of ExbB serving as a scaffold. Stage II is converted to Stage III in the presence of pmf. The conversion is reversible by the addition of CCCP to halt the pmf. Once the pmf is restored, the energization proceeds to Stage III again (Ollis et al., 2012).

Rhizobium leguminosarum

Rhizobium leguminosarum is a Gram negative bacterium commonly found in the soil. It is aerobic, motile and plays an important agricultural role in nitrogen fixation. Known to infect leguminous plants, rhizobia, as they are collectively called, form a symbiotic relationship with their host by reducing atmospheric nitrogen to the more usable NH_3 and, in turn, receiving carbohydrates from the plant (Long, 2001). For symbiosis to occur, rhizobia must first infect the root nodules of the plant – the site of nitrogen fixation. Infection occurs when rhizobia become

trapped between two root hair cell walls. Once inside the cell, rhizobia grow and secrete Nod factors necessary for nodulation (Gage et al., 2000). The expression of nodulation genes *nod, nol,* and *noe* is initiated when plant flavonoids activate the transcriptional regulator NodD, which subsequently activates the nodulation genes that are involved in the synthesis of Nod factors (Peters et al., 1986). NodD proteins bind to conserved motifs called nod boxes located at promoter regions of *nod* genes (Wang et al., 2012). After the activation and secretion of Nod factors, nodules form at the root of the legume. It is here that rhizobia convert atmospheric nitrogen into ammonia for the legume (Geurts et al., 2002).

Present Work

Previous work on *Rhizobium leguminosarum* ATCC 14479 has shown that the bacterium produces the trihydroxamate siderophore vicibactin (Wright et al., 2013). The import of Fe-vicibactin complexes through outer membrane transporters occurs via active transport. We hypothesize that the TonB-ExbB-ExbD complex is involved in providing energy to the transporters. Earlier work has shown that Δ*tonB* mutant fails to grow in iron-depleted media compared to wild type. Using radioactively-labeled ^{55}Fe bound to vicibactin, the mutant also failed to import the ^{55}Fe-vicibactin complex. Complementation with the wild type allele restored growth and ^{55}Fe import to near wild type levels (Hill, 2014). This substantiates TonB's indirect involvement in the import of the vital element. To assess the roles of ExbB and ExbD, we first identified putative *exbB* and *exbD* genes and created single and double knockouts of the genes. The mutants were then compared with wild type in the ability to grow in complex media, low iron and high iron minimal media. Due to results that differed greatly from those of Δ*tonB* assays, we postulated that the putative *exbB* and *exbD* genes we identified are rather the highly similar homologs *tolQ* and *tolR* of the Tol-Pal system. Additional sequencing downstream of

exbD/tolR revealed a putative *tolA* gene – one of seven genes belonging to the Tol-Pal system. To further characterize the putative *tolQ* and *tolR* genes, single and double knockout mutants were assayed for their susceptibility to detergents and high ionic strength media, and for their tendency to exhibit a chaining phenotype when grown in low osmolarity or high ionic strength media. We also constructed in-frame fragments of the 120 and 200 amino acid TonB C-terminus protein for further physical and chemical analyses.

CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains

The strain of Rhizobia used in this study was *Rhizobium leguminosarum* ATCC 14479 and was obtained from the American Type Culture Collection. The *Escherichia coli* strains were DH5α, SM10, and BL21 (DE3) (Novagen).

Growth Conditions

Rhizobium leguminosarum ATCC 14479 was grown in Yeast Extract Mannitol (YEM) broth, Modified Manhart and Wong (MMW) broth, and Congo Red (CR) solid media. YEM is efficient in the cultivation of Rhizobia species and consists of (w/v): 1% mannitol, 0.05% K_2HPO_4, 0.1% yeast extract, 0.01% NaCl, and 0.02% $MgSO_4*7H_2O$. The pH of the broth was adjusted to 6.8 using 12M NaOH prior to autoclaving. When required, MMW was used as minimal media and contained the following (w/v): 0.0764% K_2HPO_4, 0.1% KH_2PO_4, 0.15% Glutamate, 0.018% $MgSO_4$, 0.013% $CaSO_4*2H_2O$, and 0.6% dextrose. The pH was adjusted to 6.8 using 12M NaOH and the media was autoclaved. Prior to inoculation, filter-sterilized 1X vitamin solution was added to the media. The composition of the vitamin solution is listed in Table 1 below.

Table 1. List of Ingredients for 1000X Vitamin Solution

1000X Vitamin Solution	
Ingredient Name	**Amount (in mg/100 mL)**
H_3BO_3	145
$CuSO_4*5H_2O$	4.37
$MnCl_2*4H_2O$	4.3
$ZnSO_4*7H_2O$	108
$Na_2MoO_4*2H_2O$	250
$CoCl_2*6H_2O$	10
$Na_2EDTA*2H_2O$	550
Riboflavin	10
ρ-aminobenzoic acid	10
Nicotinic acid	10
Biotin	12
Thiamine HCl	40
Pyridoxine HCl	10
Calcium panthenate	50
Inositol	50
Vitamin B12	10

Congo Red solid media is also used for the cultivation of *Rhizobium* species as well as their detection. The congo red dye in the media is not absorbed by rhizobia very efficiently, resulting in pink to white colonies on the agar. However, other microorganisms absorb congo red

much more readily and thus colonies appear dark pink to red (Kneen et al., 1983). This facilitates in distinguishing contaminants from *Rhizobium* species. The ingredients of CR media were as follows (in w/v): 1% mannitol, 0.05% K_2HPO_4, 0.02% $MgSO_4*7H_2O$, 0.01% NaCl, 0.1% yeast extract, 3% Bacto-agar, and 0.025% congo red dye. Prior to autoclaving, 0.025% (v/v) of congo red dye was added to the media and the pH was adjusted to 6.8 using 12M NaOH.

Rhizobium leguminosarum ATCC 14479 grown on CR plates were incubated at 28° C for 48-72 hours, or until colonies formed. When grown in liquid media, MMW or YEM broths were inoculated with *Rhizobium leguminosarum* and grown in a 28° C shaking incubator at 200 rpm for 48-72 hours.

Escherichia coli DH5α, SM10, and BL21 (DE3) strains were grown on Luria-Bertani (LB) agar plates or in broth. The contents of LB are (in w/v): 1% tryptone, 0.5% yeast extract, 0.5% NaCl, and, when applicable, 1.5% Bacto agar.

When required, antibiotics were added into broth prior to inoculation with *R. leguminosarum* or *E. coli*, or into autoclaved agar media prior to pouring into plates. The concentration of each antibiotic used is listed in Table 2 below.

Table 2. List of Antibiotics Used and Their Concentrations

Antibiotic	**Working Concentration (µg/mL)**
Ampicillin	100
Carbenicillin	100
Tetracycline	10
Gentamycin	10
Kanamycin	50
Penicillin G	50
Nalidixic Acid	10

Genomic Extraction of *Rhizobium leguminosarum*

Rhizobium leguminosarum ATCC 14479 was first plated onto CR plate from a -80° C freezer stock and grown at 28° C for 2-3 days. A single colony was used to inoculate 3mL YEM broth supplemented with penicillin G to inhibit growth of possible contaminants. The inoculated broth was grown in a 28° C shaker until growth was visible, usually 48-72 hours. The culture was then transferred to a microcentrifuge tube and centrifuged for 10 minutes at 16,000 x *g*. With the supernatant poured off, the pellet was washed twice with 0.85% NaCl to remove the exopolysaccharide produced by rhizobia. One milliliter of TNE buffer (Appendix) was added, sample vortexed and centrifuged for 5 minutes at 16,000 x *g*. Then, 1.5 mL of 70% ethanol was added, sample vortexed and placed on ice for 15 minutes. The sample was then centrifuged and supernatant poured off. The cells were dried for about 5 minutes. Subsequently, cells were resuspended in 480 µL of TEST-LR buffer (Appendix) and placed on ice for one hour, followed by placement in -20°C freezer for 15 minutes. From the freezer the sample was placed in a 68 °C

water bath for 10 minutes. After 10 minutes, 53 µL of 10% sodium dodecyl sulfate (SDS) was added, contents mixed by inversion, and sample placed back in 68°C water bath for 15 minutes. Eighty-seven µL of 5M NaCl and 69 µL of CTAB/NaCl solution was added, the sample inverted to mix contents, and incubated for 15 minutes at 68°C. Afterward, the sample was placed in -20°C freezer for 30 minutes. Then, 650 µL of chloroform:isoamyl alcohol (24:1) was added, the sample inverted several times, and centrifuged for 10 minutes at 16,000 x *g*. The top layer was transferred to a new microcentrifuge tube to which 700 µL of CPI (chloroform: phenol: isoamyl alcohol) (25:24:1) was added. The contents were mixed by inversion, centrifuged, and the top layer removed to a new tube. The top layer was then precipitated with 1 mL 95% ethanol and the pellet containing genomic DNA resuspended in ddH_2O.

PCR Amplification of *exbB* and *exbD*

Because the genome of *Rhizobium leguminosarum* ATCC 14479 has not been sequenced, primers were designed to amplify both *exbB* and *exbD* based on *Rhizobium leguminosarum* WSM 2304, a strain with presumably high sequence similarity to *R. leguminosarum* ATCC 14479. HindIII and BamHI restriction sites were added to the 5' end of the forward and reverse primer, respectively. The primers were named pUC19F (forward primer) and pUC19R (reverse primer) (Appendix B).

Cloning and Sequencing

The PCR amplicon of *exbB* and *exbD* genes and the cloning plasmid pUC19 were digested using BamHI and HindIII restriction enzymes and ligated via T4 DNA polymerase. The recombinant plasmid was then transformed into *E. coli* DH5α and recovered via

NaOH/SDS/KAc plasmid prep method. The isolated recombinant plasmid was then sent for sequencing to the Molecular Biology Resource Facility at University of Tennessee.

Splicing by Overlap Extension (SOE)

A knockout construct of *exbB*, *exbD*, and *exbBD* was created using splicing by overlap extension (SOE) (Fig. 1), a method in which approximately 1000 base pairs of flanking regions of a gene are generated through PCR that eliminates a significant fragment or the entire gene of interest. One fragment contains the 5' end of a gene and a portion of its upstream sequences and the other fragment contains the 3' end of a gene and its downstream sequences while the central sequences are removed. The two fragments are joined together through a second round of PCR resulting in a knockout construct of the gene of interest, and in this work is designated with a Δ symbol preceding the name of the gene.

To create the 5' region of *exbB*, primers SOEFo and sglSOEbB Ri were used, and primers sglSOEbB Ri and SOERo for the creation of the 3' region of *exbB*. Likewise, for the creation of the 5' region of *exbD*, primers SOEFo and sglSOEbD Ri were used, and for the amplification of the 3' region of *exbD*, primers sglSOEbD Fi and SOERo were used. For the double mutant, primers SOEFo and dblSOE Ri were used for amplifying the 5' region, and dblSOE Fi and SOERo were used for amplifying the 3' region. The sequences of the primers used in creating Δ*exbB*, Δ*exbD*, and Δ*exbBD* knockout constructs and the regions where they bind to their corresponding genes are illustrated on a nucleotide map in Appendix B.

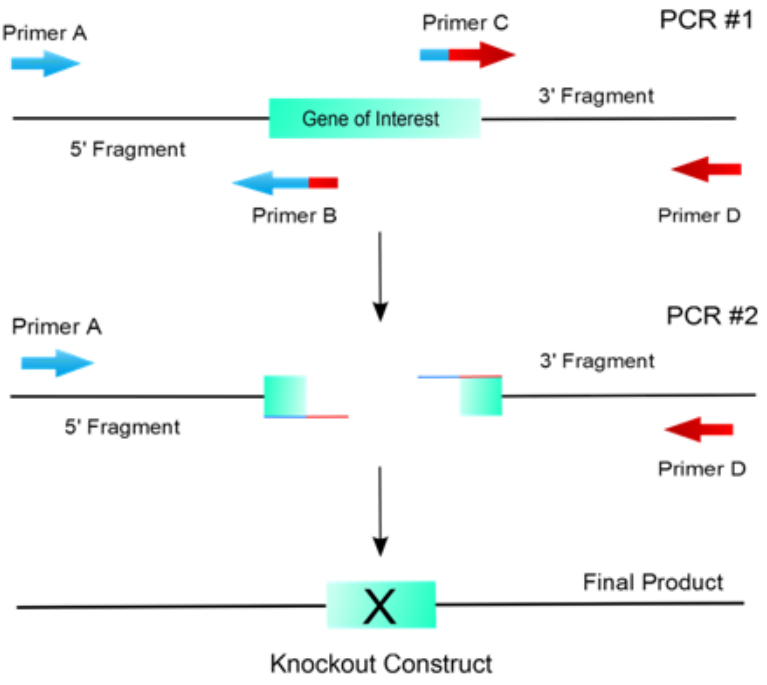


Figure 1. Illustration of Splicing by Overlap Extension

pEX18 Suicide Vector

The knockout constructs created via SOEing were ligated into the $pEX18_{Gm}$ suicide vector containing the gene for gentamycin resistance. Along with antibiotic resistance, $pEX18_{Gm}$ vectors contain a lacZα gene that allows for quick IPTG/X-Gal screening of recombinant vectors, a multiple cloning site with several common restriction sites (Hoang et al. 1998), and the counterselectable *sacB* gene that, when expressed in Gram negative bacteria in the presence of sucrose, is lethal to the cell. Once ligated, the recombinant pEX vectors were then transformed via electroporation into *E. coli* SM10λpir cells and plated onto LB_{Gm} agar plates. The plates were allowed to incubate overnight at 37°C. The following day, colonies that contained the recombinant plasmid were identified by colony PCR.

Conjugation

A single colony of SM10 that contained the recombinant vector was grown in 3 mL LB_{Gm} broth overnight on a 37° C shaker set at 200 rpm. A colony of wild type *R. leguminosarum* ATCC 14479 was grown for 48 hours in 3 mL YEM broth in 28°C on a 200 rpm rotary shaker. Both donor (SM10) and recipient (*R. leguminosarum*) cells were harvested by centrifugation and washed once with sterile 0.85% saline. With the saline supernatant decanted, the pellets were resuspended in 200 µL YEM broth. Two hundred µL of the donor was mixed with 100 µL of recipient cells and vortexed at low speed. The total volume (300µL) was then plated onto CR plates and incubated for 48 hours in a 28°C incubator. Afterward, a loopful of transconjugants was transferred to 3 mL 0.85% saline, vortexed to remove clumps and serially diluted. The dilutions were plated onto CR plates containing gentamycin and nalidixic acid ($CR_{Gm/NA}$) and incubated at 28°C until colonies appeared (approx. 5 days). Gentamycin is used to inhibit the growth of *R. leguminosarum* that has not acquired the $pEX18_{Gm}$ vector whereas nalidixic acid is used for the inhibition of *E. coli*. Once colonies were visible, colony PCR was performed to identify merodiploids. Once identified, the merodiploids were grown in 1 mL YEM broth for 6 hours on a 28°C rotary shaker to allow for a second cross-over to occur in which the mutant allele is either incorporated into the chromosome or eliminated. The cells were serially diluted and plated onto CR plates containing 5% sucrose ($CR_{5\%\ suc}$) until colonies formed (approximately 3 days). Cells that have undergone a second cross-over event will contain only one copy of the gene of interest, either the wild type or the mutant allele, and will have eliminated the $pEX18_{Gm}$ vector from the chromosome. As a result, those cells will not be susceptible to the sucrose present in the media due to the absence of the *sacB* gene and will thus survive. Surviving colonies are screened by colony PCR to identify potential mutants. Potential mutants are then

grown in YEM for approximately 48 hours, their genomic DNA isolated and used as template for PCR using primers specific for the flanking areas up- and downstream of gene of interest, and sequenced to confirm the gene knockout.

Media and Glassware Preparation for Growth Curves

All glassware used in the generation of the growth curves was treated with nitric acid to dissolve any residual iron due to hard water. To control for the amount of iron available to the mutants and wild type, 0.25mM 2,2-dipyridyl was added to all minimal media to chelate any residual Fe^{2+} remaining after nitric acid treatment. Mutants and wild type *R. leguminosarum* were grown in: Yeast Extract Mannitol, which served as complex media; Modified Manhart and Wong (MMW) minimal media supplemented with 1X vitamin solution devoid of $FeCl_3$, 0.25mM 2,2-dipyridyl and 10µM $FeCl_3$. Previous work by Wright (2010) determined 10µM $FeCl_3$ to be a sufficient concentration to serve as high iron media. For low iron media, MMW was supplemented with 1X vitamin solution, 0.25mM 2,2-dipyridyl and 0.25µM $FeCl_3$. Complex media required no nitric acid treatment or supplementation of additional iron since the concentration of iron cannot be controlled due to varying quantities of iron and other nutrients in the yeast extract.

Susceptibility to High Ionic Strength Medium

Single colonies of wild type, Δ*exbD* and Δ*exbBD Rhizobium leguminosarum* from CR plates were inoculated into 5 mL YEM seed broths each and incubated at 28°C at 250 rpm for 24-48 hours. The seed was then used to inoculate test tubes containing YEM broth of varying NaCl concentration, from 0% NaCl to 15% NaCl in 2.5% increments, or from 0% NaCl to 0.0175% NaCl in 0.0025% increments. An inoculum of each mutant and wild type in regular

YEM served as a positive control for growth. The inoculums were incubated at 28°C for 12 or 48 hours at 250 rpm. After 12 or 48 hours, OD_{600} was measured for each sample. Inoculums were then serially diluted and plated on CR until colonies formed. Once colonies were present, colony forming units (CFUs) were calculated for each inoculum.

Susceptibility to Detergent

Susceptibility of mutants and wild type to detergents was tested in the same manner as susceptibility to high ionic strength medium above. Modifications include the use of regular YEM with varying concentrations of Triton X-100 instead of NaCl. The concentrations ranged from 0% to 15% Triton X-100 in 2.5% increments. An inoculum of each mutant and wild type in regular YEM served as a positive control for growth. The inoculums were grown in YEM at 28°C for 12 or 48 hours at 250 rpm. The optical density was subsequently measured at 600 nm (OD_{600}) and CFUs calculated by serially diluting and plating inoculums onto CR plates.

Gram Stain to Detect Chaining Phenotype

Wild type, Δ*exbD* and Δ*exbBD* were grown in YEM broth containing 0.0175% NaCl for 24 hours at 28°C and 250 rpm. A loopful of each mutant and wild type from the broth was placed on a slide, allowed to dry, and heat fixed by briefly passing the slide through a flame. Crystal violet was added to the slide for one minute to cover the smear and rinsed off with dH_2O. Gram's iodine was subsequently placed on the slide for one minute and rinsed off. Then, the slide was rinsed with 95% ethanol until the runoff from the slide was clear. Finally, the smear was covered in safranin for one minute and rinsed. The slide was blot dried and visualized on the 100X oil immersion lens on the Nikon Eclipse E-200 microscope.

TonB C-terminal Expression

The 120 and 200 amino acid C-terminal fragments of TonB (Hill, 2014) were amplified via PCR using primers TonBCT and TonBR17B (Appendix B), cloned into pET17b expression vector, and transformed into BL21 (DE3) cells through electroporation. Once the fragments were confirmed to be in-frame through sequencing, colonies were grown in 3 mL LB_{amp100} broth as seed cultures at 37°C overnight. The following morning, 1 mL of each seed culture was used to inoculate 100 mL of LB_{amp100} broth until OD_{600} = 0.4-0.6. Then, IPTG was added to a final concentration of 1mM and allowed to incubate on a 37°C rotary shaker for approximately 4 hours. After induction, the cultures were then transferred to 50 mL corning tubes and centrifuged at 10,000 rpm for 5 minutes. The supernatant was discarded and the pellets were stored at -20°C until further use.

Cell Lysis and Protein Concentration Estimation

Prior to polyacrylamide gel electrophoresis, the induced BL21 (DE3) cells were lysed using B-PER Bacterial Protein Extraction Reagent (Thermo Scientific) following manufacturer's instructions or by sonication for 20 seconds followed by at least 20 seconds of cooling on ice. The procedure was repeated until the lysate was clear and no longer viscous. The lysate was centrifuged at 10,000 rpm for 15 minutes to pellet cell debris. The supernatant was transferred to new tubes and pellet discarded. The protein concentration of lysates was estimated by a Bradford assay using Bradford Reagent (Sigma-Aldrich) following manufacturer's protocol.

SDS-PAGE

TonB C-terminal fragments were visualized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). The samples were mixed with 2X Laemmli

buffer (Appendix), boiled for 5 min, and briefly centrifuged. Ten micrograms of protein was loaded onto a 12% SDS-PAGE gel (Appendix A) and run at 60V until proteins reached the bottom of stacking gel. The voltage was increased to 100V when proteins entered the resolving gel. The gel was allowed to run until loading dye reached the bottom of the gel. The gel was then stained with Coomassie Brilliant Blue overnight and destained with destaining solution containing 50:40:10 (v/v) ddH2O:methanol:acetic acid.

Western Blot

Proteins from the SDS-PAGE were electroblotted onto a PVDF membrane and probed using anti-T7 monoclonal antibodies (Novagen). After SDS-PAGE, the gel was soaked in 1X Transfer Buffer (Appendix) along with two fiber pads and Whatman No. 1 filter paper for 20 mins. Immobilon-P PVDF membrane was cut to desired size and soaked in methanol for 1 min to activate the membrane then rinsed with ddH2O prior to soaking in Transfer Buffer for 10 mins. A sandwich was then assembled in the following order: fiber pad, Whatman No. 1 filter paper, SDS-PAGE gel, Immobilon-P membrane, Whatman No.1 filter paper, fiber pad. The sandwich was clamped in a cassette, placed in the electrophoresis apparatus filled with cold 1X Transfer Buffer and an ice pack to keep the unit cool. The transfer was run at 150mA for 2 hours. Afterward, the membrane was blocked in 1X TBST with 3% BSA overnight at 4°C or at room temperature for 1 hour. The membrane was then subjected to Novagen T7 Tag® monoclonal antibody and HRP conjugate following manufacturer's protocol.

Purification of TonB C-terminal Fragment

The 120 amino acid C-terminal fragment of the TonB protein was purified using EMD Millipore's T7 Tag Affinity Purification kit. Once cells were overexpressed and pelleted, they

were resuspended in 1X Wash/Bind buffer and lysed by sonication as described above. After sonication, BL21 (DE3) *Escherichia coli* cells containing the expressed 120 amino acid fragment were pelleted by ultra-centrifugation at 30,500 rpm for 30 minutes. The supernatant was collected and filtered through a 0.45 micron filter to minimize blocking of the chromatography column. The column was equilibrated with 10 bed volumes (10 mL) of 1X Wash/Bind buffer prior to loading of the crude lysate. The lysate was loaded onto the column and the flow-through collected in a corning tube. The column was then washed with 10 bed volumes (10 mL) of 1X Wash/Bind buffer to remove unbound proteins. The bound proteins were then eluted with 5 mL 1X Elution buffer. The eluted protein was collected in 1 mL fractions in 2 mL eppendorf tubes containing 150 µL of Neutralization buffer. All flow-through and eluate was collected and analyzed on SDS-PAGE.

CHAPTER 3

RESULTS AND DISCUSSION

Sequence Analysis of *exbB* and *exbD*

Because the genome of *Rhizboium leguminosarum* ATCC 14479 has not been sequenced, the first goal of characterizing *exbB* and *exbD* was to identify and confirm that the PCR amplicons are in fact *exbB* and *exbD.* Both genes were sequenced and the sequences of putative *exbB* and *exbD* were compared with similar *Rhizobium leguminosarum* strains in the National Center for Biotechnology Information (NCBI) database using NCBI's nucleotide BLAST tool. Based on the gene sequences of *R. leguminosarum* WSM2304 strain, the expected size for the *exbB* and *exbD* amplicons was 720 and 456 base pairs, respectively. Once cloned and sequenced, the size of *R. leguminosarum* ATCC 14479 strain's *exbB* and *exbD* was 720 and 453 base pairs, respectively. When the nucleotide sequences were aligned, putative *exbB* of ATCC 14479 shared 94% identity with putative *exbB* of strain WSM2304 (Fig. 2).

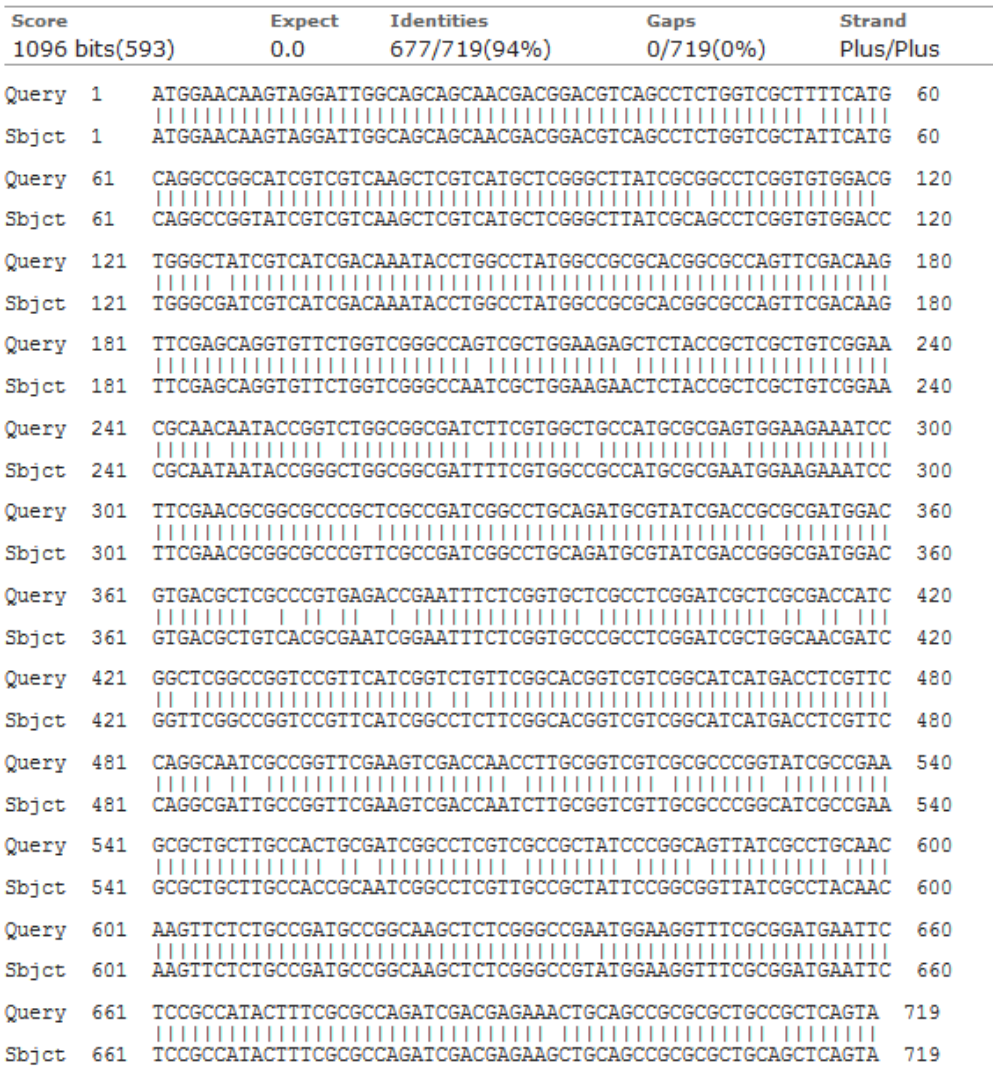


Figure 2. Nucleotide sequence alignment of putative *exbB* of *R. leguminosarum* ATCC 14479 (top) and *exbB* of *R. leguminosarum* WSM2304

Likewise, when the nucleotide sequence of putative *exbD* of *R. leguminosarum* ATCC 14479 was aligned with the putative *exbD* of strain WSM2304, the genes shared a 95% identity (Fig. 3). The expected amplicon of *exbD* was 456 base pairs, whereas the amplicon of *exbD* of *R. leguminosarum* ATCC 14479 was three base pairs shorter. The three consecutive nucleotides absent from ATCC 14479 but not strain WSM2304 in Fig. 3 accounts for this difference.

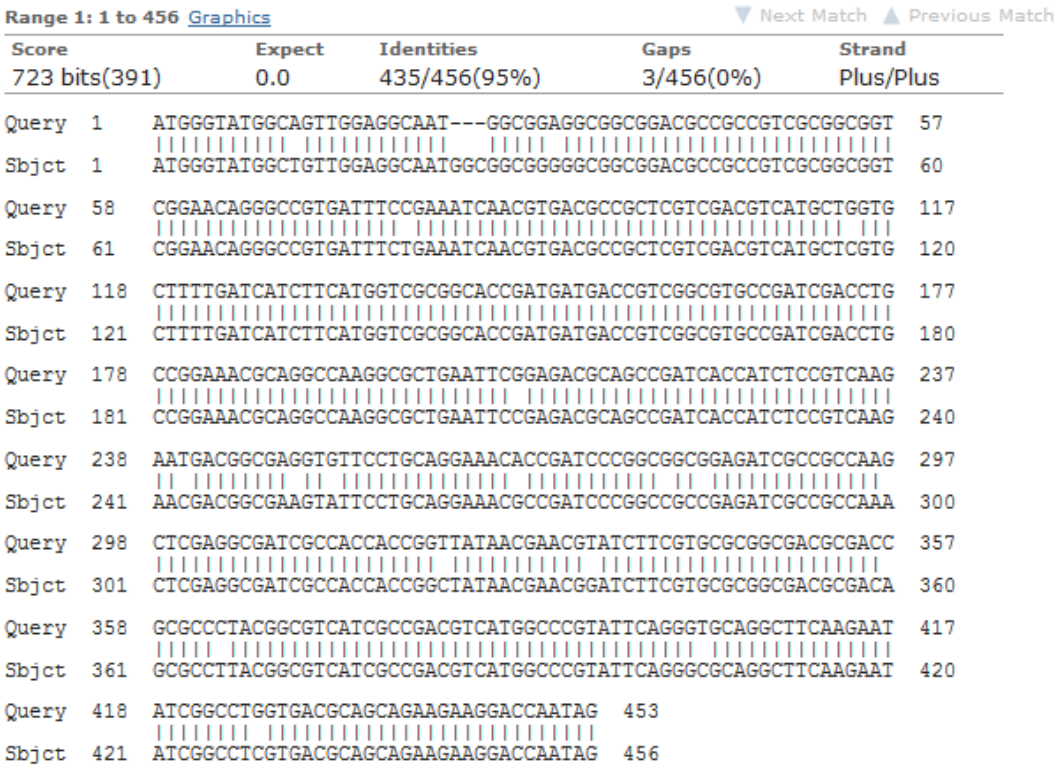


Figure 3. Nucleotide sequence alignment of putative *exbD* of *R. leguminosarum* ATCC 14479 (top) and *exbD* of *R. leguminosarum* WSM2304

The ExbB protein sequence of ATCC 14479 and strain 2304 was then aligned using ExPASy SIM alignment tool (Fig. 4). The open reading frame of *exbB* in both strains is 239 amino acids. However, the first 181 amino acids are aligned with three amino acid differences between the two strains, resulting in 98.7% identity. The remaining 58 amino acids did not share significant identity and, therefore, did not align.

```
98.7% identity in 239 residues overlap; Score: 1160.0; Gap frequency: 0.0%

14479          1 MEQVGLAAATTDVSLWSLFMQAGIVVKLVMLGLIAASVWTWAIVIDKYLAYGRARRQFDK
2304           1 MEQVGLAAATTDVSLWSLFMQAGIVVKLVMLGLIAASVWTWAIVIDKYLAYGRARRQFDK
                 ************************************************************

14479         61 FEQVFWSGQSLEELYRSLSERNNTGLAAIFVAAMREWKKSFERGARSPIGLQMRIDRAMD
2304          61 FEQVFWSGQSLEELYRSLSERNNTGLAAIFVAAMREWKKSFERGARSPIGLQMRIDRAMD
                 ************************************************************

14479        121 VTLARETEFLGARLGSLATIGSAGPFIGLFGTVVGIMTSFQAIAGSKSTNLAVVAPGIAE
2304         121 VTLSRESEFLGARLGSLATIGSAGPFIGLFGTVVGIMTSFQAIAGSKSTNLAVVAPGIAE
                 *** ** ***************************************************

14479        181 ALLATAIGLVAAIPAVIACNKFSADAGKLSGRMEGFADEFSAILSRQIDEKLQPRAAAQ
2304         181 ALLATAIGLVAAIPAVIAYNKFSADAGKLSGRMEGFADEFSAILSRQIDEKLQPRAAAQ
                 ***************** ************************************
```

Figure 4. Protein sequence alignment of ExbB of *R. leguminosarum* ATCC 14479 (top row) and ExbB of *R. leguminosarum* strain WSM2304 (bottom row) using ExPASy SIM alignment tool

Similarly with ExbD, when the protein sequences were aligned, 120 amino acids of ExbD of ATCC 14479 aligned with 121 amino acids of the ExbD of strain WSM2304. A single amino acid deletion in ATCC 14479 or an amino acid insertion in strain WSM2304 resulted in 99.3% identity between the ExbD of the two strains. Thirty residues of ExbD of ATCC 14479 and 31 residues of WSM2304 had little identity, however, and did not align.

```
99.3% identity in 151 residues overlap; Score: 731.0; Gap frequency: 0.7%

14479          1 MGMAVGGNGGGGG-RRRRGGRNRAVISEINVTPLVDVMLVLLIIFMVAAPMMTVGVPIDL
2304           1 MGMAVGGNGGGGGGRRRRGGRNRAVISEINVTPLVDVMLVLLIIFMVAAPMMTVGVPIDL
                 ************* ********************************************

14479         60 PETQAKALNSETQPITISVKNDGEVFLQETPIPAAEIAAKLEAIATTGYNERIFVRGDAT
2304          61 PETQAKALNSETQPITISVKNDGEVFLQETPIPAAEIAAKLEAIATTGYNERIFVRGDAT
                 ************************************************************

14479        120 APYGVIADVMARIQGAGFKNIGLVTQQKKDQ
2304         121 APYGVIADVMARIQGAGFKNIGLVTQQKKDQ
                 ********************************
```

Figure 5. Protein sequence alignment of ExbD of *R. leguminosarum* ATCC 14479 (top row) and ExbD of *R. leguminosarum* WSM2304 (bottom row) using ExPASy SIM alignment tool

Because little is known about the TonB complex in rhizobia, ExbB and ExbD were compared with the well-studied *Escherichia coli* to determine if any structural similarities can be observed using DomPred – a bioinformatics tool by University College London Department of Computer Science (UCL-CS) that predicts secondary structure of proteins. DomPred's predicted secondary structure of *R. leguminosarum*'s ExbB (Fig. 6) shows a protein consisting of α helices and coils but no β strands. Similarly in *E. coli*, ExbB is predicted to consist of mainly helices (Fig. 7). However, the singular difference between the two predicted protein structures is the possibility of a β strand at residue 134 in *E. coli*. It should be noted, however, that the blue bar indicating confidence of prediction at this residue is small. Likewise, when comparing the predicted secondary structure of ExbD in *R. leguminosarum* and *E. coli*, there are no structural differences observed between the two organisms (Fig. 8 and 9). The location of the helices and strands varies slightly due to the difference in the length of the protein. ExbD of *E. coli* is 9 amino acids shorter than that of *R. leguminosarum* ATCC 14479.

Figure 6. DomPred predicted secondary structure of ExbB in *R. leguminosarum*

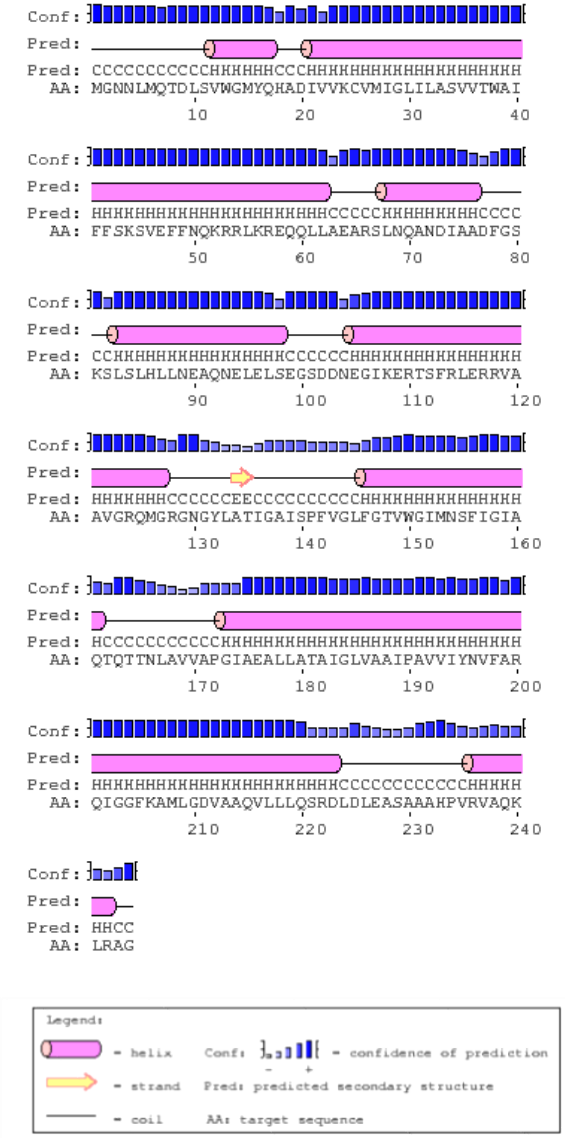


Figure 7. DomPred predicted secondary structure of ExbB in *E. coli*

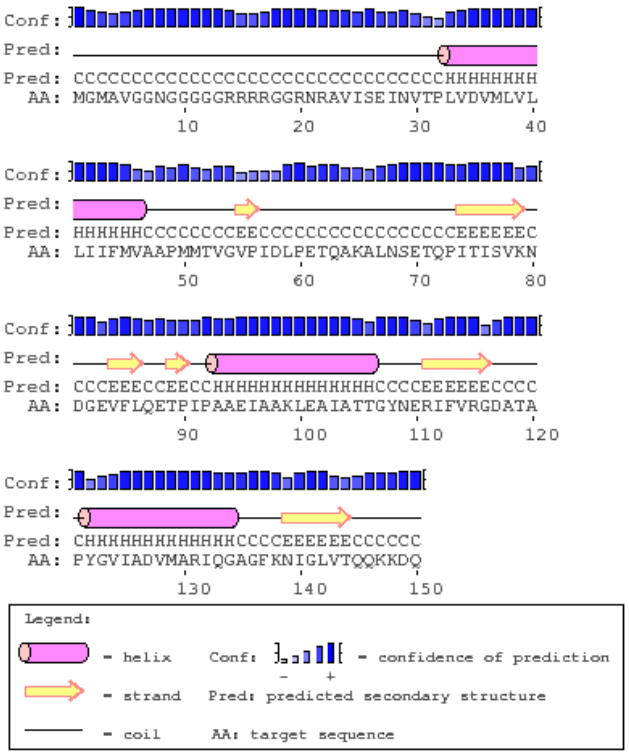


Figure 8. DomPred predicted secondary structure of ExbD in *R. leguminosarum*

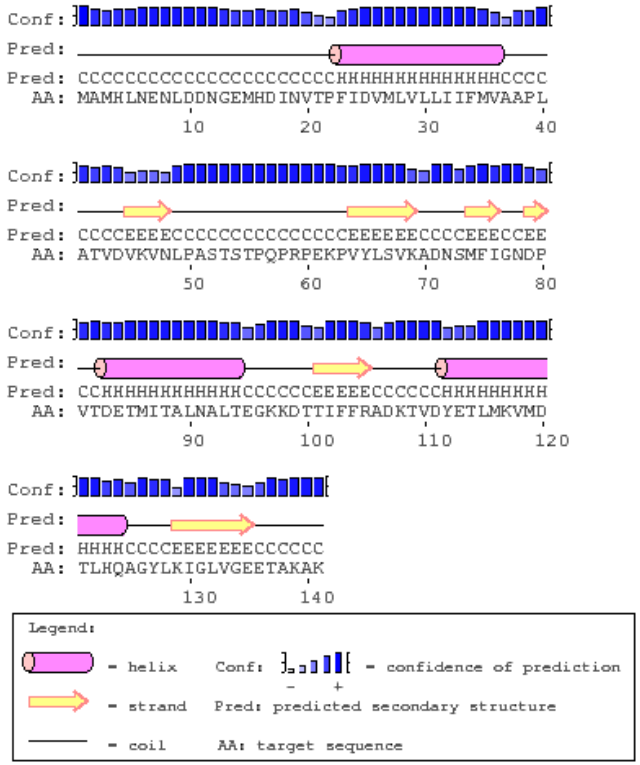


Figure 9. DomPred predicted secondary structure of ExbD in *E. coli*

Confirmation of Δ*exbD* and Δ*exbBD* Mutants

Once potential Δ*exbD* and Δ*exbBD* mutants (Fig. 10 and Fig. 11, respectively) were identified by colony PCR, they were confirmed by sequencing their genomic DNA using primers SOEFo and SOERo (Appendix B). Genomic extraction was performed as described above. Conjugations of SM10 cells harboring the pEX18$_{Gm}$ vector containing Δ*exbB* with wild type *R. leguminosarum* resulted in merodiploid cells. However, no Δ*exbB* mutants were identified. All merodiploids reverted to wild type.

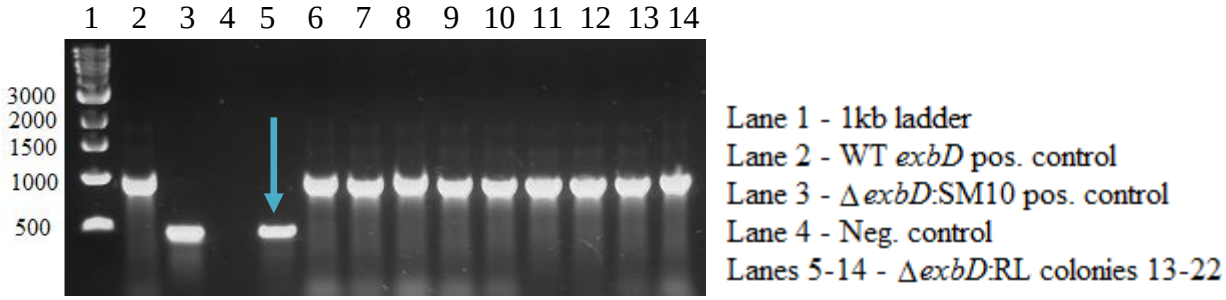


Figure 10. 1% TBE agarose gel showing the identification of a potential Δ*exbD* mutant (blue arrow)

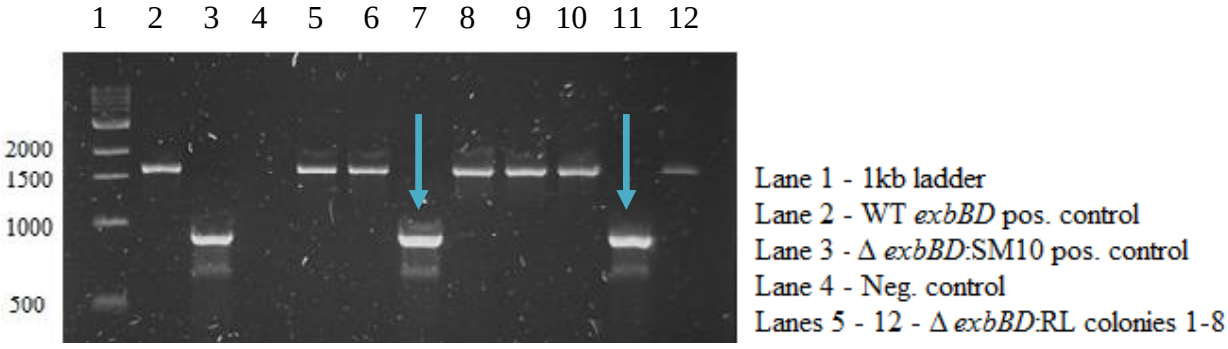


Figure 11. 1% TBE agarose gel showing the identification of potential Δ*exbBD* mutants (blue arrows)

Primers SOE2DshortF and SOERo (Appendix B) were used in screening for Δ*exbD* mutants via colony PCR. Using these primers, the expected size of the wild type amplicon was

924 base pairs and 498 base pairs for the mutant. For the screening of Δ*exbBD* mutant, primers SOE2BshortF and SOERo (Appendix B) were used. The expected amplicons were 1665 base pairs for the wild type and 890 base pairs for the mutant. Bands in Figures 10 and 11 for mutant and wild type alleles appear to be the expected size.

Δ*exbD* Complex Media Growth Curve

To assess the ability of the mutant to grow in media containing a variety of nutrients, including iron, Δ*exbD* mutant and wild type were grown in complex media for 72 hours. Figure 12 shows the growth curve of the mutant and wild type in complex media during the 72 hour incubation period. No significant difference can be observed between the growth of the mutant and the wild type. A likely explanation for the same pattern of growth for both strains is the composition of the complex media. Yeast extract is a common ingredient in rich media and contains a variety of nutrients for bacterial cultivation, including iron. Therefore, iron concentrations in complex media cannot be controlled. Enough iron may be present in complex media that microorganisms need not utilize siderophore-mediated iron transport.

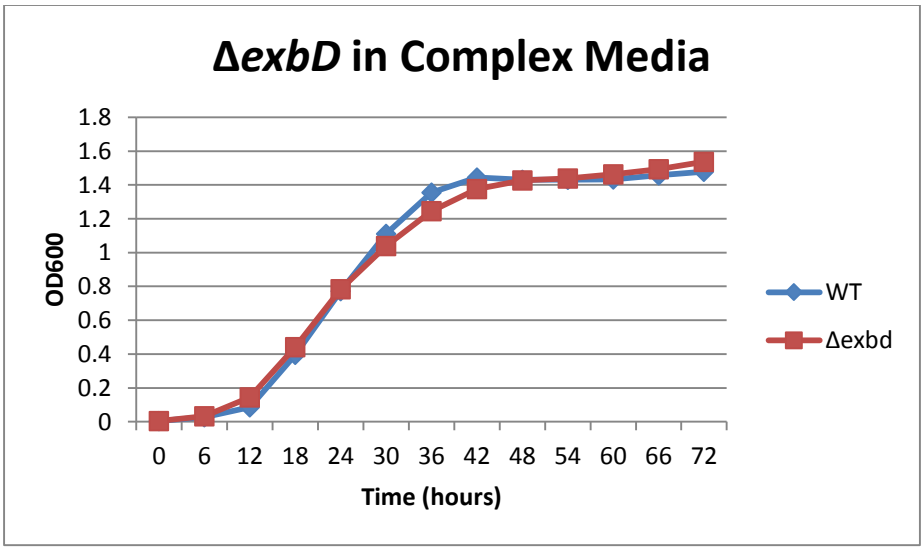


Figure 12. Growth curve of wild type and Δ*exbD* in complex media

Δ*exbD* High Iron Minimal Media Growth Curve

To further test the mutant's ability to grow in media with a limited supply of iron, mutant and wild type were each grown in minimal media containing a high concentration of iron (10 µM). Cultures were measured at OD_{600} every 6 hours for 72 hours total. The growth curve of wild type and mutant is shown in Figure 13.

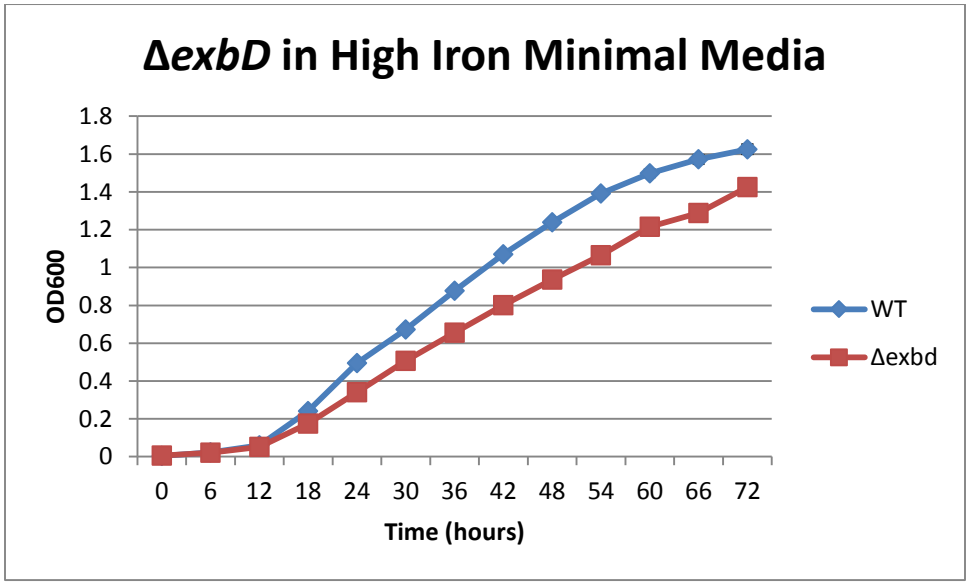


Figure 13. Growth curve of wild type and Δ*exbD* in high iron minimal media

As opposed to complex media growth curve where both mutant and wild type grew at the same rate, in high iron minimal media the mutant grew slightly slower than wild type. Because the supplemented iron in the media is in excess concentration and thus greater than intracellular concentrations, passive diffusion may be occurring in which iron does not need to be transported intracellularly through outer membrane transporters. Therefore, mutants defective in any gene(s) involved in TonB-dependent transport may still able to survive and grow when iron concentrations in the media are high enough for diffusion to occur.

Δ*exbD* Low Iron Minimal Media Growth Curve

To test the growth of the mutant when subjected to low iron conditions, mutant and wild type were grown in minimal media containing a low concentration of iron (0.25 µM). The absorbance was measured every six hours for 72 hours total and plotted, as shown in Figure 14 below.

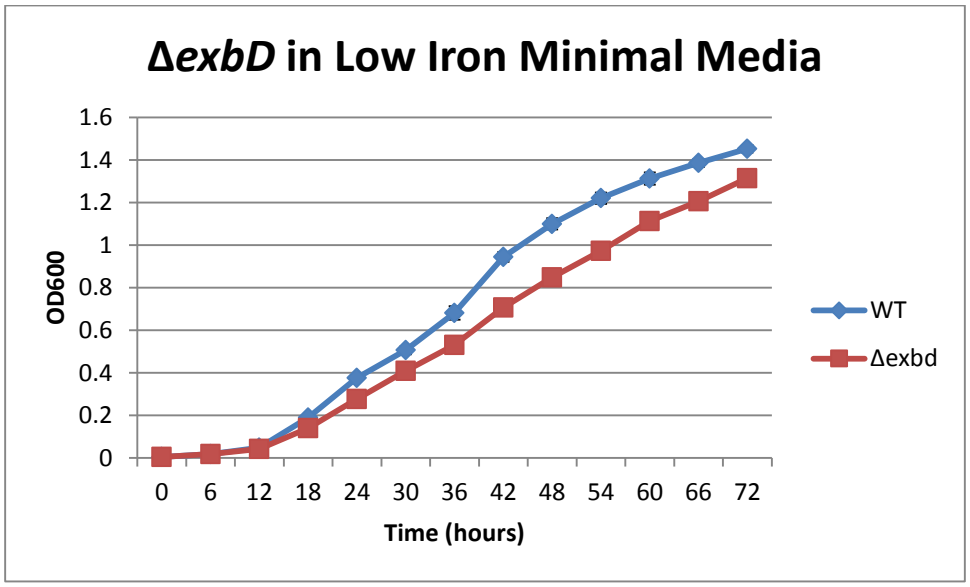


Figure 14. Growth curve of wild type and Δ*exbD* in low iron minimal media

Interestingly, the growth of the mutant was not severely affected as anticipated. The growth pattern is very similar to that of the growth in high iron media. The mutant grew only slightly slower than wild type. Because the concentration of iron in the media is too low for passive diffusion to occur, one explanation of the result may be that since only a portion of the gene has been deleted, enough of the gene remains to retain partial function. In *E. coli* studies it is known that the C-terminal of ExbD interacts with the C-terminal of TonB. Perhaps part of the C-terminal of the mutant is still able to form an interaction with TonB. Of course, other plausible explanations may be that ExbD is not as vital in *R. leguminosarum* ATCC 14479 or that another

copy of the gene is present. In fact, the presence of two copies of the same gene has been observed in various organisms, including *Xanthomonas campestris*, *Vibrio alginolyticus*, and *Pseudomonas aeruginosa* (Wang et al., 2008; Wiggerich et al., 2000; Zhao, 2000). Many species of *Vibrio*, including *V. fischerii, V. cholerae,* and *V. anguillarum*, have an additional copy of one or more of the genes of the TonB complex. In *V. alginolyticus*, two sets of *tonB-exbB-exbD* genes have been identified. Both sets of genes are involved in iron acquisition and virulence (Wang et al., 2008). Therefore, it is possible that *R. leguminosarum* may have more than one copy of the *exbB* and/or *exbD* gene. The presence of another copy of the *tonB* gene is unlikely, however. Growth and ^{55}Fe transport assays show that Δ*tonB* mutants fail to grow in low iron media (Hill, 2014). If a second *tonB* gene was present, it would be expected that the second gene would be able to at least partially compensate for the knocked-out gene. If *R. leguminosarum* does not have two copies of *exbB, exbD,* or *tonB* genes, perhaps it may have paralogs of such genes. In other organisms, ExbB and ExbD share homologies with flagellar proteins MotA and MotB, as well as with proteins TolQ and TolR – two proteins that are part of the Tol-Pal system and function to provide energy to outer membrane processes (Teleha et al., 2013). Therefore, genes may have been duplicated throughout the course of evolution and the new copies evolved functions unrelated to outer membrane energy transduction. It is also likely that the gene annotations in the NCBI database which were used to design primers were incorrect, and thus the genes have other functions unrelated to iron transport.

Δ*exbBD* Complex Media Growth Curve

After the completion of Δ*exbD* growth assay, an Δ*exbBD* double mutant growth assay was performed following same procedures used for the previous mutant, including preparation of glassware and the use of same media and concentrations of vitamin solution, iron, and 2,2-

dipyridyl. The wild type and double mutant were grown for 72 hours and their absorbance at OD_{600} measured every six hours. The plot of the complex media growth curve is shown in Fig. 15 below.

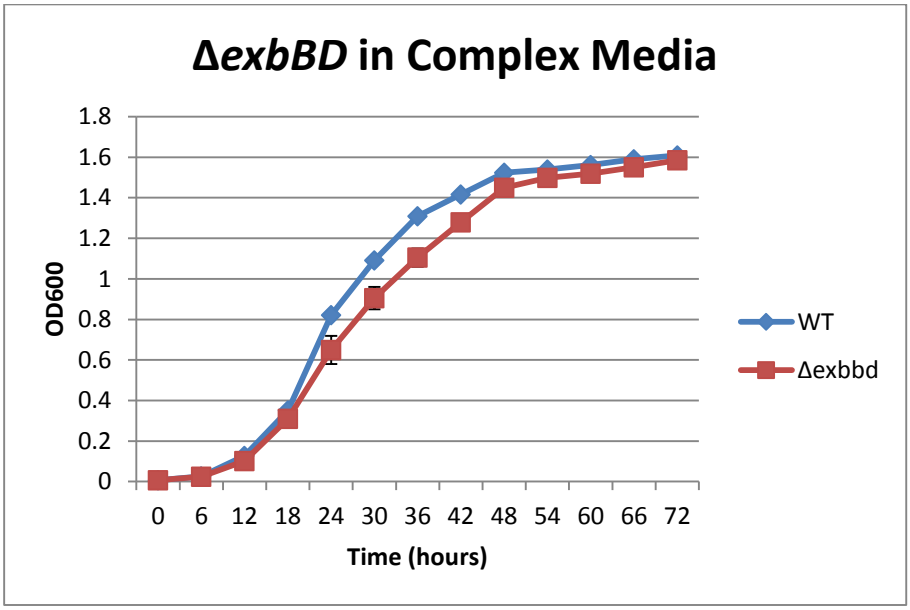


Figure 15. Growth curve of wild type and Δ*exbBD* in complex media

Much like the Δ*exbD* mutant grown in complex media, the double mutant has an almost identical growth pattern as the wild type. This is not surprising since the same conjecture can be applied to the double mutant as to the single mutant. That is, the concentrations of nutrients, including iron, cannot be controlled in a complex medium such as Yeast Extract Mannitol (YEM) broth due to the presence of yeast extract. Since the iron content of complex media is high, passive diffusion of iron occurs and may thus eliminate the need for siderophore-mediated iron acquisition.

Δ*exbBD* High Iron Minimal Media Growth Curve

When grown in minimal media containing a high concentration (10μM) of $FeCl_3$, the wild type outgrew the Δ*exbBD* mutant only slightly (Fig. 16). This pattern of growth was also observed in the Δ*exbD* growth curve (Fig. 13).

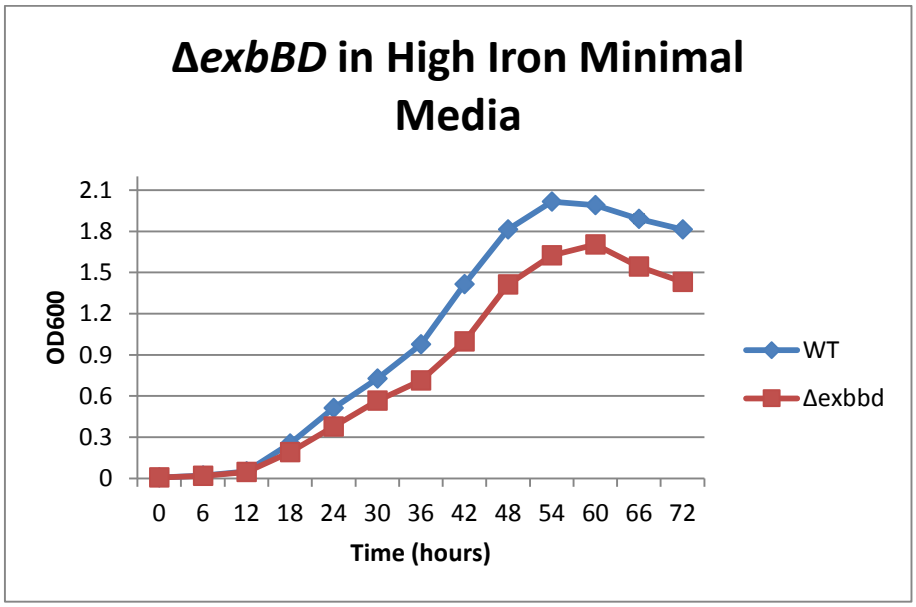


Figure 16. Growth curve of wild type and Δ*exbBD* in high iron minimal media

The double mutant grew to a slightly lower OD_{600} than the wild type. Both reached log and stationary phase at about the same time whereas cell death occurred a few hours sooner in wild type than in the mutant. In the Δ*exbD* growth curve, both wild type and mutant had not reached cell death or stationary phase after 72 hours. The results can be explained based on the same arguments given in the case of the Δ*exbD* growth curve. That is, the high concentration of iron in the media results in passive diffusion of the iron across the cell membrane. As a result, the *tonB-exbB-exbD* genes are repressed and not utilized for siderophore-mediated iron transport.

Δ*exbBD* Low Iron Minimal Media Growth Curve

Contrary to the results obtained from the growth curve of the Δ*tonB* mutant grown in low iron minimal media (Hill, 2014), the Δ*exbBD* double mutant did not fail to grow when grown in minimal media containing a low concentration (0.25μM) of $FeCl_3$ (Fig. 17). Much like the high iron minimal media growth curve (Fig. 16), the mutant in low iron media grew only slightly less than the wild type. One observable difference between high and low iron growth curves is rate of growth. Both wild type and Δ*exbBD* in high iron media entered stationary phase and subsequently cell death sooner than the wild type and Δ*exbBD* in low iron media. In fact, at the 72 hour time point, neither wild type nor Δ*exbBD* had entered stationary phase yet. Since *exbB* and *exbD* genes in other Gram negative organisms are vital to the function of the TonB complex, the growth curve in Fig. 17 is rather surprising. The low concentration of iron in the minimal media eliminates the possibility of passive diffusion occurring, as was the assumption in previous growth curves. Therefore, another alternative explanation must exist to justify the growth curve observed.

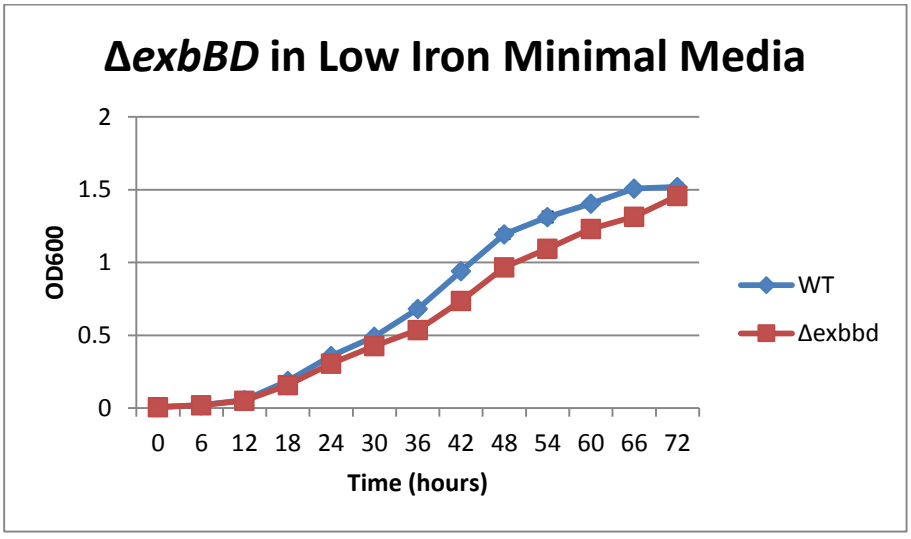


Figure 17. Growth curve of wild type and Δ*exbBD* in low iron minimal media

When constructed, the Δ*exbBD* PCR construct retained part of the 5' end of *exbB* and part of the 3' end of *exbD*. This resulted in the construct containing the start codon of *exbB* and the stop codon of *exbD* while a significant portion of the remaining sequences between the start and stop codons was eliminated. Instead of a complete elimination of both *exbB* and *exbD*, the knock-out construct retained less than a third of *exbB* and slightly less than a half of *exbD*. Therefore, it could be that the truncated protein retained partial function to interact with and transduce energy to TonB, although unlikely. The reason for the unlikelihood of such an occurrence is that neither *exbB* nor *exbD* can function independently in *E. coli* (Held et al., 2002). Both genes are part of the *exb* operon in *E. coli*, and sequence analysis of *R. leguminosarum*'s *exbB* and *exbD* genes reveals only 18 base pairs between the stop codon of *exbB* and the start codon of *exbD*. This suggests that *exbB* and *exbD* of *R. leguminosarum* may be part of an operon as well. Therefore, even if one gene remains intact while the other gene is knocked out, energy transduction still fails to occur.

Since growth is not severely affected in the Δ*exbD* or Δ*exbBD* mutant when grown under low iron conditions, as was initially expected, this raises many questions and concerns as to why growth of mutants is not hindered. The least likely possibility has been mentioned above – that part of the knocked out gene(s) retains enough nucleotide sequences to yield a partially or completely functional protein. A second, more likely possibility is that there is more than one *exbB* and/or *exbD* gene present, as was mentioned earlier. Several Gram negative organisms have been reported to contain more than one copy of the *exbB-exbD-tonB* genes. If such is the case in *R. leguminosarum*, then the intact copy of the gene is partially compensating for the mutated gene. Since the growth of both mutants in minimal media was only slightly hindered, both copies of the gene(s), if present, are most likely required for optimal growth. Or, each copy

of the gene is indirectly responsible for the transport of iron from different sources. If iron transport occurs via different transporters, it is possible that one copy or set of *exbB-exbD-tonB* genes interacts with one type of transporter and another set with a different type of transporter. In addition to iron-siderophore transport, the TonB complex is also involved in cyanocobalamin transport in *E. coli* via the BtuB outer membrane transporter (Cadieux et al. 2000). Therefore, different nutrients in addition to iron may be transported by TonB-dependent transporters (TBDTs) in *Rhizobium leguminosarum*. Currently no TBDTs have been characterized in *R. leguminosarum*. Consequently, it remains unclear whether *exbB* and/or *exbD* genes are involved in the transport of other nutrients.

Since low concentrations of iron in minimal media do not affect Δ*exbD* and Δ*exbBD* mutants' growth, iron transport across the outer and inner membrane must still be occurring to some extent, as evident by Fig. 17. Therefore, since a significant mutation in the two genes which may play a key role in siderophore-mediated iron transport bears no significant consequence to the organism's survival and growth, other factors must be taken into consideration to explain the results obtained. A few possibilities have been mentioned above, such as presence of more than one copy of each or both genes, or the possibility that the genes are energizing transporters involved in the transport of some nutrient other than iron. However, further inspection of the sequences obtained for *exbB* and *exbD* reveal a new insight which may explain the oddities in the data. When aligned with most similar sequences in the NCBI database using BLAST, nucleotide sequence of *exbB* in *R. leguminosarum* ATCC 14479 most closely aligns with a "putative TolQ protein uptake system component" of *R. leguminosarum* biovar viciae, strain 3841. However, the second most similar sequence alignment is with the "biopolymer transporter ExbB" of *R. leguminosarum* biovar trifolii, strain WSM1689.

Alignments of *R. leguminosarum* ATCC 14479 with strain 3841 and strain WSM1689 both share 96% identity and zero gaps, yet the genes are functionally very different. Likewise, amino acid sequence alignment of ExbB shows most similarity to TolQ and ExbB proteins with 99% identity (Fig. 18).


TolQ protein uptake system component [Rhizobium leguminosarum bv. viciae 3841]
Sequence ID: ref|YP_769550.1| Length: 239 Number of Matches: 1
See 4 more title(s)
MULTISPECIES: biopolymer transporter ExbB [Rhizobium]
Sequence ID: ref|WP_003542733.1|
putative TolQ protein uptake system component [Rhizobium leguminosarum bv. viciae 3841]
Sequence ID: emb|CAK09463.1|
TolQ protein [Rhizobium leguminosarum bv. viciae WSM1455]
Sequence ID: gb|EJC67957.1|
biopolymer transporter ExbB [Rhizobium leguminosarum bv. trifolii WSM1689]
Sequence ID: gb|AHF85516.1|
Range 1: 1 to 239 GenPept Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
471 bits(1212)	3e-166	Compositional matrix adjust.	238/239(99%)	238/239(99%)	0/239(0%)

```
Query  1    MEQVGLAAATTDVSLWSLFMQAGIVVKLVMLGLIAASVWTWAIVIDKYLAYGRARRQFDK  60
            MEQVGLAAATTDVSLWSLFMQAGIVVKLVMLGLIAASVWTWAIVIDKYLAYGRARRQFDK
Sbjct  1    MEQVGLAAATTDVSLWSLFMQAGIVVKLVMLGLIAASVWTWAIVIDKYLAYGRARRQFDK  60

Query  61   FEQVFWSGQSLEELYRSLSERNNTGLAAIFVAAMREWKKSFERGARSPIGLQMRIDRAMD  120
            FEQVFWSGQSLEELYRSLSERNNTGLAAIFVAAMREWKKSFERGARSPIGLQMRIDRAMD
Sbjct  61   FEQVFWSGQSLEELYRSLSERNNTGLAAIFVAAMREWKKSFERGARSPIGLQMRIDRAMD  120

Query  121  VTLARETEFLGARLGSLATIGSAGPFIGLFGTVVGIMTSFQAIAGSKSTNLAVVAPGIAE  180
            VTLARETEFLGARLGSLATIGSAGPFIGLFGTVVGIMTSFQAIAGSKSTNLAVVAPGIAE
Sbjct  121  VTLARETEFLGARLGSLATIGSAGPFIGLFGTVVGIMTSFQAIAGSKSTNLAVVAPGIAE  180

Query  181  ALLATAIGLVAAIPAVIACNKFSADAGKLSGRMEGFADEFSAILSRQIDEKLQPRAAAQ  239
            ALLATAIGLVAAIPAVIA NKFSADAGKLSGRMEGFADEFSAILSRQIDEKLQPRAAAQ
Sbjct  181  ALLATAIGLVAAIPAVIAYNKFSADAGKLSGRMEGFADEFSAILSRQIDEKLQPRAAAQ  239
```

Figure 18. NCBI protein BLAST of ExbB from *R. leguminosarum* ATCC 14479 (top) with other most similar sequences

Additionally, when the nucleotide and amino acid sequence of *exbD* of *R. leguminosarum* ATCC 14479 is aligned with other most similar sequences, it aligns most closely with both *exbD* and *tolR* genes (or proteins) of various *R. leguminosarum* strains with a 99% identity (Fig. 19). It has been reported that TolQ and TolR share structural homologies to ExbB and ExbD and to the flagellar proteins MotA and MotB. Therefore, it is likely that *exbB* shares significant sequence similarity with *tolQ* and *exbD* with *tolR* genes. If the sequences between *exbB/exbD* and

tolQ/tolR are similar enough that the primers used to amplify *exbB* and *exbD* were able to instead bind to *tolQ* and *tolR*, then the PCR product of *exbB* and/or *exbD* would not be discernible from *tolQ* and/or *tolR*. Neither ExbB/ExbD nor TolQ/TolR has been extensively studied in rhizobia. Therefore, the gene assignments for *R. leguminosarum* in the NCBI database are currently putative. If the homologs are highly similar to each other, it is possible that the gene assignments are incorrect, or were incorrect at the time when primers were designed. Genes labeled as *exbB* and *exbD* may, in fact, be *tolQ* and *tolR*.

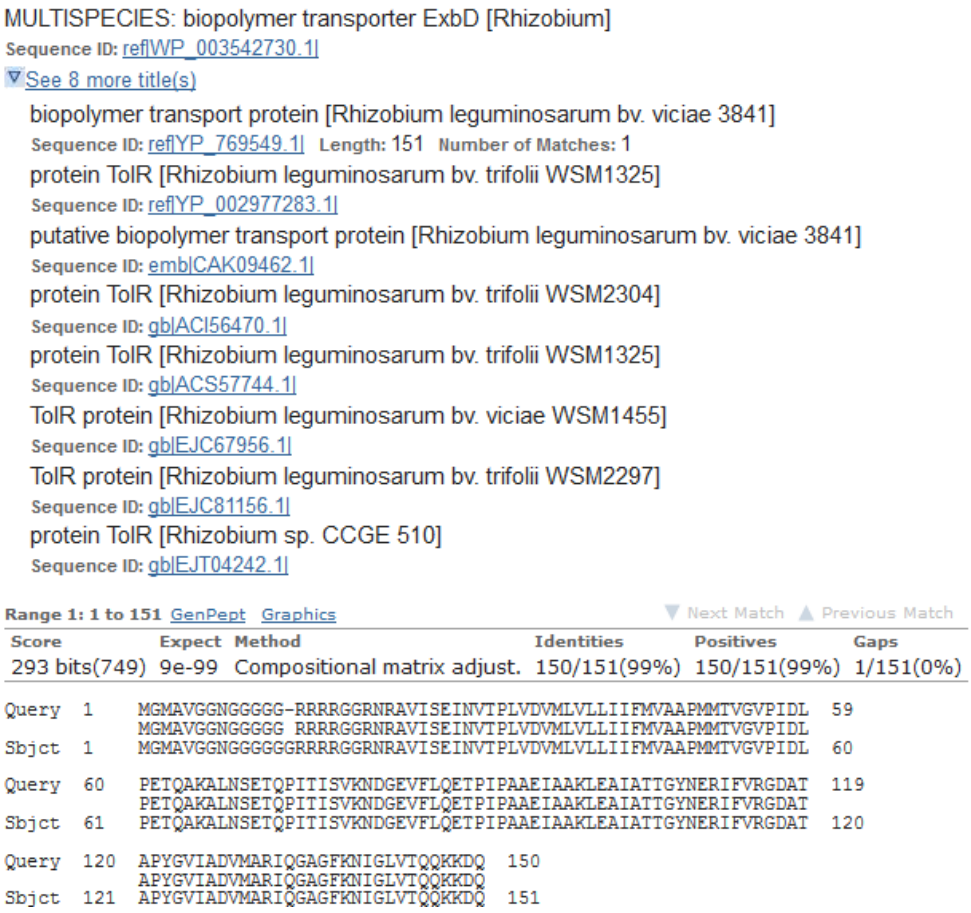


Figure 19. NCBI protein BLAST of ExbD from *R. leguminosarum* ATCC 14479 (top) with other most similar sequences

When the potential *exbB* and *exbD* genes were first identified in *Rhizobium leguminosarum* ATCC 14479 via PCR, the forward primer was located a significant portion upstream of the potential *exbB* transcriptional start codon. This was done so that the genes in their entirety are amplified without the risk of mistaking a nearby start codon with the correct one, and thus reducing the actual size of the gene(s). As a result of the primer binding significantly upstream of what was believed to be *exbB*, the gene upstream was sequenced as well. When compared against the NCBI database, the gene shows significant identity to an acyl-coenzyme A (CoA) thioesterase gene and a 4-hydroxybenzoyl-CoA thioesterase gene in various *Rhizobium* strains, including *R. leguminosarum* and *R. etli*. The gene codes for a 225 amino acid protein found in many Gram negative organisms. Acyl-CoA thioesterases catalyze the hydrolysis of acyl-CoA into a free fatty acid and CoA (Hunt et al., 2002), whereas 4-hydroxybenzoyl CoA thioesterases hydrolyze 4-hydroxybenzoyl CoA into 4-hydroxybenzoate and CoA (Song et al., 2012). A review of recent literature pertaining to bacterial acyl coenzyme A thioesterases indicates that they may be part of the conserved *tol-pal* system found in Gram-negative bacteria. *ybgC* is one of seven genes belonging to the *tol-pal* gene cluster that has been identified as having thioesterase activity toward acyl-CoA (Zhuang et al., 2002). This gene is found directly upstream of *tolQ* in various Gram negative organisms, including *Haemophilus infuenzae*, *Pseudomonas aeruginosa, Helicobacter pylori,* and *Agrobacterium vitis*. Therefore, it is very likely that the putative *exbB* and *exbD* genes initially identified in *Rhizobium leguminosarum* ATCC 14479 are instead *tolQ* and *tolR.* As mentioned above, this may be due to incorrect gene assignments in the NCBI database which resulted in incorrect primers binding to the highly similar *tolQ* and *tolR*.

Sequencing of TolA

Since the growth of Δ*exbD* and Δ*exbBD* mutants is not affected under low-iron conditions, and due to the presence of a Tol-Pal-associated acyl-CoA thioesterase gene upstream of *exbB,* additional sequencing of genes downstream of *exbD* was necessary to confirm the identity of the putative *exbB* and *exbD* genes. A primer binding to the 3' region of the putative *exbD* was designed and used to sequence the gene downstream. About 540 reliable base pairs were sequenced and analyzed using NCBI's nucleotide BLAST tool. Out of nine highly similar alignments, eight show similarity to an unknown "hypothetical protein" or "signal peptide protein" in various *Rhizobium* species. However, one alignment shows 93% identity to a "putative TolA outer membrane protein" in *Rhizobium leguminosarum* bv. viciae 3841 (Fig. 20).

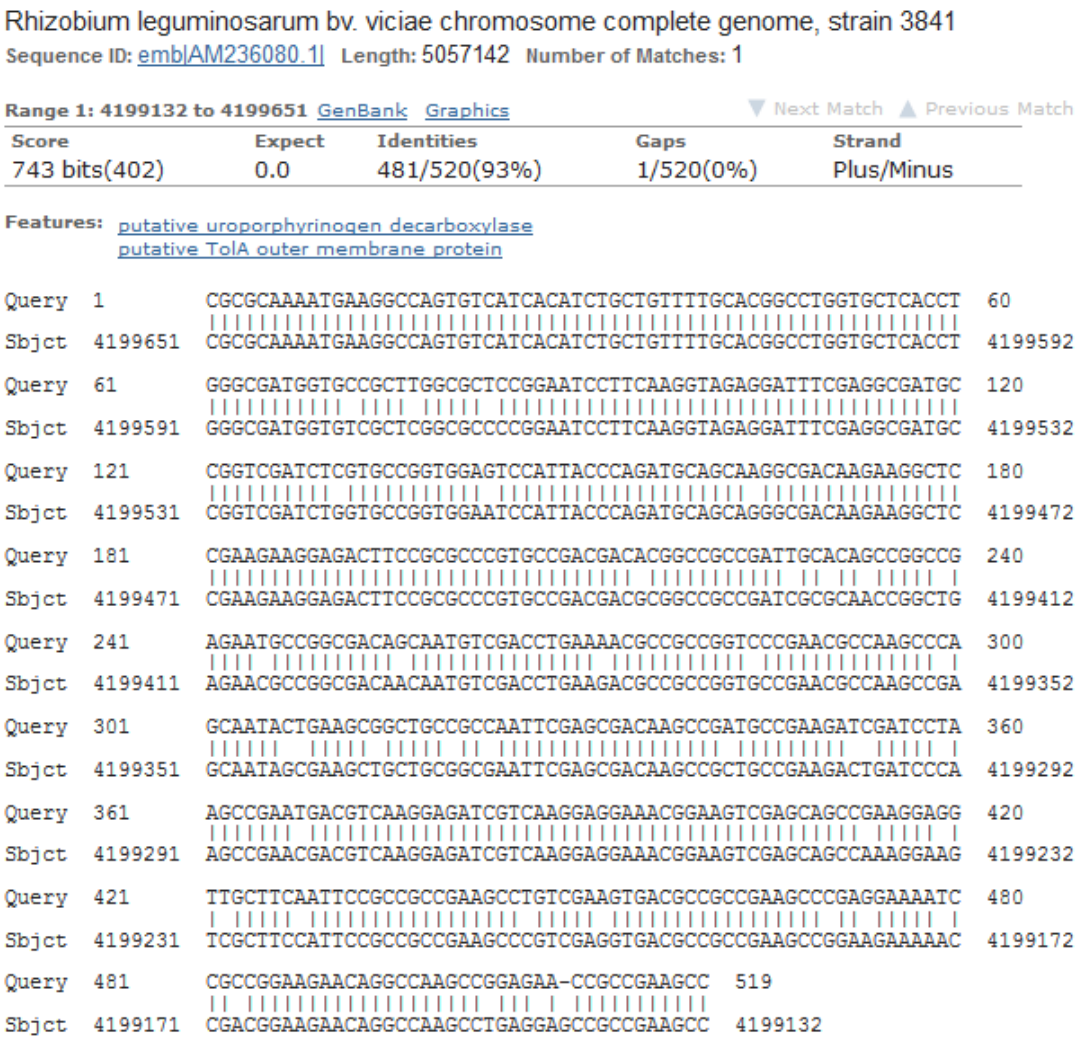


Figure 20. Nucleotide BLAST of putative *tolA* of *R. leguminosarum* ATCC 14479 with putative *tolA* of *R. leguminosarum* WSM2304

A protein BLAST revealed similar results, with majority of alignments identifying a similarity to a hypothetical protein. As with the nucleotide BLAST, one alignment shows a 96% protein similarity with a "putative TolA outer membrane protein" in *R. leguminosarum* bv. viciae 3841. Several other alignments listing a putative TolA protein ranging from 51% to 92% identity are found in *R. etli, R. rubi, Ensifer adhaerens,* and *Agrobacterium tumefaciens*. All four species belong to the Rhizobiaceae family. Literature review and NCBI's protein database show TolA as a membrane-anchored protein of the TolQ-TolR-TolA complex belonging to the conserved Tol-

Pal system. Taken together, growth curves and sequence analyses of the two *tolpal*-associated genes *ybgC* and *tolA* suggest that the identified putative *exbB* and *exbD* genes may instead be *tolQ* and *tolR.* Additional experiments are required to confirm the identity of the genes. However, throughout this work, wild type and mutant *exbB* and *exbD* genes will now be referred to as *tolQ* and *tolR*, respectively.

Tol-Pal System

The Tol-Pal system is a conserved protein complex in Gram negative bacteria consisting of TolQ, TolR, TolA, TolB, Pal (peptidoglycan associated lipoprotein), YbgC, and YbgF proteins. The Tol-Pal system plays a role in outer membrane integrity and group A colicin and bacteriophage transport (Derouiche, 1995). In *E. coli*, *tolB, pal,* and *ybgF* are arranged in one gene cluster and the remaining four genes in a second cluster (Gerding et al., 2007). TolA is a single transmembrane domain protein with a cytoplasm-anchored N-terminus, a periplasmic central region and a C-terminus involved in colicin transport. TolQ contains three transmembrane domains, a periplasmic N-terminus and a cytoplasmic C-terminus. TolR is a single transmembrane domain protein with a short cytoplasmic N-terminus and a periplasmic C-terminus (Derouiche, 1995). The topology of TolA, TolQ and TolR is identical to that of TonB, ExbB and ExbD, respectively, in the number of transmembrane domains and the arrangement of the N- and C-terminal domains. TolAQR interact with each other via their transmembrane domains, forming a complex, while the outer membrane-anchored Pal interacts with the periplasmic TolB as well as the C-terminal of TolA, forming a second complex (Lahiri et al., 2011, Santos et al., 2014). When assembled, the two complexes bridge together the outer membrane, periplasm, and inner membrane through the interaction of TolA with Pal (Santos et al., 2014, Cascales et al., 2000). YbgC and YbgF are cytoplasmic and periplasmic proteins,

respectively, of the Tol-Pal complex. However, deletions of either *ybgC* or *ybgF* do not result in a *tol* phenotype (Cascales et al., 2004).

The Tol-Pal complex has been implicated in a variety of functions, including maintenance of outer membrane integrity, transport of bacteriophages and group A colicins, motility, and proper invagination of the outer membrane prior to cell division (Gerding et al., 2007). A recent study demonstrated the importance of an intact Tol-Pal complex for polar localization of chemoreceptors in *E. coli*, as well as its role in cell motility and chemotaxis (Santos et al., 2014). Mutants defective in all Tol and Pal proteins were found to have disturbed localization of chemoreceptors and an increase in the tumbling frequency of cells. Swimming and swarming assays also revealed a decrease in motility of each single-gene *tol* and *pal* mutant (Santos et al., 2014). Furthermore, the Tol-Pal complex is required for the proper outer membrane invagination during binary fission, and single-gene mutants defective in *tolA* and *pal*, as well as mutants lacking all Tol and Pal proteins, exhibit a chaining phenotype indicative of the inability of the outer membrane to fully invaginate and the cell to separate. The tendency for *tol* and/or *pal* mutants to form chains is observed when mutants are grown in low osmolarity or high ionic strength rich medium. The phenotype is reversed when osmolarity in the medium is increased (Gerding et al., 2007). In addition to perturbations in chemotaxis, motility, cell division, and chemoreceptor localization, mutations in any *tol* or *pal* genes result in a compromise of outer membrane integrity, and thus leakage of periplasmic proteins and susceptibility to detergents and other toxic compounds (Lahiri et al., 2011).

To further characterize the roles of the putative *tolQ* and *tolR* genes of *Rhizobium leguminosarum*, the phenotypic characteristics of Δ*tolR* and Δ*tolQR* mutants were assessed and their susceptibility to detergents and changes in osmolarity tested.

Gram Stain of Δ*tolR* and Δ*tolQR*

To determine whether a chaining phenotype can be observed in Δ*tolR* and Δ*tolQR* mutants, each mutant and wild type was grown in YEM broth with 0.0175% NaCl until mid-log phase. The aforementioned concentration of NaCl was chosen based on the first susceptibility to high ionic strength medium assay. Because growth of mutants and wild type was severely affected at 2.5% NaCl and above, the NaCl concentration was reduced to 0.0175%. The cultures of each mutant and wild type were subsequently used to perform a Gram stain. The slides were visualized on Nikon Eclipse E-200 100X oil immersion lens.

When Gram stained, wild type *R. leguminosarum* was observed as mostly a single rod shaped cell (bacillus), and each cell was approximately the same length and width (Fig. 21A). Each Δ*tolR* mutant cell also appeared as a bacillus of uniform size and length, much like the wild type (Fig. 21B). No chaining phenotype was observed which suggests the mutant's ability for each cell to separate during binary fission is unaffected. This could be due to an inadequate concentration of NaCl to induce the chaining phenotype, or the mutation of the gene has no effect on the outer membrane invagination and cell separation. The Gram stain of the double mutant also showed no discernible difference from the single mutant or wild type (Fig. 21C).

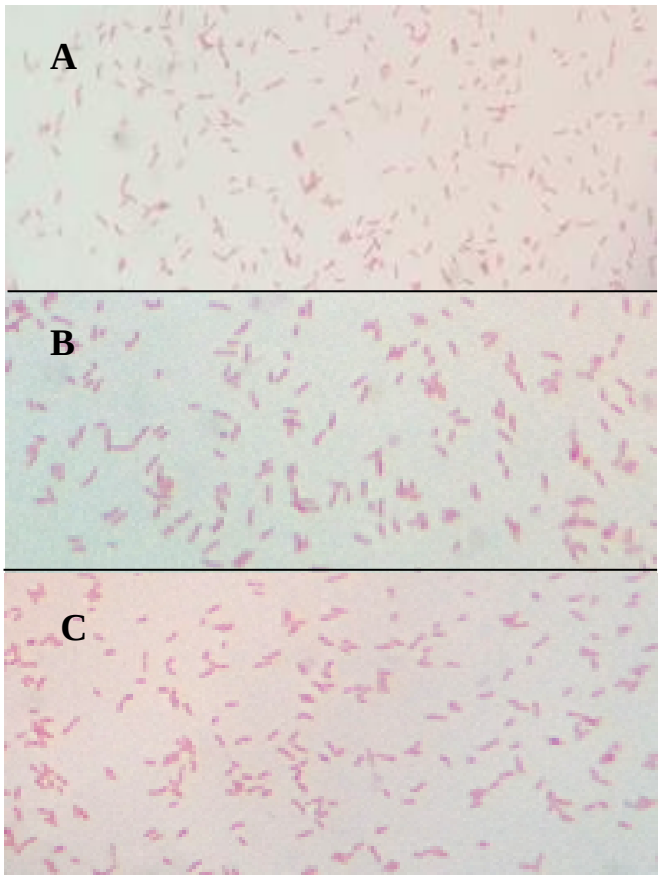


Figure 21. Gram stain of A) wild type, B) Δ*tolR*, and C) Δ*tolQR R. leguminosarum* grown in YEM with 0.0175% NaCl added

Because the Tol-Pal proteins bridge the outer and inner membrane through the interaction of Pal with TolA, mutations in *tolA* and/or *pal* may affect septation. *E. coli* Δ*tolA* mutants form long chains of cells when grown in low osmolarity or high ionic strength rich medium (Meury, 1999). Likewise, Δ*pal* and Δ*tolpal* (mutant defective in all *tolpal* genes, excluding *ybgC* and *ybgF*) mutants form long multi-septate chains of cells when grown in Luria-Bertani (LB) broth containing no added NaCl (Gerding et al., 2007). This chaining phenotype has been observed in *Vibrio cholerae, Pseudomonas putida,* and *Erwinia chrysanthemi* (Heilpern et al., 2000; Llamas et al., 2000). However, no research on the Tol-Pal system in *Rhizobium* has been published. The

formation of multi-septate chains may not be a characteristic of Δ*tolpal* mutants in *R. leguminosarum*. Or, TolQ and/or TolR mutations might not affect the septation process since the interaction of TolA with Pal may be independent of TolQ and TolR in rhizobia. In other organisms, such as *E. coli*, the conformational change in TolA that allows for its interaction with Pal is dependent upon TolQ, TolR, and pmf (Germon et al., 2001). The three proteins interact with each other through their transmembrane domains, and this interaction is required to couple and transduce the pmf of the cytoplasmic membrane to TolA. Since crosstalk between TolQ-TolR-TolA and ExbB-ExbD-TonB has been observed (Braun et al., 1993), it is possible that ExbB and ExbD are complementing the Δ*tolR* and Δ*tolQR* mutants. In *E. coli*, group A and group B colicins are imported by the TolQ-TolR-TolA and ExbB-ExbD-TonB complexes, respectively. However, when Δ*tolQR* mutants are transformed with a plasmid containing *exbB* and *exbD*, the mutants become sensitive to group A colicins, suggesting an interaction of ExbB and ExbD with TolA. Likewise, in Δ*exbBD* mutants carrying a plasmid containing *tolQ* and *tolR*, sensitivity to group B colicins is increased, indicating that TolQ and TolR are interacting with TonB (Braun et al., 1993). Since this complementation occurs in *E. coli*, it is likely that it may also occur in *R. leguminosarum*, especially since the sequence similarities between the two transport systems is substantial (Fig. 18 and Fig. 19). As a result, the phentotype characteristic of Tol-Pal mutants, such as the formation of long multi-septate chains, would not be observed if the interaction of TolA with Pal is assisted by ExbB and ExbD.

To assess whether low osmolarity medium would trigger a chaining phenotype, mutant and parental strains were grown in YEM devoid of all salts (NaCl, $MgSO_4$, and K_2HPO_4). The cultures were grown under the same conditions as those in high ionic strength medium above. A Gram stain was performed on all three strains. No differences were observed between the strains

grown in YEM media containing no salts and those grown in YEM with 0.0175% added NaCl (data not shown). This further suggests that mutations in the TolQR proteins do not prevent the separation of cells during binary fission.

Susceptibility to Detergents

Because the Tol-Pal system has been shown to be involved in outer membrane integrity, mutations in *tolpal* genes lead to sensitivities to detergents and to environments with low osmolarity or high ionic strength. To assess the susceptibility of Δ*tolR* and Δ*tolQR* mutants to detergents, mutants and wild type were inoculated into YEM broth containing 0% to 15% Triton X-100 detergent in 2.5% increments. All inoculums were adjusted to have approximately the same initial OD_{600}. The inoculums were incubated for 48 hours at 250 rpm and 28°C. Subsequently their OD_{600} was measured and dilutions of inoculums plated onto CR agar plates. Once colonies appeared, the CFUs were calculated.

The OD_{600} measurements were approximately the same for wild type and mutant strains (Fig. 22A). The growth of each strain decreased with increasing concentration of Triton X-100; however, there was no significant difference between wild type and mutant strains. Since absorbance measurements do not distinguish between live and dead cells, the inoculums were serially diluted and plated onto CR plates to determine the number of viable cells. Figure 22B shows no significant difference between the viability of wild type and mutant strains when grown in the presence of 5% to 15% detergent. The number of viable cells in media containing 12.5% and 15% Triton X-100 is too small to be visible in Fig. 22B. However, the viability was approximately the same for parental and mutant strains.

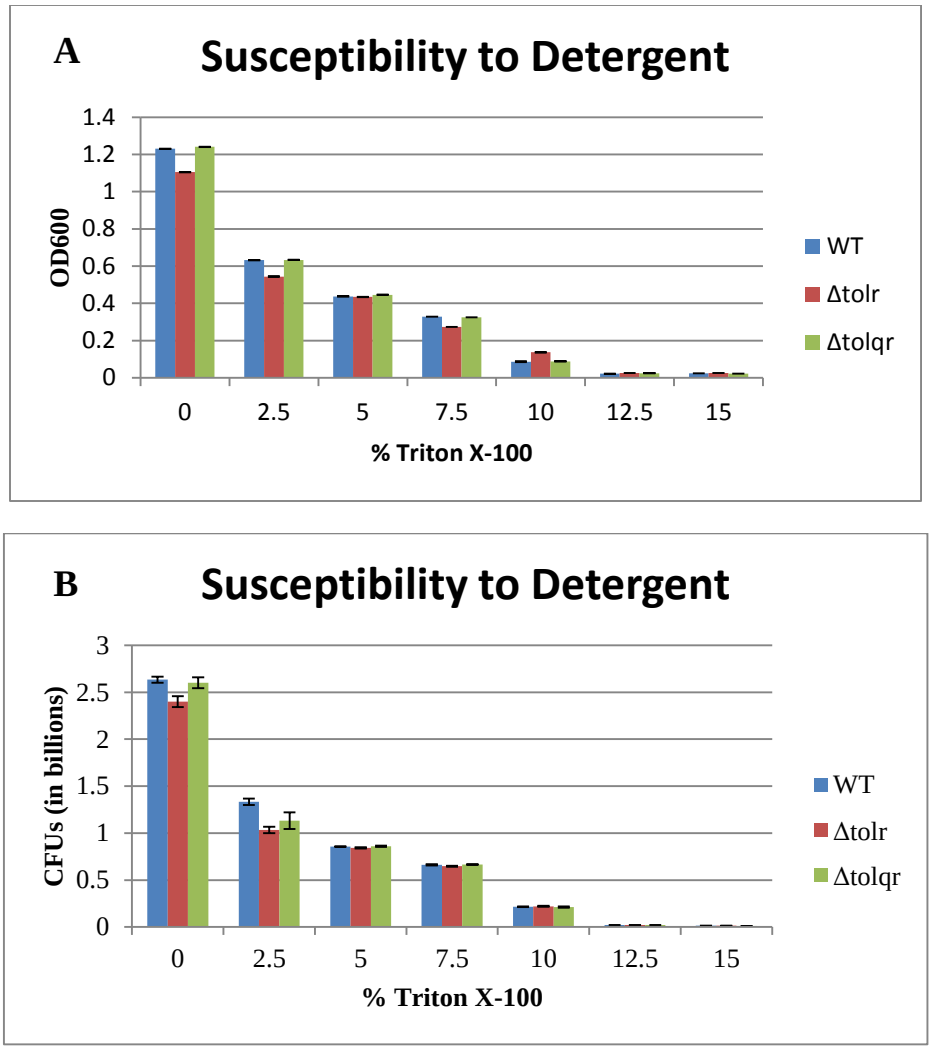


Figure 22. Graph of the A) the OD_{600} measurements and B) colony forming units (CFUs) of wild type (blue), Δ*tolR* (red), and Δ*tolQR* (green) grown in media containing varying concentrations of Triton X-100. Error bars represent standard error

Because there was no considerable difference between wild type and mutant strains, it was thought that the vast amount of exopolysaccharide (EPS) typically produced by *R. leguminosarum* may be protecting the cell from the damaging effects of detergents. To determine if exopolysaccharide production plays a role in the mutants' resistance to Triton X-100, cells were washed twice with 0.85% saline to remove the EPS. The washed cells were resuspended in ddH_2O and inoculated into tubes containing the appropriate concentration of Triton X-100. The cultures were grown for 48 hours with measurements taken at 12 and 48 hour time points.

Twelve hour aborbance measurements and CFU calculations were performed since the amount of EPS production would be significantly less than at 48 hours (Fig. 23A and B).

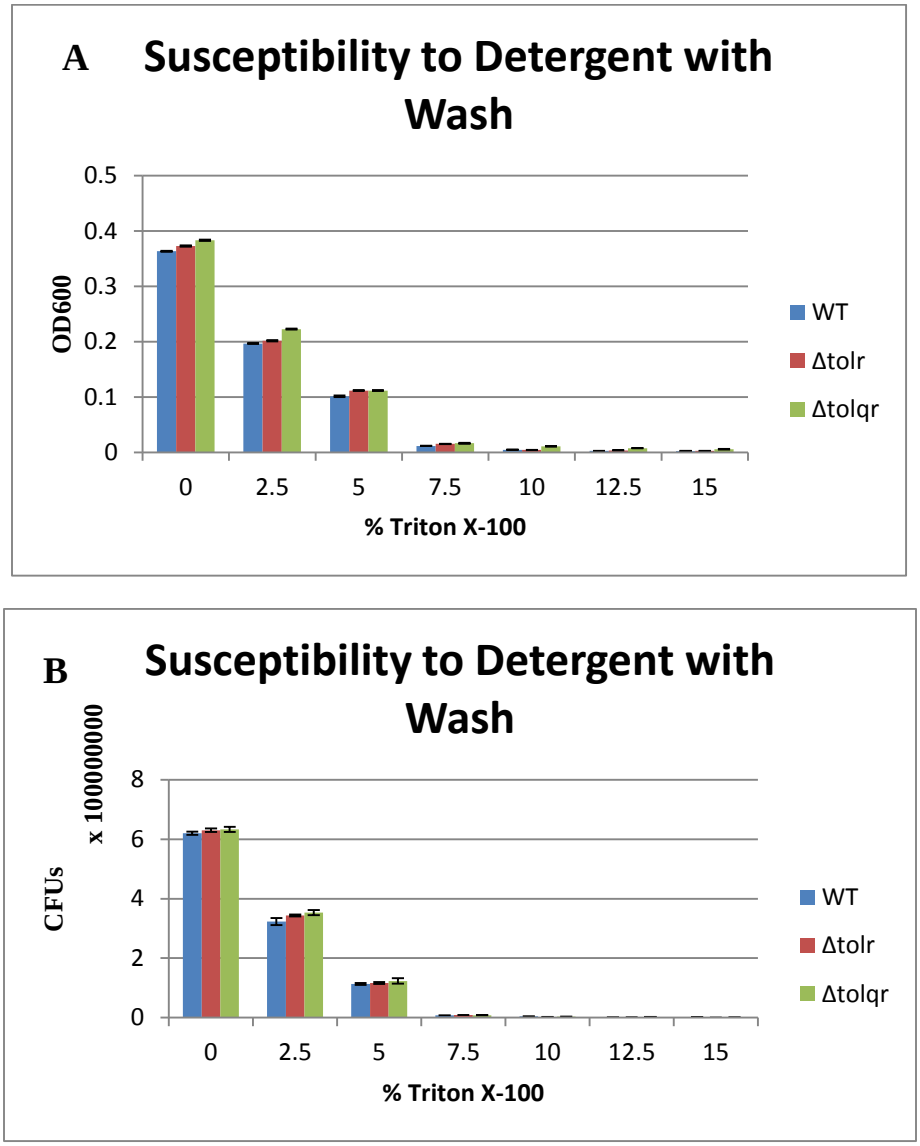


Figure 23. Graph of the A) OD_{600} and B) colony forming units (CFUs) of washed wild type (blue), Δ*tolR* (red), and Δ*tolQR* (green) cells grown in media containing varying concentrations of Triton X-100. Error bars represent standard error

Absorbance was measured for 48 hour cultures as well, but no difference was observed between wild type and mutants (data not shown). There was no difference in growth or the

number of surviving cells between parental and mutant strains when the EPS has been removed. These results indicate that EPS is most likely not a factor contributing to the survival of mutants in media containing detergent. It was expected that mutants would be more susceptible to higher levels of detergent than wild type since all *E. coli* Tol and Pal mutants exhibit characteristics similar to Lpp (Braun's lipoprotein) mutants, such as formation of outer membrane vesicles and shedding of periplasmic proteins (Bernadac et al., 1998). This characteristic, however, is not common to all Gram negative bacteria. *Salmonella enterica* subspecies *enterica* serovar Typhimurium Δ*tolA* mutants confer resistance to Triton X-100 to the level of the wild type, growing at even 12% Triton X-100 concentrations. The Δ*tolA* mutant of serovar Typhi is much more sensitive to the detergent, however. Its growth is inhibited at 2% Triton X-100 (Lahiri et al., 2011). Therefore, sensitivity to detergents of Δ*tolR* and Δ*tolQR* mutants in *R. leguminosarum* may be more comparable to those observed in *S. enterica* subsp. *enterica* serovar Typhimurium than *E. coli*. Without the Pal-TolA interaction that links the outer and inner membranes, expansion of the periplasmic space occurs at sites of constriction in *E. coli* (Gerding et al., 2007). This expansion is the result of the constriction of the outer membrane lagging behind the inner membrane since the bridge that links the two membranes together is interrupted due to mutations in TolA, Pal, or both. Since TolQ and TolR are not directly involved in the linkage of inner and outer membranes, mutations in either gene may not yield the same result as a Δ*tolA* mutant, for example. This may especially be true if ExbB and ExbD are complementing TolQ and TolR, respectively.

Susceptibility to High Ionic Strength Medium

As mentioned above, *tolpal* mutants are susceptible to conditions of high ionic strength, such as an increase in NaCl concentrations in growth media. To determine whether incremental

increases of NaCl in YEM broth have any effect on the growth or survivability of mutants, each mutant and wild type was inoculated into YEM broth containing 0% to 15% NaCl in 2.5% increments and incubated at 250 rpm and 28°C for 48 hours. The OD_{600} was measured at the end of 48 hour incubation period, and cultures were serially diluted and plated onto CR agar plates. The results of OD_{600} measurements are shown in Figure 24.

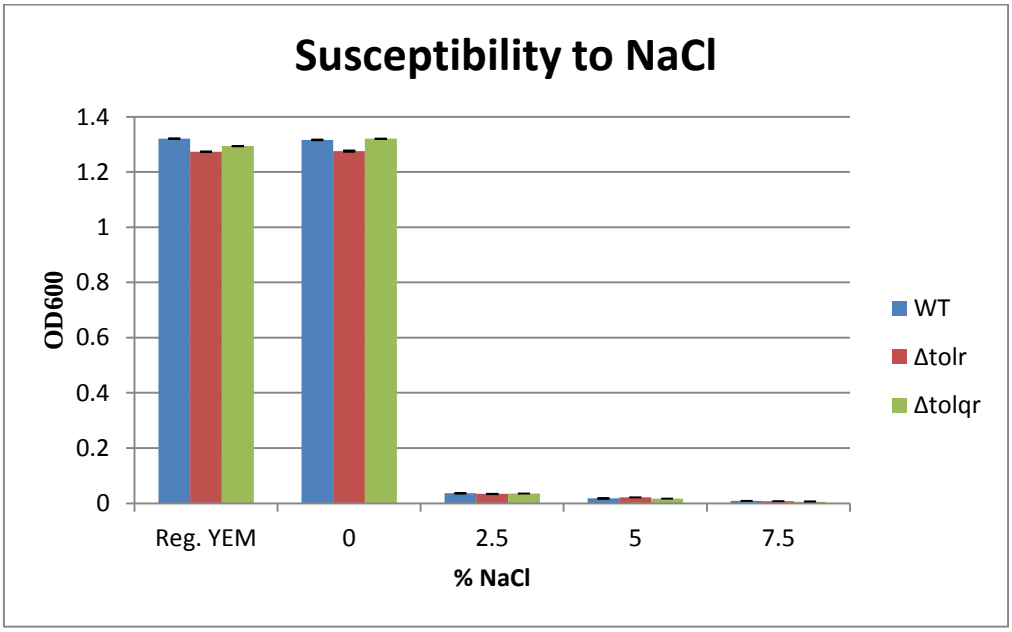


Figure 24. Graph of the susceptibility of wild type (blue), Δ*tolR* (red), and Δ*tolQR* (green) mutants to different concentrations of NaCl. Wild type and mutants failed to grow at 10% and higher concentrations of NaCl, and are therefore not shown. Error bars represent standard error

As a positive control for growth, mutants and parental strains were grown in regular YEM broth. In broth containing 0% NaCl, there was no difference between mutants and wild type and all three strains grew to the same optical density as the positive control (Reg. YEM). At 2.5% NaCl, the growth of mutants and parental strains is significantly inhibited and no growth occurs at concentrations of 10% NaCl and above. As a result, those values are not included in Fig. 24. Because 2.5% NaCl concentration significantly impacts growth, the assay was repeated using NaCl concentrations ranging from 0% to 0.0175% in 0.0025% increments. This range was

chosen based on the standard concentration of 0.01% NaCl in regular YEM broth. The assay was performed using washed cells to eliminate the possibility of EPS protecting the membrane integrity of mutants and wild type. Strains were incubated for 48 hours with measurements taken at the 12 hour time point to minimize production of EPS. Absorbance at 600 nm was measured and CFUs were calculated, as shown in Figure 25A and B, respectively. Measurements of cultures at 48 hours are not shown. However, there was no observable difference between wild type and mutants.

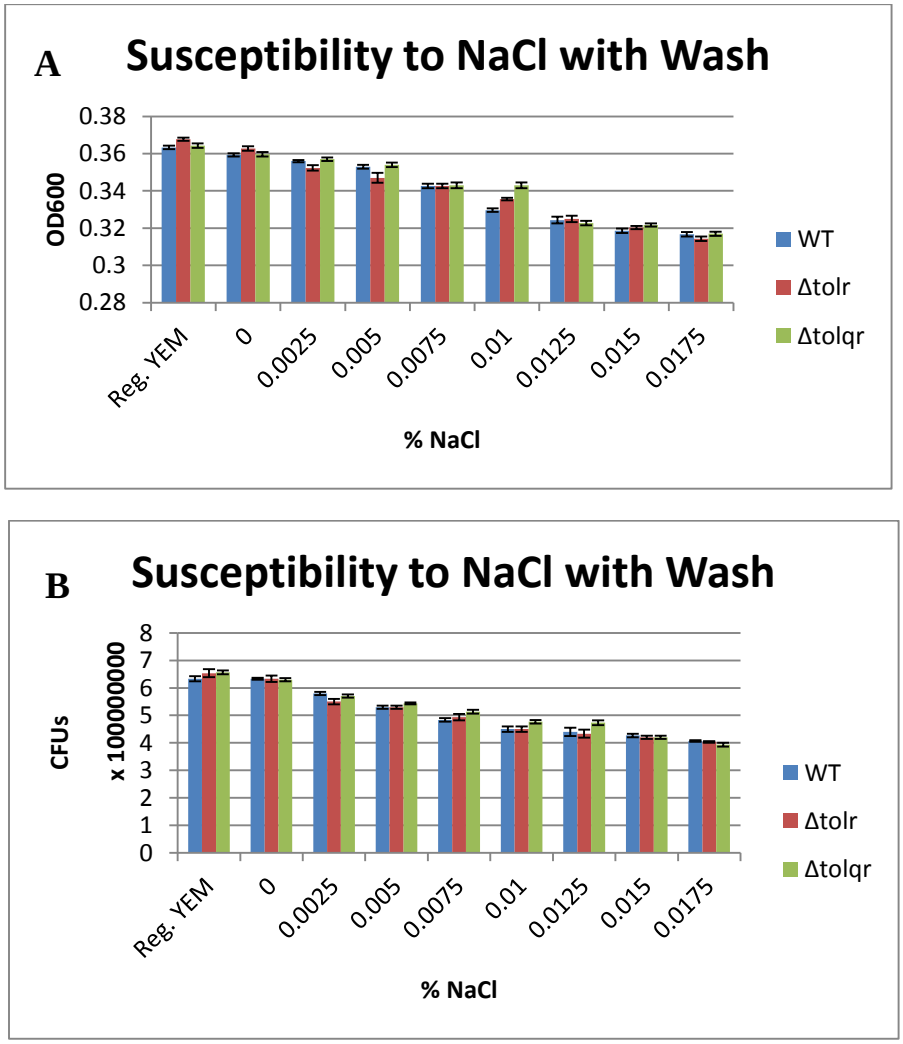
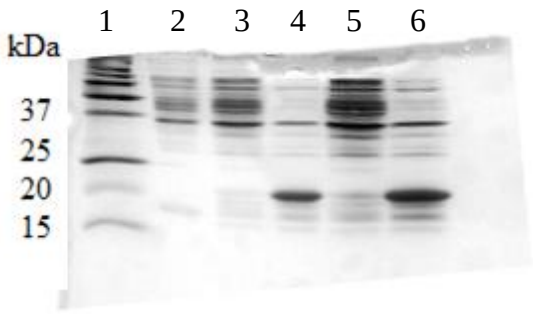


Figure 25. Graph of the A) OD_{600} measurements and B) colony forming units (CFUs) of washed wild type (blue), Δ*tolR* (red), and Δ*tolQR* (green) cells grown in media containing varying concentrations of NaCl. Error bars represent standard error

Much like the "Susceptibility to Detergent" assay (Fig. 22), there was no difference in growth between mutant and parental strains. Absorbance values (Fig. 25A) were relatively similar for wild type and Δ*tolR/ΔtolQR* mutants, as were the number of viable cells (Fig. 25B). Based on these results, we can conclude that mutations in putative *tolQ* and *tolR* do not confer a *tol* phenotype in *R. leguminosarum*, such as the formation of long multi-septate chains and sensitivity to detergent or high ionic strength medium.

Expression of TonB C-Terminal Protein Fragment

The 120 and 200 amino acid C-terminal fragments of TonB were constructed and heterologously expressed in *Escherichia coli* BL21 (DE3) cells. To visualize protein expression, induced and uninduced samples were run on an SDS-PAGE and stained with Coomassie Blue dye. The expression of the 200 amino acid fragment of TonB was not detected, however. Reasons for this are unknown, but factors may include inadequate optimization of conditions, such as length of time of induction or the concentration of IPTG, or degradation of protein by host proteases. The presence of a proline-rich region in the 200 amino acid fragment may also play a role in its stability and expression. This region is not present in the 120 amino acid fragment. The 120 amino acid fragment was successfully expressed, however (Fig. 26), and confirmed via Western Blot (Fig. 27).



Lane 1 – MW marker
Lane 2 – pET17b induced
Lane 3 – TonB 120 aa C-terminal uninduced (5µg)
Lane 4 – TonB 120 aa C-terminal induced (5µg)
Lane 5 – TonB 120 aa C-terminal uninduced (10µg)
Lane 6 – TonB 120 aa C-terminal induced (10µg)

Figure 26. SDS-PAGE of unexpressed and expressed 120 amino acid fragment of TonB C-terminal

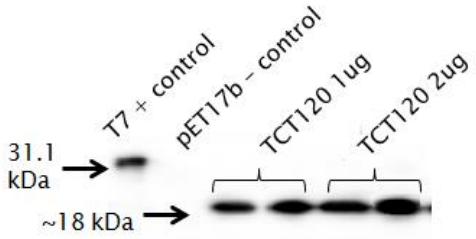


Figure 27. Western Blot of expressed 120 amino acid fragment of TonB C-terminal

The TonB C-terminal protein fragment was expected to be approximately 16.6 kDa. However, since the T7 tag of the pET17b vector that is transcribed along with the protein is 11 amino acids long, the molecular weight of the protein with the tag was expected to be approximately 17.8 kDa. The expected molecular weight corresponded to the size of the band in Figure 26, lanes 4 and 6.

Purification of TonB C-terminal Protein Fragment

After confirmation of expression of the TonB C-terminal protein fragment by SDS-PAGE and Western Blot, the protein of interest was purified via EMD Millipore's T7•Tag Affinity Purification Kit following manufacturer's protocol. The protein of interest was eluted with 5 mL of 1X Elution Buffer in 1 mL increments. The eluted protein and all flow-through collected were run on an SDS-PAGE to determine the presence of a pure protein (Fig. 28).

Unfortunately, much of the protein remained in the crude extract flow-through suggesting over-saturation of the column. As a result, the concentration of purified protein was very low. To determine if the cause for poor binding was perhaps due to the protein being trapped in inclusion bodies, another purification was performed using urea to solubilize the inclusion bodies. Urea treatment and subsequent purification steps were performed as suggested by the manufacturer. However, the amount of protein binding was the same as the purification without urea treatment. Since the proteins in the crude extract are in their native state, it may be that the T7 tag at the N-terminus of the fusion protein is obstructed by the native folding of the protein, thus not exposed enough to bind to the agarose resin antibodies. Or, the amount of resin in the column was not sufficient to bind all of the protein in the crude extract. Loading less protein onto the column to avoid over-saturation may alleviate this issue. Since the purification yielded a low concentration of purified protein, the protein was concentrated using Amicon Ultra Centrifugal Filters (Millipore) (Fig. 28, lane 10).

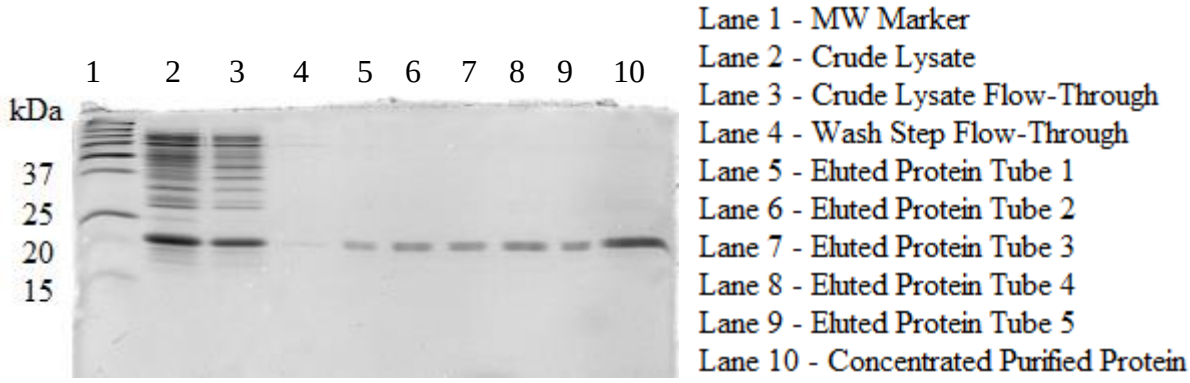


Figure 29. SDS-PAGE of TonB C-terminal pre- and post-purification. Five µg of protein loaded in lanes 2-9, except lane 4 (10 µL of sample) and lane 10 (10 µg)

CHAPTER 4

CONCLUSION

When we set out to identify *exbB* and *exbD* genes in the unsequenced *Rhizobium leguminosarum* ATCC 14479, we relied on previously sequenced strains to design primers. Because our strain is known to infect the red clover, *Trifolium pretense*, we chose *exbB* and *exbD* genes of the highly similar *R. leguminosarum* bv. trifolii WSM2304 strain as the genomic template for primer design. Once the genes were amplified, cloned into a plasmid and sequenced, the sequence was analyzed using BLAST. Nucleotide and protein BLAST results showed a significant identity to ExbB and ExbD of other *R. leguminosarum* strains. The sequence for *exbB*, however, also showed identity to TolQ. The predicted secondary structure generated by DomPred was compared to the predicted structure of *E. coli*'s ExbB and ExbD. Proteins of both strains were almost identical in terms of the predicted arrangement and number of α helices and β sheets.

To determine whether putative ExbB and ExbD proteins are involved in iron transport, single and double gene knockout mutants were created. Unfortunately, an *exbB* single mutant could not be identified. Using the Δ*exbD* and Δ*exbBD* mutant and wild type strains, growth assays in complex, low-iron, and high-iron media were performed and data plotted to generate a growth curve. Growth curves for both mutants were very similar to each other and both were able to grow in low-iron media.

Since neither an *exbB* nor *exbD* mutant in *E. coli* is able to survive under low-iron conditions, we hypothesized that another copy of the set of genes was present and compensating for the knocked out genes. After all, it is not uncommon for microorganisms to have two sets of *exbb/exbd* or *tonb/exbb/exbd* genes (Wiggerich and Puhler, 2000; Zhao and Poole, 2000).

However, when primers to amplify *exbB* were designed, they were constructed so that they bound significantly upstream of the 5' end of *exbB*. As a result, the neighboring gene was also cloned and sequenced. Sequence analysis predicted the gene codes for an acyl coenzyme A thioesterase – a protein belonging to the Tol-Pal complex. Additional bioinformatics research revealed substantial sequence similarities between putative ExbB/ExbD and putative TolQ/TolR in *R. leguminosarum*. It is likely that when primers were designed based on *exbB* and *exbD* of *R. leguminosarum* WSM2304, the gene assignment in the NCBI database was incorrect since *exbB/exbD* and *tolQ/tolR* of many *R. leguminosarum* strains are listed as putative genes in the database. As a result, the primers instead may have bound to the highly similar *tolQ* and *tolR*.To determine whether the putative *exbB* and *exbD* genes were instead *tolQ* and *tolR*, additional sequencing of neighboring genes was performed to identify a *tolA* gene that is found downstream of *tolR* in other Gram negative organisms. Sequence results of the gene located downstream of the 3' end of *exbD* identified the gene as a putative *tolA* gene coding for the TolA protein– one of seven proteins belonging to the Tol-Pal system. With TolA and acyl CoA thioesterase (coded by the *ybgC* gene) proteins identified and the genes arranged in the same order as the *tolpal* gene clusters of various other Gram negative organisms, it became apparent that the *exbB* and *exbD* genes could be the Tol-Pal associated *tolQ* and *tolR*. Suddenly, the unusual growth curves of the mutants were no longer peculiar. Because our results did not indicate that putative ExbB and ExbD function in iron transport, the possibility that the the gene annotations in the NCBI database were incorrect and may potentially be those of the Tol-Pal system required further testing.

The Tol-Pal system, unlike the TonB-ExbB-ExbD complex, has not been reported to be involved in iron transport. Instead, its roles are vaster, ranging from outer membrane integrity to

cell division. Since previous work on the Tol-Pal system in *E. coli* and *S. enterica* showed *tolpal* mutants are sensitive to detergents, high salt concentrations, and form multi-septate chains under low osmolarity or high ionic strength conditions, we performed sensitivity assays and Gram stains to determine if the same is true for *R. leguminosarum*. Gram stains of parental and mutants strains in low osmolarity and high ionic strength medium showed no differences between the mutants and wild type and no formation of multi-septate chains indicative of the cell's inability to fully separate. Detergent and high salt sensitivity assays also showed no differences between mutants and wild type. Exopolysaccharide production characteristic of *R. leguminosarum* was determined to have no effect on the survivability of mutants or wild type. Our current results do not suggest that the putative genes are *tolQ* and *tolR* since their gene assignment in the NCBI database has not been verified. If the genes are *tolQ* and *tolR*, perhaps they are not directly involved in outer membrane integrity or in separation of cells during binary fission, or other proteins, such as the highly similar ExbB and ExbD, are complementing TolQ and TolR. Additonal testing is required to determine if the initially identified putative genes *exbB* and *exbD* are instead *tolQ* and *tolR* and to characterize their roles in *R. leguminosarum*.

We were also able to successfully express and purify the 120 amino acid fragment of the TonB C-terminus. Expression of the 200 amino acid fragment was unsuccessful, however. Difficulties in its expression may be attributed to the proline-rich region not present in the 120 amino acid fragment. This region is believed to provide rigidity to TonB and assist in its extension across the cytoplasmic space (Krewulak et al., 2007). It may be that the proline-rich region is affecting the stability of the protein. The larger protein fragment may be less stable and prone to degradation by proteases.

Further work is necessary to elucidate the exact roles of putative TolQ and TolR in *R. leguminosarum*. Four genes, *ybgC, tolQ, tolR,* and *tolA*, have been identified and sequenced. Additional sequencing is needed to determine the identity and location of the remaining *tolpal* genes. To determine if TolQ and/or TolR play a role in motility, swimming, swarming, and twitching motility assays need to be performed. The absence of zones of growth from the site of inoculation would suggest a defect in motility. Also, once a Δ*tolQ* mutant is created, the functions of each TolQ and TolR protein could be assessed. The creation of additional *tolpal* mutants would assist in characterizing the role of each Tol-Pal protein in *R. leguminosarum*. Because no research on the Tol-Pal system in any strain of *Rhizobium* has been published to date, this characterization would be the first to shed light on its role in the nitrogen-fixing bacterium. Because no phenotypic or growth-rate differences can be observed between mutants and wild type, expression studies would need to be performed to determine if any of the *tolpal* genes are being transcribed.

The identification and sequencing of *exbB* and *exbD* would assist in determining their similarity to *tolQ* and *tolR*. Because the lack of difference in growth and sensitivity to detergent and high salt concentrations between parental and mutant strains may be due to complementation by ExbB and ExbD, single and double *exbB/exbD* mutants would need to be created and susceptibility assays repeated to determine if they are complementing TolQ and TolR.

The purification of the 120 amino acid fragment of the TonB C-terminal yielded low levels of protein. Therefore, additional expression and purification is necessary to attain a sufficient protein concentration for downstream applications. The putative TonB protein identified by Hill (2014) is significantly larger than TonB of *E. coli* and has not been confirmed to be TonB via protein sequencing. Therefore, N-terminal sequencing of the purified C-terminus

fragment would confirm the identity of the putative TonB protein. Nuclear magnetic resonance (NMR) spectroscopy would provide additional information on its physical and chemical properties.

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APPENDICES

Appendix A

Media and Buffers

Luria Broth (LB)

Tryptone	10.0g
Yeast Extract	5.0g
NaCl	10.0g
ddH_2O	to 1L

Dissolve ingredients and autoclave.

For LB agar plates:

Agar	15.0g

Dissolve ingredients and autoclave.

Modified Manhart and Wong (MMW)

Dextrose	6.0g
Glutamate	1.5g
KH_2PO_4	1.0g
K_2HPO_4	0.764g
$MgSO_4$	0.18g
CaSo4*2H2O	0.13g
ddH2O	to 1L

Dissolve ingredients, adjust pH to 6.8, and autoclave.

TNE Buffer

0.1 M Tris-HCl (pH 8.0)
0.15 M NaCl
20 mM EDTA

TEST-LR Buffer

0.1 M Tris (pH 8.0)
20 mM EDTA
0.6 M Sucrose
1% Triton X-100
24 µg/mL lysozyme
0.8 µg/mL RNase A

Yeast Extract Mannitol Broth (YEM)/Congo Red Agar (CR)

Mannitol	4.0g
K_2HPO_4	0.2g
$MgSO_4$	0.08g
NaCl	0.04g
Yeast Extract	0.4g
ddH_2O	to 400mL

Dissolve ingredients, adjust pH to 6.8 and autoclave.

For Congo Red agar plates, add:

Agar	12.0g
1% Congo Red dye	1mL

Dissolve ingredients, adjust pH to 6.8 and autoclave.

Vitamin Solution	**(mg)**
$Na_2EDTA*2H_2O$	550
$Na_2MoO_4*2H_2O$	250
H_3BO_3	145
$ZnSO_4*7H_2O$	108
Calcium Panthenate	50
Inositol	50
Thiamine HCl	40
Biotin	12
$CoCl_2*6H_2O$	10
Riboflavin	10
p-aminobenzoic Acid	10
Nicotinic Acid	10
Pyridoxine HCl	10
Vitamin B_{12}	10
$CuSO_4*5H_2O$	4.37
$MnCl_2*4H_2O$	4.3
ddH_2O	to 1L

Dissolve ingredients and filter sterilize.

SDS-PAGE Solutions

Stacking Gel Buffer (pH 6.8)
0.5M Tris
ddH_2O

Resolving Gel Buffer (pH 8.8)
1.5M Tris
ddH_2O

Tris-Glycine-SDS Buffer (10X)

Ingredient	Final Concentration
Tris	0.25 M
Glycine	1.92 M
Sodium Dodecyl Sulfate (SDS)	1.0% (w/v)
ddH_2O	to 1 L

SDS-PAGE gel

Ingredient	Stacking	Resolving (12%)
30% bis-acrylamide	0.66 mL	3 mL
Stacking Gel Buffer	1.26 mL	-
Running Gel Buffer	-	1.88 mL
ddH_2O	3 mL	2.52 mL
10% SDS	50 µL	75 µL
10% APS	25 µL	37.5 µL
TEMED	5 µL	3.75 µL

Coomassie Blue Staining Solution

Ingredient	Final concentration
Coomassie R-250	0.1% (w/v)
Methanol	50% (v/v)
Glacial acetic acid	10% (v/v)
ddH_2O	40% (v/v)

SDS-PAGE Destaining Solution

Ingredient	Final concentration
Methanol	50% (v/v)
Glacial acetic acid	10% (v/v)
ddH_2O	40% (v/v)

2X Laemmli Buffer

Ingredient	Final concentration
Sodium dodecyl sulfate	4% (w/v)
Glycerol	20% (v/v)
1M Tris pH 6.8	120 mM
Bromophenol blue	0.02% (w/v)
ddH_2O	

Western Blot Solutions

Transfer Buffer (10X)

Ingredient	Final concentration
Tris	0.25 M
Glycine	1.92 M
ddH_2O	

For 1X working stock, dilute with ddH_2O and add methanol to 20% final concentration

1X TBST Wash Buffer

Ingredient	Final concentration
Tris	50 mM
NaCl	150 mM
Tween	0.1%
ddH_2O	

Blocking Buffer

Ingredient	Final concentration
Bovine serum albumin	3% (w/v)
TBST	1X

Dissolve BSA and filter sterilize.

Appendix B

Primers

Primer Name	Primer Sequence 5’ – 3’
pUC19 F	GCGCGGATCCCTTCATCACATCGTCTCCTG
pUC19 R	GCGCAAGCTTCGGCTTGGCCTGTTCTTCCG
ExbB pET F	GCGCAAGCTTGATGGAACAAGTAGGATTGGCAG
ExbB pET R	GCGCGGATCCGGCCATACCCATGTGAGAATAC
ExbD pET F	GCGCAAGCTTGATGGGTATGGCAGTTGGAGG
ExbD pET R	GCGCGGATCCGCAGCAGATGTGATGACACTGG
DCT F	GCGCAAGCTTGATGGTCGGCGTGCCGATCGAC
SOE Fo	GCGCGGATCCGCTGATTGGTCGCTCATTATCG
SOE Ro	GCGCAAGCTTGCTTCGACAGGCTTCGGCG
sglSOEbB Ri	GATGGTCGCGAGCGATCCGATCCAAAATCCCCGAATCC
sglSOEbB Fi	GGATTCGGGGATTTTGGATCGGATCGCTCGCGACCATC
sglSOEbD Ri	CCTTCATTTTGCGCGCTATTGCATGTGAGAATACTCCGTTGG
sglSOEbD Fi	CCAACGGAGTATTCTCACATGCAATAGCGCGCAAAATGAAGG
dblSOE Ri	CTGCAGGAACACCTCGCCCTGGCCCGACCAGAACAC
dblSOE Fi	GTGTTCTGGTCGGGCCAGGGCGAGGTGTTCCTGCAG
SOE2Bshort F	GGATTCGGGGATTTTGGATCA
SOE2Dshort F	CCAACGGAGTATTCTCACATG
TonBCT	GCGCAAGCTTGATGGTGAACGGCCAGGACG
TonBR17B	GCGCGAATTCGATGCCTGATATCGCGCAGG

Primer Map

Nucleotide sequence map showing the location of each primer used in generation of wild type and mutant *exbB* and *exbD* genes of *R. leguminosarum* ATCC 14479.

gactggaacggggatccttcatcacatcgtctcctgattggtcgctcattatcgggaatagttaggaattgtgac base pairs
ctgaccttgcccctaggaagtagtgtagcagaggactaaccagcgagtaatagcccttatcaatccttaacactg 1 to 75

aatcattcaatgcttgcctgtcgggaagcgttgcgccaaacagggcacctgagtttggagaaaattatgacggaa base pairs
ttagtaagttacgaacggacagcccttcgcaacgcggtttgtcccgtggactcaaacctcttttaatactgcctt 76 to 150

cgccctttttccatttcgggagagctgacggaagccggacaccgcctcgttcagcgggtctattatgaagatact base pairs
gcgggaaaaaggtaaagccctctcgactgccttcggcctgtggcggagcaagtcgcccagataatacttctatga 151 to 225

gacttttccggcctggtctatcacgcccgctacctgcatttcctggagcgcggccgtaccgattatctgcgctgc base pairs
ctgaaaaggccggaccagatagtgcgggcgatggacgtaaaggacctcgcgccggcatggctaatagacgcgacg 226 to 300

ctcggcgtcgagcagcgcgaactcgtcagcgccgatgaggagggcctcgttttcgttgtccaccgcatggagatc base pairs
gagccgcagctcgtcgcgcttgagcagtcgcggctactcctcccggagcaaaagcaacaggtggcgtacctctag 301 to 375

gacttcaagagcccggcgcgcatggacgatgtgctgacgatcctgacacacacggagaaagccggcggcgccaag base pairs
ctgaagttctcgggccgcgcgtacctgctacacgactgctaggactgtgtgtgcctctttcggccgccgcggttc 376 to 450

atggtgctcaatcagcagatccgctcgggcgagacgctgctgatcgccgccaaggtgatcatcgccgtcatcaac base pairs
taccacgagttagtcgtctaggcgagcccgctctgcgacgactagcggcggttccactagtagcggcagtagttg 451 to 525

gcccgggggcggccaaggcggttgccggaaacactggcagcgaaattcctggaaggcagcatgccgctacaggcc base pairs
cgggcccccgccggttccgccaacggcctttgtgaccgtcgctttaaggaccttccgtcgtacggcgatgtccgg 526 to 600

gaattgccgaaaaatcggaccttgtcaaaacttgaactttatgtgaaggaattcccgcgccgcaggatgagcgct base pairs
cttaacggctttttagcctggaacagttttgaacttgaaatacacttccttaagggcgcggcgtcctactcgcga 601 to 675

ctttcggcagccggaccttgctccggtcaagtaatccatggaacgaaatcttctaaatacaggcttactgtcaca base pairs
gaaagccgtcggcctggaacgaggccagttcattaggtaccttgctttagaagatttatgtccgaatgacagtgt 676 to 750

gtccggcactaacgatctattaaccataatggtgtcttactgggaaagtcggagtttgtgcggtgcacgccttcc base pairs
caggccgtgattgctagataattggtattaccacagaatgacccttttcagcctcaaacacgccacgtgcggaagg 751 to 825

tttgaccaaatttgacggcaagaaaggcggaagatgagcttggaagtttgaccagggctttgggcggcggccgcc base pairs
aaactggtttaaactgccgttctttccgccttctactcgaaccttcaaactggtcccgaaacccgccgccggcgg 826 to 900

sglSOEbB Ri

ggacacttcttgcgagatcagtttgtgccgcccggtccagcaccggggcgtttggattcggggattttggatcaa base pairs
cctgtgaagaacgctctagtcaaacacggcgggccaggtcgtggccccgcaaacctaagcccctaaaacctagtt 901 to 975

tggaacaagtaggattggcagcagcaacgacggacgtcagcctctggtcgcttttcatgcaggccggcatcgtcg base pairs
accttgttcatcctaaccgtcgtcgttgctgcctgcagtcggagaccagcgaaaagtacgtccggccgtagcagc 976 to 1050

tcaagctcgtcatgctcgggcttatcgcggcctcggtgtggacgtgggctatcgtcatcgacaaatacctggcct base pairs
agttcgagcagtacgagcccgaatagcgccggagccacacctgcacccgatagcagtagctgtttatggaccgga 1051 to 1125

dblSOE Ri

atggccgcgcacggcgccagttcgacaagttcgagcaggtgttctggtcgggccagtcgctggaagagctctacc base pairs
taccggcgcgtgccgcggtcaagctgttcaagctcgtccacaagaccagcccggtcagcgaccttctcgagatgg 1126 to 1200

gctcgctgtcggaacgcaacaataccggtctggcggcgatcttcgtggctgccatgcgcgagtggaagaaatcct base pairs
cgagcgacagccttgcgttgttatggccagaccgccgctagaagcaccgacggtacgcgctcaccttctttagga 1201 to 1275

tcgaacgcggcgcccgctcgccgatcggcctgcagatgcgtatcgaccgcgcgatggacgtgacgctcgcccgtg base pairs
agcttgcgccgcgggcgagcggctagccggacgtctacgcatagctggcgcgctacctgcactgcgagcgggcac 1276 to 1350

sglSOEbB Fi

agaccgaatttctcggtgctcgcctcggatcgctcgcgaccatcggctcggccggtccgttcatcggtctgttcg base pairs
tctggcttaaagagccacgagcggagcctagcgagcgctggtagccgagccggccaggcaagtagccagacaagc 1351 to 1425

gcacggtcgtcggcatcatgacctcgttccaggcaatcgccggttcgaagtcgaccaaccttgcggtcgtcgcgc base pairs
cgtgccagcagccgtagtactggagcaaggtccgttagcggccaagcttcagctggttggaacgccagcagcgcg 1426 to 1500

ccggtatcgccgaagcgctgcttgccactgcgatcggcctcgtcgccgctatcccggcagttatcgcctgcaaca base pairs
ggccatagcggcttcgcgacgaacggtgacgctagccggagcagcggcgatagggccgtcaatagcggacgttgt 1501 to 1575

agttctctgccgatgccggcaagctctcgggccgaatggaaggtttcgcggatgaattctccgccatactttcgc base pairs
tcaagagacggctacggccgttcgagagcccggcttaccttccaaagcgcctacttaagaggcggtatgaaagcg 1576 to 1650

sglSOEbD Ri

gccagatcgacgagaaactgcagccgcgcgctgccgctcagtaaccaacggagtattctcacatgggtatggcag base pairs
cggtctagctgctctttgacgtcggcgcgcgacggcgagtcattggttgcctcataagagtgtacccataccgtc 1651 to 1725

ttggaggcaatggcggaggcggcggacgccgccgtcgcggcggtcggaacagggccgtgatttccgaaatcaacg base pairs
aacctccgttaccgcctccgccgcctgcggcggcagcgccgccagccttgtcccggcactaaaggctttagttgc 1726 to 1800

tgacgccgctcgtcgacgtcatgctggtgcttttgatcatcttcatggtcgcggcaccgatgatgaccgtcggcg base pairs
actgcggcgagcagctgcagtacgaccacgaaaactagtagaagtaccagcgccgtggctactactggcagccgc 1801 to 1875

tgccgatcgacctgccggaaacgcaggccaaggcgctgaattcggagacgcagccgatcaccatctccgtcaaga base pairs
acggctagctggacggcctttgcgtccggttccgcgacttaagcctctgcgtcggctagtggtagaggcagttct 1876 to 1950

dblSOE Fi

atgacggcgaggtgttcctgcaggaaacaccgatcccggcggcggagatcgccgccaagctcgaggcgatcgcca base pairs
tactgccgctccacaaggacgtcctttgtggctagggccgccgcctctagcggcggttcgagctccgctagcggt 1951 to 2025

ccaccggttataacgaacgtatcttcgtgcgcggcgacgcgaccgcgccctacggcgtcatcgccgacgtcatgg base pairs
ggtggccaatattgcttgcatagaagcacgcgccgctgcgctggcgcgggatgccgcagtagcggctgcagtacc 2026 to 2100

sglSOEbD Fi

cccgtattcagggtgcaggcttcaagaatatcggcctggtgacgcagcagaagaaggaccaatagcgcgcaaaat base pairs
gggcataagtcccacgtccgaagttcttatagccggaccactgcgtcgtcttcttcctggttatcgcgcgtttta 2101 to 2175

gaaggccagtgtcatcacatctgctgttttgcacggcctggtgctcacctgggcgatggtgccgcttggcgctcc base pairs
cttccggtcacagtagtgtagacgacaaaacgtgccggaccacgagtggacccgctaccacggcgaaccgcgagg 2176 to 2250

ggaatccttcaaggtagaggatttcgaggcgatgccggtcgatctcgtgccggtggagtccattacccagatgca base pairs
ccttaggaagttccatctcctaaagctccgctacggccagctagagcacggccacctcaggtaatgggtctacgt 2251 to 2325

gcaaggcgacaagaaggctccgaagaaggagacttccgcgcccgtgccgacgacacggccgccgattgcacagcc base pairs
cgttccgctgttcttccgaggcttcttcctctgaaggcgcgggcacggctgctgtgccggcggctaacgtgtcgg 2326 to 2400

ggccgagaatgccggcgacagcaatgtcgacctgaaaacgccgccggtcccgaacgccaagcccagcaatactga base pairs
ccggctcttacggccgctgtcgttacagctggacttttgcggcggccagggcttgcggttcgggtcgttatgact 2401 to 2475

agcggctgccgccaattcgagcgacaagccgatgccgaagatcgatcctaagccgaatgacgtcaaggagatcgt base pairs
tcgccgacggcggttaagctcgctgttcggctacggcttctagctaggattcggcttactgcagttcctctagca 2476 to 2550

SOE Ro

caaggaggaaacggaagtcgagcagccgaaggaggttgcttcaattccgccgccgaagcctgtcgaagtgacgcc base pairs
gttcctcctttgccttcagctcgtcggcttcctccaacgaagttaaggcggcggcttcggacagcttcactgcgg 2551 to 2625

pUC19R

gccgaagcccgaggaaaatccgccggaagaacaggccaagccggagaaccgccgaagcctgagattgct base pairs
cggcttcgggctccttttaggcggccttcttgtccggttcggcctcttggcggcttcggactctaacga 2626 to 2694

Sequence Results

BLAST sequence alignment of *exbb* and *exbd* in *R. leguminosarum* ATCC 14479 with most similar sequence in other *R. leguminosarum* strains. Top strand is ATCC 14479 and bottom strand is strain 3841.

Rhizobium leguminosarum bv. viciae chromosome complete genome, strain 3841
Sequence ID: emb|AM236080.1| Length: 5057142 Number of Matches: 1

Range 1: 4199652 to 4200845 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1958 bits(1060)	0.0	1150/1194(96%)	3/1194(0%)	Plus/Minus

Features: putative uroporphyrinogen decarboxylase
putative biopolymer transport protein

```
Query  1        ATGGAACAAGTAGGATTGGCAGCAGCAACGACGGACGTCAGCCTCTGGTCGCTTTTCATG  60
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4200845  ATGGAACAAGTAGGATTGGCAGCAGCAACGACGGACGTCAGCCTCTGGTCGCTTTTCATG  4200786

Query  61       CAGGCCGGCATCGTCGTCAAGCTCGTCATGCTCGGGCTTATCGCGGCCTCGGTGTGGACG  120
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Sbjct  4200785  CAGGCCGGCATCGTCGTCAAGCTCGTCATGCTCGGGCTCATCGCAGCCTCGGTGTGGACG  4200726

Query  121      TGGGCTATCGTCATCGACAAATACCTGGCCTATGGCCGCGCACGGCGCCAGTTCGACAAG  180
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Sbjct  4200725  TGGGCGATCGTCATCGACAAATACCTGGCCTATGGCCGCGCACGGCGCCAGTTCGACAAG  4200666

Query  181      TTCGAGCAGGTGTTCTGGTCGGGCCAGTCGCTGGAAGAGCTCTACCGCTCGCTGTCGGAA  240
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Sbjct  4200665  TTCGAACAGGTGTTCTGGTCGGGCCAGTCGCTGGAAGAACTCTACCGCTCGCTGTCGGAA  4200606

Query  241      CGCAACAATACCGGTCTGGCGGCGATCTTCGTGGCTGCCATGCGCGAGTGGAAGAAATCC  300
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Sbjct  4200605  CGCAACAATACCGGGCTCGCGGCGATCTTCGTGGCCGCCATGCGTGAATGGAAGAAATCC  4200546

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Query  361      GTGACGCTCGCCCGTGAGACCGAATTTCTCGGTGCTCGCCTCGGATCGCTCGCGACCATC  420
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Sbjct  4200485  GTGACGCTCGCCCGTGAGACCGAATTTCTCGGCGCCCGCCTGGGATCGCTCGCGACCATC  4200426

Query  421      GGCTCGGCCGGTCCGTTCATCGGTCTGTTCGGCACGGTCGTCGGCATCATGACCTCGTTC  480
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Sbjct  4200425  GGCTCGGCCGGGCCGTTCATCGGTCTGTTCGGCACGGTCGTCGGCATCATGACCTCGTTC  4200366

Query  481      CAGGCAATCGCCGGTTCGAAGTCGACCAACCTTGCGGTCGTCGCGCCCGGTATCGCCGAA  540
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Sbjct  4200365  CAGGCGATTGCCGGTTCGAAGTCGACCAACCTTGCGGTTGTCGCGCCCGGTATCGCCGAA  4200306

Query  541      GCGCTGCTTGCCACTGCGATCGGCCTCGTCGCCGCTATCCCGGCAGTTATCGCCTGCAAC  600
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Sbjct  4200305  GCGCTGCTTGCCACCGCGATCGGCCTCGTCGCCGCTATCCCGGCGGTCATCGCCTACAAC  4200246

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Sbjct  4200185  TCCGCCATACTTTCGCGCCAGATCGACGAGAAACTGCAGCCTCGCGCTGCAGCTCAGTAA  4200126

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Query  778      CGCCGCCGTCGCGGCGGTCGGAACAGGGCCGTGATTTCCGAAATCAACGTGACGCCGCTC  837
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Query  838      GTCGACGTCATGCTGGTGCTTTTGATCATCTTCATGGTCGCGGCACCGATGATGACCGTC  897
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Sbjct  4200005  GTCGACGTCATGCTGGTGCTTTTGATCATCTTCATGGTCGCGGCACCGATGATGACTGTC  4199946

Query  898      GGCGTGCCGATCGACCTGCCGGAAACGCAGGCCAAGGCGCTGAATTCGGAGACGCAGCCG  957
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Sbjct  4199945  GGCGTGCCGATCGACCTGCCGGAAACGCAGGCCAAGGCGCTGAATTCCGAGACGCAGCCG  4199886

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Query  1018     GCGGAGATCGCCGCCAAGCTCGAGGCGATCGCCACCACCGGTTATAACGAACGTATCTTC  1077
                || |||||||||||| ||||||||||||||||||| ||||| ||||||||||||||||||
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Query  1078     GTGCGCGGCGACGCGACCGCGCCCTACGGCGTCATCGCCGACGTCATGGCCCGTATTCAG  1137
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Sbjct  4199765  GTGCGCGGCGACGCGACCGCGCCTTACGGCGTCATCGCCGATGTCATGGCCCGTATTCAG  4199706

Query  1138     GGTGCAGGCTTCAAGAATATCGGCCTGGTGACGCAGCAGAAGAAGGACCAATAG  1191
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Sbjct  4199705  GGTGCAGGCTTCAAGAATATCGGCCTCGTGACGCAGCAGAAGAAGGACCAATAG  4199652
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CLUSTAL 2.1 multiple sequence alignment of wild type *exbd* (top) and Δ*exbd* (bottom) in *R. leguminosarum* ATCC 14479

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ExbD_WT          CTTCATCACATCGTCTCCTGATTGGTCGCTCATTATCGGGAATAGTTAGGAATTGTGACA
ExbD_mutant      CTTCATCACATCGTCTCCTGATTGGTCGCTCATTATCGGGAATAGTTAGGAATTGTGACA
                 ************************************************************

ExbD_WT          ATCATTCAATGCTTGCCTGTCGGGAAGCGTTGCGCCAAACAGGGCACCTGAGTTTGGAGA
ExbD_mutant      ATCATTCAATGCTTGCCTGTCGGGAAGCGTTGCGCCAAACAGGGCACCTGAGTTTGGAGA
                 ************************************************************

ExbD_WT          AAATTATGACGGAACGCCCTTTTTCCATTTCGGGAGAGCTGACGGAAGCCGGACACCGCC
ExbD_mutant      AAATTATGACGGAACGCCCTTTTTCCATTTCGGGAGAGCTGACGGAAGCCGGACACCGCC
                 ************************************************************

ExbD_WT          TCGTTCAGCGGGTCTATTATGAAGATACTGACTTTTCCGGCCTGGTCTATCACGCCCGCT
ExbD_mutant      TCGTTCAGCGGGTCTATTATGAAGATACTGACTTTTCCGGCCTGGTCTATCACGCCCGCT
                 ************************************************************

ExbD_WT          ACCTGCATTTCCTGGAGCGCGGCCGTACCGATTATCTGCGCTGCCTCGGCGTCGAGCAGC
ExbD_mutant      ACCTGCATTTCCTGGAGCGCGGCCGTACCGATTATCTGCGCTGCCTCGGCGTCGAGCAGC
                 ************************************************************

ExbD_WT          GCGAACTCGTCAGCGCCGATGAGGAGGGCCTCGTTTTCGTTGTCCACCGCATGGAGATCG
ExbD_mutant      GCGAACTCGTCAGCGCCGATGAGGAGGGCCTCGTTTTCGTTGTCCACCGCATGGAGATCG
                 ************************************************************

ExbD_WT          ACTTCAAGAGCCCGGCGCGCATGGACGATGTGCTGACGATCCTGACACACACGGAGAAAG
ExbD_mutant      ACTTCAAGAGCCCGGCGCGCATGGACGATGTGCTGACGATCCTGACACACACGGAGAAAG
                 ************************************************************

ExbD_WT          CCGGCGGCGCCAAGATGGTGCTCAATCAGCAGATCCGCTCGGGCGAGACGCTGCTGATCG
ExbD_mutant      CCGGCGGCGCCAAGATGGTGCTCAATCAGCAGATCCGCTCGGGCGAGACGCTGCTGATCG
                 ************************************************************

ExbD_WT          CCGCCAAGGTGATCATCGCCGTCATCAACGCCCGGGGGCGGCCAAGGCGGTTGCCGGAAA
ExbD_mutant      CCGCCAAGGTGATCATCGCCGTCATCAACGCCCGGGGGCGGCCAAGGCGGTTGCCGGAAA
                 ************************************************************

ExbD_WT          CACTGGCAGCGAAATTCCTGGAAGGCAGCATGCCGCTACAGGCCGAATTGCCGAAAAATC
ExbD_mutant      CACTGGCAGCGAAATTCCTGGAAGGCAGCATGCCGCTACAGGCCGAATTGCCGAAAAATC
                 ************************************************************

ExbD_WT          GGACCTTGTCAAAACTTGAACTTTATGTGAAGGAATTCCCGCGCCGCAGGATGAGCGCTC
ExbD_mutant      GGACCTTGTCAAAACTTGAACTTTATGTGAAGGAATTCCCGCGCCGCAGGATGAGCGCTC
                 ************************************************************

ExbD_WT          TTTCGGCAGCCGGACCTTGCTCCGGTCAAGTAATCCATGGAACGAAATCTTCTAAATACA
ExbD_mutant      TTTCGGCAGCCGGACCTTGCTCCGGTCAAGTAATCCATGGAACGAAATCTTCTAAATACA
                 ************************************************************

ExbD_WT          GGCTTACTGTCACAGTCCGGCACTAACGATCTATTAACCATAATGGTGTCTTACTGGGAA
ExbD_mutant      GGCTTACTGTCACAGTCCGGCACTAACGATCTATTAACCATAATGGTGTCTTACTGGGAA
                 ************************************************************
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ExbD_WT          AGTCGGAGTTTGTGCGGTGCACGCCTTCCTTTGACCAAATTTGACGGCAAGAAAGGCGGA
ExbD_mutant      AGTCGGAGTTTGTGCGGTGCACGCCTTCCTTTGACCAAATTTGACGGCAAGAAAGGCGGA
                 ************************************************************

ExbD_WT          AGATGAGCTTGGAAGTTTGACCAGGGCTTTGGGCGGCGGCCGCCGGACACTTCTTGCGAG
ExbD_mutant      AGATGAGCTTGGAAGTTTGACCAGGGCTTTGGGCGGCGGCCGCCGGACACTTCTTGCGAG
                 ************************************************************

ExbD_WT          ATCAGTTTGTGCCGCCCGGTCCAGCACCGGGGCGTTTGGATTCGGGGATTTTGGATCATG
ExbD_mutant      ATCAGTTTGTGCCGCCCGGTCCAGCACCGGGGCGTTTGGATTCGGGGATTTTGGATCATG
                 ************************************************************

ExbD_WT          GAACAAGTAGGATTGGCAGCAGCAACGACGGACGTCAGCCTCTGGTCGCTTTTCATGCAG
ExbD_mutant      GAACAAGTAGGATTGGCAGCAGCAACGACGGACGTCAGCCTCTGGTCGCTTTTCATGCAG
                 ************************************************************

ExbD_WT          GCCGGCATCGTCGTCAAGCTCGTCATGCTCGGGCTTATCGCGGCCTCGGTGTGGACGTGG
ExbD_mutant      GCCGGCATCGTCGTCAAGCTCGTCATGCTCGGGCTTATCGCGGCCTCGGTGTGGACGTGG
                 ************************************************************

ExbD_WT          GCTATCGTCATCGACAAATACCTGGCCTATGGCCGCGCACGGCGCCAGTTCGACAAGTTC
ExbD_mutant      GCTATCGTCATCGACAAATACCTGGCCTATGGCCGCGCACGGCGCCAGTTCGACAAGTTC
                 ************************************************************

ExbD_WT          GAGCAGGTGTTCTGGTCGGGCCAGTCGCTGGAAGAGCTCTACCGCTCGCTGTCGGAACGC
ExbD_mutant      GAGCAGGTGTTCTGGTCGGGCCAGTCGCTGGAAGAGCTCTACCGCTCGCTGTCGGAACGC
                 ************************************************************

ExbD_WT          AACAATACCGGTCTGGCGGCGATCTTCGTGGCTGCCATGCGCGAGTGGAAGAAATCCTTC
ExbD_mutant      AACAATACCGGTCTGGCGGCGATCTTCGTGGCTGCCATGCGCGAGTGGAAGAAATCCTTC
                 ************************************************************

ExbD_WT          GAACGCGGCGCCCGCTCGCCGATCGGCCTGCAGATGCGTATCGACCGCGCGATGGACGTG
ExbD_mutant      GAACGCGGCGCCCGCTCGCCGATCGGCCTGCAGATGCGTATCGACCGCGCGATGGACGTG
                 ************************************************************

ExbD_WT          ACGCTCGCCCGTGAGACCGAATTTCTCGGTGCTCGCCTCGGATCGCTCGCGACCATCGGC
ExbD_mutant      ACGCTCGCCCGTGAGACCGAATTTCTCGGTGCTCGCCTCGGATCGCTCGCGACCATCGGC
                 ************************************************************

ExbD_WT          TCGGCCGGTCCGTTCATCGGTCTGTTCGGCACGGTCGTCGGCATCATGACCTCGTTCCAG
ExbD_mutant      TCGGCCGGTCCGTTCATCGGTCTGTTCGGCACGGTCGTCGGCATCATGACCTCGTTCCAG
                 ************************************************************

ExbD_WT          GCAATCGCCGGTTCGAAGTCGACCAACCTTGCGGTCGTCGCGCCCGGTATCGCCGAAGCG
ExbD_mutant      GCAATCGCCGGTTCGAAGTCGACCAACCTTGCGGTCGTCGCGCCCGGTATCGCCGAAGCG
                 ************************************************************

ExbD_WT          CTGCTTGCCACTGCGATCGGCCTCGTCGCCGCTATCCCGGCAGTTATCGCCTGCAACAAG
ExbD_mutant      CTGCTTGCCACTGCGATCGGCCTCGTCGCCGCTATCCCGGCAGTTATCGCCTGCAACAAG
                 ************************************************************

ExbD_WT          TTCTCTGCCGATGCCGGCAAGCTCTCGGGCCGAATGGAAGGTTTCGCGGATGAATTCTCC
ExbD_mutant      TTCTCTGCCGATGCCGGCAAGCTCTCGGGCCGAATGGAAGGTTTCGCGGATGAATTCTCC
                 ************************************************************
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ExbD_WT          GCCATACTTTCGCGCCAGATCGACGAGAAACTGCAGCCGCGCGCTGCCGCTCAGTAACCA
ExbD_mutant      GCCATACTTTCGCGCCAGATCGACGAGAAACTGCAGCCGCGCGCTGCCGCTCAGTAACCA
                 ************************************************************

ExbD_WT          ACGGAGTATTCTCACATGGGTATGGCAGTTGGAGGCAATGGCGGAGGCGGCGGACGCCGC
ExbD_mutant      ACGGAGTATTCTCACATG------------------------------------------
                 ******************

ExbD_WT          CGTCGCGGCGGTCGGAACAGGGCCGTGATTTCCGAAATCAACGTGACGCCGCTCGTCGAC
ExbD_mutant      ------------------------------------------------------------

ExbD_WT          GTCATGCTGGTGCTTTTGATCATCTTCATGGTCGCGGCACCGATGATGACCGTCGGCGTG
ExbD_mutant      ------------------------------------------------------------

ExbD_WT          CCGATCGACCTGCCGGAAACGCAGGCCAAGGCGCTGAATTCGGAGACGCAGCCGATCACC
ExbD_mutant      ------------------------------------------------------------

ExbD_WT          ATCTCCGTCAAGAATGACGGCGAGGTGTTCCTGCAGGAAACACCGATCCCGGCGGCGGAG
ExbD_mutant      ------------------------------------------------------------

ExbD_WT          ATCGCCGCCAAGCTCGAGGCGATCGCCACCACCGGTTATAACGAACGTATCTTCGTGCGC
ExbD_mutant      ------------------------------------------------------------

ExbD_WT          GGCGACGCGACCGCGCCCTACGGCGTCATCGCCGACGTCATGGCCCGTATTCAGGGTGCA
ExbD_mutant      ------------------------------------------------------------

ExbD_WT          GGCTTCAAGAATATCGGCCTGGTGACGCAGCAGAAGAAGGACCAATAGCGCGCAAAATGA
ExbD_mutant      ----------------------------------------CAATAGCGCGCAAAATGA
                                                         ******************

ExbD_WT          AGGCCAGTGTCATCACATCTGCTGTTTTGCACGGCCTGGTGCTCACCTGGGCGATGGTGC
ExbD_mutant      AGGCCAGTGTCATCACATCTGCTGTTTTGCACGGCCTGGTGCTCACCTGGGCGATGGTGC
                 ************************************************************

ExbD_WT          CGCTTGGCGCTCCGGAATCCTTCAAGGTAGAGGATTTCGAGGCGATGCCGGTCGATCTCG
ExbD_mutant      CGCTTGGCGCTCCGGAATCCTTCAAGGTAGAGGATTTCGAGGCGATGCCGGTCGATCTCG
                 ************************************************************

ExbD_WT          TGCCGGTGGAGTCCATTACCCAGATGCAGCAAGGCGACAAGAAGGCTCCGAAGAAGGAGA
ExbD_mutant      TGCCGGTGGAGTCCATTACCCAGATGCAGCAAGGCGACAAGAAGGCTCCGAAGAAGGAGA
                 ************************************************************

ExbD_WT          CTTCCGCGCCCGTGCCGACGACACGGCCGCCGATTGCACAGCCGGCCGAGAATGCCGGCG
ExbD_mutant      CTTCCGCGCCCGTGCCGACGACACGGCCGCCGATTGCACAGCCGGCCGAGAATGCCGGCG
                 ************************************************************

ExbD_WT          ACAGCAATGTCGACCTGAAAACGCCGCCGGTCCCGAACGCCAAGCCCAGCAATACTGAAG
ExbD_mutant      ACAGCAATGTCGACCTGAAAACGCCGCCGGTCCCGAACGCCAAGCCCAGCAATACTGAAG
                 ************************************************************
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ExbD_WT          CGGCTGCCGCCAATTCGAGCGACAAGCCGATGCCGAAGATCGATCCTAAGCCGAATGACG
ExbD_mutant      CGGCTGCCGCCAATTCGAGCGACAAGCCGATGCCGAAGATCGATCCTAAGCCGAATGACG
                 ************************************************************

ExbD_WT          TCAAGGAGATCGTCAAGGAGGAAACGGAAGTCGAGCAGCCGAAGGAGGTTGCTTCAATTC
ExbD_mutant      TCAAGGAGATCGTCAAGGAGGAAACGGAAGTCGAGCAGCCGAAGGAGGTTGCTTCAATTC
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ExbD_WT          CGCCGCCGAAGCCTGTCGAAG
ExbD_mutant      CGCCGCCGAAGCCTGTCGAAG
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CLUSTAL 2.1 multiple sequence alignment of wild type *exbbd* (top) and Δ*exbbd* (bottom) in *R. leguminosarum* ATCC14479

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ExbBD_WT         ATGGAACAAGTAGGATTGGCAGCAGCAACGACGGACGTCAGCCTCTGGTCGCTTTTCATG
ExbBD_mutant     ATGGAACAAGTAGGATTGGCAGCAGCAACGACGGACGTCAGCCTCTGGTCGCTTTTCATG
                 ************************************************************

ExbBD_WT         CAGGCCGGCATCGTCGTCAAGCTCGTCATGCTCGGGCTTATCGCGGCCTCGGTGTGGACG
ExbBD_mutant     CAGGCCGGCATCGTCGTCAAGCTCGTCATGCTCGGGCTTATCGCGGCCTCGGTGTGGACG
                 ************************************************************

ExbBD_WT         TGGGCTATCGTCATCGACAAATACCTGGCCTATGGCCGCGCACGGCGCCAGTTCGACAAG
ExbBD_mutant     TGGGCTATCGTCATCGACAAATACCTGGCCTATGGCCGCGCACGGCGCCAGTTCGACAAG
                 ************************************************************

ExbBD_WT         TTCGAGCAGGTGTTCTGGTCGGGCCAGTCGCTGGAAGAGCTCTACCGCTCGCTGTCGGAA
ExbBD_mutant     TTCGAGCAGGTGTTCTGGTCGGGCCAG--------------------------------
                 ***************************

ExbBD_WT         CGCAACAATACCGGTCTGGCGGCGATCTTCGTGGCTGCCATGCGCGAGTGGAAGAAATCC
ExbBD_mutant     ------------------------------------------------------------


ExbBD_WT         TTCGAACGCGGCGCCCGCTCGCCGATCGGCCTGCAGATGCGTATCGACCGCGCGATGGAC
ExbBD_mutant     ------------------------------------------------------------


ExbBD_WT         GTGACGCTCGCCCGTGAGACCGAATTTCTCGGTGCTCGCCTCGGATCGCTCGCGACCATC
ExbBD_mutant     ------------------------------------------------------------


ExbBD_WT         GGCTCGGCCGGTCCGTTCATCGGTCTGTTCGGCACGGTCGTCGGCATCATGACCTCGTTC
ExbBD_mutant     ------------------------------------------------------------


ExbBD_WT         CAGGCAATCGCCGGTTCGAAGTCGACCAACCTTGCGGTCGTCGCGCCCGGTATCGCCGAA
ExbBD_mutant     ------------------------------------------------------------


ExbBD_WT         GCGCTGCTTGCCACTGCGATCGGCCTCGTCGCCGCTATCCCGGCAGTTATCGCCTGCAAC
ExbBD_mutant     ------------------------------------------------------------
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ExbBD_WT         AAGTTCTCTGCCGATGCCGGCAAGCTCTCGGGCCGAATGGAAGGTTTCGCGGATGAATTC
ExbBD_mutant     ------------------------------------------------------------

ExbBD_WT         TCCGCCATACTTTCGCGCCAGATCGACGAGAAACTGCAGCCGCGCGCTGCCGCTCAGTAA
ExbBD_mutant     ------------------------------------------------------------

ExbBD_WT         CCAACGGAGTATTCTCACATGGGTATGGCAGTTGGAGGCAATGGCGGAGGCGGCGGACGC
ExbBD_mutant     ------------------------------------------------------------

ExbBD_WT         CGCCGTCGCGGCGGTCGGAACAGGGCCGTGATTTCCGAAATCAACGTGACGCCGCTCGTC
ExbBD_mutant     ------------------------------------------------------------

ExbBD_WT         GACGTCATGCTGGTGCTTTTGATCATCTTCATGGTCGCGGCACCGATGATGACCGTCGGC
ExbBD_mutant     ------------------------------------------------------------

ExbBD_WT         GTGCCGATCGACCTGCCGGAAACGCAGGCCAAGGCGCTGAATTCGGAGACGCAGCCGATC
ExbBD_mutant     ------------------------------------------------------------

ExbBD_WT         ACCATCTCCGTCAAGAATGACGGCGAGGTGTTCCTGCAGGAAACACCGATCCCGGCGGCG
ExbBD_mutant     ----------------------GGCGAGGTGTTCCTGCAGGAAACACCGATCCCGGCGGCG
                                       ************************************

ExbBD_WT         GAGATCGCCGCCAAGCTCGAGGCGATCGCCACCACCGGTTATAACGAACGTATCTTCGTG
ExbBD_mutant     GAGATCGCCGCCAAGCTCGAGGCGATCGCCACCACCGGTTATAACGAACGTATCTTCGTG
                 ************************************************************

ExbBD_WT         CGCGGCGACGCGACCGCGCCCTACGGCGTCATCGCCGACGTCATGGCCCGTATTCAGGGT
ExbBD_mutant     CGCGGCGACGCGACCGCGCCCTACGGCGTCATCGCCGACGTCATGGCCCGTATTCAGGGT
                 ************************************************************

ExbBD_WT         GCAGGCTTCAAGAATATCGGCCTGGTGACGCAGCAGAAGAAGGACCAATAGCGCGCAAAA
ExbBD_mutant     GCAGGCTTCAAGAATATCGGCCTGGTGACGCAGCAGAAGAAGGACCAATAGCGCGCAAAA
                 ************************************************************

ExbBD_WT         TGAAGGCCAGTGTCATCACATCTGCTGTTTTGCACGGCCTGGTGCTCACCTGGGCGATGG
ExbBD_mutant     TGAAGGCCAGTGTCATCACATCTGCTGTTTTGCACGGCCTGGTGCTCACCTGGGCGATGG
                 ************************************************************

ExbBD_WT         TGCCGCTTGGCGCTCCGGAATCCTTCAAGGTAGAGGATTTCGAGGCGATGCCGGTCGATC
ExbBD_mutant     TGCCGCTTGGCGCTCCGGAATCCTTCAAGGTAGAGGATTTCGAGGCGATGCCGGTCGATC
                 ************************************************************

ExbBD_WT         TCGTGCCGGTGGAGTCCATTACCCAGATGCAGCAAGGCGACAAGAAGGCTCCGAAGAAGG
ExbBD_mutant     TCGTGCCGGTGGAGTCCATTACCCAGATGCAGCAAGGCGACAAGAAGGCTCCGAAGAAGG
                 ************************************************************

ExbBD_WT         GACTTCCGCGCCCGTGCCGACGACACGGCCGCCGATTGCACAGCCGGCCGAGAATGCCG
ExbBD_mutant     AGACTTCCGCGCCCGTGCCGACGACACGGCCGCCGATTGCACAGCCGGCCGAGAATGCCG
                 ************************************************************
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ExbBD_WT         GCGACAGCAATGTCGACCTGAAAACGCCGCCGGTCCCGAACGCCAAGCCCAGCAATACTG
ExbBD_mutant     GCGACAGCAATGTCGACCTGAAAACGCCGCCGGTCCCGAACGCCAAGCCCAGCAATACTG
                 ************************************************************

ExbBD_WT         AAGCGGCTGCCGCCAATTCGAGCGACAAGCCGATGCCGAAGATCGATCCTAAGCCGAATG
ExbBD_mutant     AAGCGGCTGCCGCCAATTCGAGCGACAAGCCGATGCCGAAGATCGATCCTAAGCCGAATG
                 ************************************************************

ExbBD_WT         CGTCAAGGAGATCGTCAAGGAGGAAACGGAAGTCGAGCAGCCGAAGGAGGTTGCTTCAA
ExbBD_mutant     ACGTCAAGGAGATCGTCAAGGAGGAAACGGAAGTCGAGCAGCCGAAGGAGGTTGCTTCAA
                 ************************************************************

ExbBD_WT          TTCCGCCGCCGAAGCCTGTCGAAG
ExbBD_mutant      TTCCGCCGCCGAAGCCTGTCGAAG
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```

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