A previously uncharacterized tetratricopeptide-repeat-containing protein is involved in cell envelope function in *Rhizobium leguminosarum*

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*Rhizobium leguminosarum* is a soil bacterium that is an intracellular symbiont of leguminous plants through the formation of nitrogen-fixing root nodules. Due to the changing environments that rhizobia encounter, the cell is often faced with a variety of cell altering stressors that can compromise the cell envelope integrity. A previously uncharacterized operon (RL3499–RL3502) has been linked to proper cell envelope function, and mutants display pleiotropic phenotypes including an inability to grow on peptide-rich media. In order to identify functional partners to the operon, suppressor mutants capable of growth on complex, peptide-rich media were isolated. A suppressor mutant of a non-polar mutation to RL3500 was chosen for further characterization. Transposon mutagenesis, screening for loss of the suppressor phenotype, led to the identification of a Tn5 insertion in an uncharacterized tetratricopeptide-repeat-containing protein RL0936. Furthermore, RL0936 had a 3.5-fold increase in gene expression in the suppressor strain when compared with the WT and a 1.5-fold increase in the original RL3500 mutant. Mutation of RL0936 decreased desiccation tolerance and lowered the ability to form biofilms when compared with the WT strain. This work has identified a potential interaction between RL0936 and the RL3499–RL3502 operon that is involved in cell envelope development in *R. leguminosarum*, and has described phenotypic activities to a previously uncharacterized conserved hypothetical gene.

INTRODUCTION

*Rhizobium leguminosarum* is a Gram-negative bacterium that can form a symbiotic relationship with legumes such as peas and lentils. *R. leguminosarum* infects the plant root through an infection thread, enters root cells through endocytosis and differentiates into bacteroids culminating in the formation of a root nodule structure. Within the nodule the bacteroids supply fixed atmospheric nitrogen to the plant, while the plant supplies carbon to the bacteroids (Gibson *et al.*, 2008). Free-living rhizobia can be found in the rhizosphere, where they experience a dynamic growth environment including fluctuations in water availability, temperature and nutrient limitation due to competition with other microbes. Since bacteria often must respond to changing environments, they have evolved a complex cell envelope that adapts under stressful conditions to maintain cell envelope integrity in order to sustain its primary function of permitting the select passage of nutrients and waste products (Silhavy *et al.*, 2010; Storz & Hengge-Aronis, 2000).

The genetic network involved in cell envelope development and stress adaptation is only partially described in rhizobia, despite its function for cell survival in the soil and during symbiosis. Using genetic approaches, we have initiated a programme to identify novel genes involved in cell envelope development. Our earlier work and that of others has identified a link between *Rhizobium* genes involved in cell envelope development and their requirement for growth on complex peptide rich media such as tryptone-yeast extract medium (TY). Specific examples include genes involved in the synthesis of the very long chain fatty acid attached to the lipid A of the outer membrane lipopolysaccharide (Vanderlinde *et al.*, 2009), a gene with homology to the cell-envelope-associated carboxyl-terminal protease (ctpA) (Gilbert *et al.*, 2007), a two-component system that functions in biofilm formation and survival in the rhizosphere (Orur et al., 2009).
sensor histidine kinase (ctpA) (Gibson et al., 2008), a
two-component signal transduction system (chvG/chvI)
(Bélanger et al., 2009; Foreman et al., 2010; Vanderlinde &
Yost, 2012) and a broadly conserved four gene operon with a
MoxR AAA+ and von Wilderbrand A domain containing
gene (RL3499–RL3502) (Vanderlinde et al., 2011). The
shared mutant phenotype of an inability to grow on complex
peptide rich media suggests a possible network between these
cell envelope related genes, and further experimentation is
needed to identify potential interaction networks.

Vanderlinde et al. (2011) identified a link between the
uncharacterized, broadly conserved MoxR AAA+ operon,
and proper cell envelope function in R. leguminosarum.
The genes in this operon are subsequently referred to as
cell complex media growth deficient (cmdA–cmdD), based on
the mutant phenotype of an inability to grow on complex
peptide rich growth media. Mutations in the operon
resulted in a number of phenotypes related to envelope
disfunction and cell morphology (Vanderlinde et al., 2011).
The large MoxR AAA+ family contains a diverse group of
ATPases that are found throughout bacteria and are
suggested to have chaperone-like activities (Snider &
Houry, 2006; Wong & Houry, 2012). These MoxR
AAA+ proteins often function together with VWA-
domain-containing proteins to form a chaperone system
involved in the folding and activation of proteins through
the insertion of metal cofactors into the substrate
molecules (Snider & Houry, 2006). The general nature of
MoxR related genes and the pleiotropic phenotypes
observed in the R. leguminosarum mutants suggests the
operon may be connected to a network of envelope related
genes. By identifying interacting partners with the cmdA–
cmdD operon, we can begin to elucidate the complex
genetic network responsible for proper cell envelope
development in rhizobia.

Isolation of suppressor mutants is a well-established
approach to identify functional partners and elucidate
novel gene functions (Clementz et al., 1997; Driscoll &
Finan, 1993; Li et al., 2004; Ogura et al., 1991; Oresnik et al.,
1994; Wells & Long, 2003), and has been used to define
functional networks involved in cell envelope development
and function (Alonso-Monge et al., 2001; Fields et al.,
noted that culturing cmdA–cmdD mutants on TY agar at
high cell density (10⁸ c.f.u. ml⁻¹) resulted in the appear-
ance of suppressor mutants capable of growth on the TY
agar.

In this study, suppressor mutants capable of growth on TY
medium were successfully isolated for strains with muta-
tions in the cmdA–cmdD operon. A suppressor mutant in
the non-polar cmdB (RL3500) deletion mutant background
was chosen for further characterization. Transposon
mutagenesis of the suppressor mutant and screening for
loss of growth on TY was used to attempt identification of
the suppressor locus. This approach lead to the isolation of
a mutant carrying a Tn5 insertion in an uncharacterized
tetrucopeptide repeat (TPR)-containing protein, origin-
ally annotated as RL0936 (Young et al., 2006), and hence-
forth termed ttpA (tetrucopeptide repeat protein). The
results herein characterize this previously uncharacterized
ttpA gene, coding for a conserved hypothetical protein, and
provide evidence suggesting that ttpA may interact with the
cmdA–cmdD operon.

METHODS

Plasmids, PCR primers, bacterial strains and growth condi-
tions. All plasmids, strains and primers used in this study are listed in
Table 1. R. leguminosarum strains were grown using either Vincent’s
minimal media (VMM) and 10 mM mannitol as the carbon source
(Vincent, 1970), or tryptone-yeast extract (TY) media (Beringer,
1974). These strains were cultured with the following antibiotic
concentrations when appropriate (µg ml⁻¹): streptomycin, 500;
gentamicin, 30; tetracycline, 5; and neomycin, 100. All Escherichia
coli strains were grown using LB medium and the following antibiotic
concentrations when appropriate (µg ml⁻¹): spectinomycin, 100;
gentamicin, 15; tetracycline, 10; ampicillin, 100; and kanamycin, 50.

Isolation of suppressor mutants in cmdA–cmdD operon
mutants. Deletion of genes in the cmdA–cmdD operon results in a
mutant’s inability to grow on TY media or VMM supplemented with
3 mM glycine. Suppressor mutants that restored the ability of a cmdB
mutant (38cmdB) to grow on VMM supplemented with 3 mM glycine
were isolated by performing serial dilutions of 38cmdB (10⁶–10⁸)
and plating on VMM with 3 mM glycine, as well as VMM as a control.
Colonies that were able to grow on the glycine plates were purified and
PCR was performed to verify the original cmdB mutation remained
present in the suppressor mutants. Suppressor strains were then
streaked onto TY to confirm restored growth on TY. A suppressor
mutant matching these criteria was used for further study (38cmdB-S).

Transposon mutagenesis of suppressor mutants. The mini-Tn5
derivative found on plasmid ptGN (Tang et al., 1999) was used for
insertional mutagenesis of the suppressor mutant 38cmdB-S. The
transposon was introduced into 38cmdB-S via conjugation using E.
coli S17-1 containing ptGN as the donor strain and incubation at
30 °C for 48 h on VMM plates containing 1 mM proline. Mutants
were screened for sensitivity to 3 mM glycine by tooth-picking
mutants onto VMM containing 3 mM glycine, and using VMM with
streptomycin and neomycin as a positive control. Mutants that were
unable to grow on the glycine plates were also screened for an
inability to grow on the peptide rich complex media TY, which is the
phenotype in the original 38cmdB genetic background. To identify
the transposon insertion site, genomic DNA was isolated and used to
perform the thermal asymmetrical interlaced (TAIL)-PCR protocol as
described by Liu & Huang (1998). The TAIL-PCR product was sent
for sequencing and location of the Tn insertion was identified using the
RhizoDB database (http://www.xbase.ac.uk/rhizodb/) and a
BLASTN search (Altschul et al., 1997).

Molecular techniques. An alkaline lysis method as described by
Sambrook & Russell (2001) was used to perform all plasmid
preparations. A QIAEX II gel extraction kit (Qiagen) was used to
extract PCR products from agarose gels whenever needed. All
restriction enzymes were purchased from Invitrogen and were used
according to the supplier’s instructions.

Insertional mutagenesis of ttpA. To further characterize ttpA in a
WT genetic background, a single crossover mutant was constructed in
strain 3841. Insertional mutagenesis via allelic exchange using the

http://mic.sgmjournals.org
Table 1. Summary of all plasmids, strains and primers used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source</th>
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<tbody>
<tr>
<td><strong>E. coli</strong></td>
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<tr>
<td>JM109</td>
<td>F- episome recA- endA-, Amp&lt;sup&gt;R&lt;/sup&gt;, Tc&lt;sup&gt;R&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Promega</td>
</tr>
<tr>
<td>DH5x</td>
<td>endA1 hisD17 supE44 thi-1 recA1 gyrA96 relA1 D(argF-lacZ) DlacU169 B01lacZDM15</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>S17-1</td>
<td>RP4 tra region, mobilizer strain recA derivative of MM294A with RP4-2 (Tc::Mu::Km::Tn7)</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>R. leguminosarum</strong></td>
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<td>3841</td>
<td>Spontaneous streptomycin-resistant derivative of <em>R. leguminosarum</em> bv. <em>viciae</em> strain 300, Sm&lt;sup&gt;R&lt;/sup&gt;, Ty&lt;sup&gt;+&lt;/sup&gt;, Gly&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Glenn et al. (1980), Johnston &amp; Beringer (1975)</td>
</tr>
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<td>38cmdA</td>
<td>3841 RL3499 (cmdA) deletion incapable of growth on TY media or VMM with 3 mM glycine, Sm&lt;sup&gt;R&lt;/sup&gt;, Ty&lt;sup&gt;+&lt;/sup&gt;, Gly&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Vanderlinde et al. (2011)</td>
</tr>
<tr>
<td>38cmdA-S</td>
<td>cmdA suppressor mutant, restored growth on TY and in presence of 3 mM glycine, Sm&lt;sup&gt;R&lt;/sup&gt;, Ty&lt;sup&gt;+&lt;/sup&gt;, Gly&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>38cmdB</td>
<td>3841 RL3500 (cmdB) deletion incapable of growth on TY media or VMM with 3 mM glycine, Sm&lt;sup&gt;R&lt;/sup&gt;, Ty&lt;sup&gt;+&lt;/sup&gt;, Gly&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Vanderlinde et al. (2011)</td>
</tr>
<tr>
<td>38cmdB-S</td>
<td>cmdB suppressor mutant, restored growth on TY and in presence of 3 mM glycine, Sm&lt;sup&gt;R&lt;/sup&gt;, Ty&lt;sup&gt;+&lt;/sup&gt;, Gly&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>38cmdB-S/ttpA::Tn5</td>
<td>38cmdB-S background, RL0936 (ttpA) mutant due to Tn5 insertion 290 bp into ORF, Sm&lt;sup&gt;R&lt;/sup&gt; Nm&lt;sup&gt;R&lt;/sup&gt; Gm&lt;sup&gt;R&lt;/sup&gt;, Ty&lt;sup&gt;+&lt;/sup&gt;, Gly&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td>38ttpA</td>
<td>3841 RL0936 (ttpA) single crossover insertional mutant containing pJQ200SK suicide vector, Sm&lt;sup&gt;R&lt;/sup&gt; Gm&lt;sup&gt;R&lt;/sup&gt;, Ty&lt;sup&gt;+&lt;/sup&gt;, Gly&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td><strong>Plasmids</strong></td>
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<td>pTG1</td>
<td>Tn5 derivative, Gm&lt;sup&gt;R&lt;/sup&gt; Amp&lt;sup&gt;R&lt;/sup&gt;, promoter-less nptII, gfp</td>
<td>Tang et al. (1999)</td>
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<td>pGem-T easy vector</td>
<td>pGem TA cloning vector, 3015 bp, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pJQ200SK</td>
<td>Suicide vector, P15a ori mob sacB, Gm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Quandt &amp; Hynes (1993)</td>
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<td>pH41</td>
<td>IncP broad-host-range vector with par stabilization, Tc&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Cheng &amp; Walker (1998)</td>
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<td>pRL0936-comp</td>
<td>pH41 with a 2195 bp PCR fragment containing the entire ttpA region including the promoter, Tc&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pfus1par</td>
<td>pFus1 par with plocus of RK2</td>
<td>Reeve et al. (1999); Yost et al. (2004)</td>
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<td>pfus::ttpA</td>
<td>ttpA promoter fragment cloned into pfus1par with KpnI and EcoRI</td>
<td>This study</td>
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<td><strong>Primers</strong></td>
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<tr>
<td>AD-1</td>
<td>5′-AGWGNAGWANWACAWAGG-3′</td>
<td>Liu &amp; Huang (1998)</td>
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<td>Gm-TAIL-1</td>
<td>5′-CAAACGGCGCTTGGTTGCTAATTGTGAT-3′</td>
<td>Vanderlinde et al. (2010)</td>
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<td>Gm-TAIL-2</td>
<td>5′-AGTTGGCCATACGGGAAAGAAGATG-3′</td>
<td>Vanderlinde et al. (2010)</td>
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<td>RL0936A</td>
<td>5′-AGTCTTAGAGTGACTCTTGCATCGAGA-3′</td>
<td>This study</td>
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<td>RL0936B</td>
<td>5′-GTTCCGGACAACAGGTTGTCAC-3′</td>
<td>This study</td>
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<tr>
<td>RL0936C</td>
<td>5′-ATCTGCAAGATCCGGTGTCT-3′</td>
<td>This study</td>
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<tr>
<td>RL0936H</td>
<td>5′-AGTCTAGACAGCAGCATATCAGGACAGATGA-3′</td>
<td>This study</td>
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<tr>
<td>RL0936F</td>
<td>5′-AGACTAGGACAGCGCTGACACACTTCTT-3′</td>
<td>This study</td>
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<tr>
<td>RL0936R</td>
<td>5′-AGCCCGGGTGGTCTATTGCGCTTGTGTCG-3′</td>
<td>This study</td>
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<tr>
<td>pfusRL0936F</td>
<td>5′-AGGTTACCGGGCATCCATTACGGGAAACAT-3′</td>
<td>This study</td>
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<tr>
<td>pfusRL0936R</td>
<td>5′-GGAGAAAGTGGTCAGCAGTGT-3′</td>
<td>This study</td>
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</table>

suicide vector pJQ200SK was performed using a method described by Quandt & Hynes (1993). A 600 bp internal fragment of ttpA was amplified using primers RL0936F with a SpeI linker and RL0936R with a SmaI linker (Table 1). PCRs used 1 U of Taq DNA polymerase (UBI) and the following reaction conditions: 2 mM MgSO<sub>4</sub>, 0.2 mM dNTPs, 1× reaction buffer and 0.2 μM of each primer. Using a BioRad thermocycler the PCR was run at 94°C for 5 min, followed by 30 cycles of 95°C for 30 s, 68°C for 30 s and 72°C for 1 min 30 s with a final extension of 72°C for 5 min. The PCR fragment was cloned into a pGem-T Easy Vector (Promega) as per the supplier’s instructions. Restriction enzymes SpeI and SmaI were used to excise the ttpA fragment from the pGem-T Easy Vector, which was then ligated into pJQ200SK using complementary restriction enzyme sites to create pJQRL0936. The new construct was first transformed into DH5α competent cells to confirm proper insertion, and subsequently transformed into the mobilizer strain S17-1. Conjugation was performed over 48 h with the WT strain on VMM containing 1 mM proline. Single crossover mutants were isolated by plating the conjugation mixture onto VMM containing streptomycin and gentamicin to select for insertion of the pJQ200SK vector. PCR was used to confirm the correct disruption of ttpA.

Construction of a broad-host-range plasmid containing the RL0936 gene. Genetic complementation of ttpA mutants used a vector constructed to contain the WT ttpA gene. The ttpA gene with the upstream promoter region was PCR amplified from the WT strain...
of *R. leguminosarum* bv. *viceae* 3841 using primers RL0936A and RL0936H (Table 1), both containing an XbaI restriction linker at the 5’ end. PCR used 1 U of *Taq* DNA polymerase (UBI) and the following reaction conditions: 2 mM MgSO₄, 0.2 mM dNTPs, 1 × reaction buffer and 0.2 μM of each primer. Using a Bio-Rad thermocycler the reaction was run at 94 ºC for 5 min, followed by 30 cycles of 95 ºC for 30 s, 66 ºC for 30 s and 72 ºC for 3 min 30 s, with a final extension of 72 ºC for 5 min. The PCR fidelity was confirmed though DNA sequencing of the PCR amplicon. The PCR fragment was then cloned into a pGem-T Easy Vector (Promega) and digested using XbaI. The digested fragment was then ligated into the broad-host-range plasmid pHC41 using the same restriction enzyme to cut the vector. The ligation was transformed into the mobilizer strain S17-1 to make pRL0936comp and was used for conjugation to complement *tpfA* in the mutant strain 38tpfA (Table 1). The pRL0936comp plasmid was also conjugated into 38cmdB. Recipient colonies were tested for growth on TY and VMM containing 3 mM glycine.

**RNA extraction and quantitative reverse transcription PCR (RT-qPCR) to measure mRNA levels of *tpfA***. RNA was extracted using cells that were grown on VMM with erythritol (15 mM) to an OD₆₀₀ of 0.5. A volume of 0.4 was added of 5% acid phenol in ethanol to the cells, which were then kept on ice for 30 min. Samples were centrifuged at 8200 × g for 5 min at 4 ºC. RNA extraction was performed using an miRNeasy mini kit (Qiagen) following the manufacturer’s protocol with a few modifications. Briefly, cell pellets were resuspended in 100 μL TE with lysozyme (50 mg mL⁻¹) and 20 μL protease K (Qiagen). Samples were incubated at room temperature for 5 min, vortexing for 10 s every 2 min. One millilitre of pre-heated QiAzo1 reagent (65 ºC) was added, samples were vortexed for high for 3 min, and were incubated at room temperature for 5 min. Chloroform was added (200 μl) with vigorous mixing for 5 s, and then incubated at room temperature for 3 min. Samples were centrifuged at 12,000 × g for 15 min at 4 ºC. Aqueous phases were transferred to new microfuge tubes and extraction was completed using an miRNeasy mini kit, starting with the ethanol addition step (Qiagen). DNase treatment was conducted using Turbo DNase (Life Technologies). Reverse transcription reactions were carried out according to a previously described protocol (Manzon et al., 2007), with modifications described by Vanderlinde et al. (2009, 2011). Quantitative PCR was conducted on an Applied Biosystems StepOne Real-Time PCR System using SYBR Green PCR Master Mix (Life Technologies) as per manufacturer’s instructions and using primers RL0936B and RL0936C (Table 1).

**Construction of an *tpfA::gusA* transcriptional fusion.** A 245 bp fragment upstream of RL0936 was amplified with the primers pfusRL0936F and pfusRL0936R under standard PCR conditions. The fragment was subsequently cloned into a pGem-T easy vector as per manufacturer’s instructions. The cloned promoter region was then excised using *Eco*R and *Eco*I and ligated into the pFus1par vector containing the promoterless *gusA* reporter gene in a par-stabilized plasmid. The PCR fidelity was confirmed through sequencing of the PCR amplicon. The resulting plasmid pFusRL0936 was then conjugated into the *R. leguminosarum* WT strain 3841 and into the mutant strains 38cmdB, 38cmdB-S and 38cmdB-S-tpfA::Tn5 to measure gene expression using the β-galactosidase reporter gene assay of Miller (1972), with modifications described by Yost et al. (2004). All assays were carried out on cultures in the late-exponential growth phase.

**DNA sequence analysis.** DNA sequencing was conducted by Quintarabio (Albany, CA, USA). Sequence data were analysed using EnzymeX [version 3.1; (http://nucleobyt.es/index.php? enzymeX)] and AFRAX software [version 1.7.2; (http://nucleobyt.es/index.php?AFRAX)]. OLEG 4.0 software (National Biosciences) was used to design all primers. Sequence alignments were done using CLUSTAL W (Thompson et al., 1994). Phylogenies were reconstructed using MEGA 5.05 (Tamura et al., 2011).

**Desiccation assay.** Due to the importance of a functional cell envelope in desiccation tolerance (Gilbert et al., 2007; Vanderlinde et al., 2009, 2010), desiccation tolerance of the mutants was investigated. A filtration method described by Ophir & Gutnick (1994) and modified by Gilbert et al. (2007) was used to perform desiccation sensitivity assays. Cultures were first grown in VMM broth for 48 h and diluted to 50 ml using sterile VMM broth. The dilution was filtered using a Millipore Vacuum Manifold, Microfil Filtration funnels, and S-Pak 0.45 μM type HA membranes, according to the manufacturer’s description (Millipore). Filter membranes were cut in half and one half was placed on water agar (1.25%), while the other was placed in a sterile empty Petri dish. All plates were incubated at 30 ºC for 48 h. After incubation, each filter was put in separate vials containing 2 ml of water and vortexed for 5 min to remove cells. The cell suspension was serially diluted and spotted on VMM to determine the number of culturable bacteria and the percentage survival was calculated using the ratio of c.f.u. ml⁻¹ (desiccated) to c.f.u. ml⁻¹ (hydrated).

**Biofilm assay.** A functional cell envelope is important for biofilm formation in *R. leguminosarum* (Vanderlinde et al., 2010); therefore, the biofilm capabilities of various mutants were tested. A method adapted from Fujishige et al. (2006) and O'Toole & Kolter (1998) was used to culture biofilms in microtitre plates. Cells were first scraped off from VMM plates using a sterile cotton swab and were suspended in double-distilled water (ddH₂O). To obtain an approximate starting inoculum of about 1 × 10⁸ c.f.u. ml⁻¹, the suspensions were diluted 1 in 15 using VMM broth containing 1% mannitol. From this dilution, 150 μl was added to each well of a flat bottom Corning CellBIND Surface brand 96-well plate. The plates were sealed using Parafilm and were incubated on a gyratory shaker set to 100 r.p.m. for 48 h at 30 ºC. Biofilm-containing wells were rinsed with 200 μl ddH₂O and stained with 200 μl 0.4% crystal violet in each well for 15 min at room temperature. The wells were then rinsed three times with 200 μl ddH₂O and the remaining crystal violet was solubilized with 200 μl 95% ethanol. Absorbance was measured at 550 nm.

**Antibiotic sensitivity assay.** Sensitivity to hydrophobic antibiotics can relay information regarding cell envelope integrity. Sensitivity assays were conducted on VMM plates containing appropriate antibiotics. Strain suspensions were added to 400 μl 0.75% agar VMM overlay tubes in and poured onto individual plates. The overlay was allowed to solidify, and 10 μl of either 10 mg erythromycin ml⁻¹ or 0.1 mg tetracycline ml⁻¹ was added to sterilized 7 mm discs of blotting paper. The antibiotic-containing discs were placed on the overlay plates and allowed to incubate for 48 h at 30 ºC. Zones of inhibition were measured at the end of the incubation period.

**NPN assay.** To determine outer membrane permeability changes in the *R. leguminosarum* mutant strains, uptake of the fluorescent probe 1-N-phenylphthalamimine (NPN) was measured using a method described by Helander & Mattila-Sandholm (2000). An intact outer membrane prevents binding of the NPN; therefore, an increase in fluorescence indicates decreased structural integrity of the outer membrane. Cells were added to a microtitre plate in HEPES buffer at a concentration of 5 mmol l⁻¹, pH 7.2, with 50 μM NPN (40 μmol l⁻¹ in HEPES buffer). The fluorescence was measured using a fluorescence microplate spectrophotometer with an excitation wavelength at 355 nm and detection of an emission wavelength at 405 nm.
RESULTS AND DISCUSSION

Isolation of suppressor mutants and subsequent transposon mutagenesis

Suppressor mutants can be valuable in identifying interactions between genes when studying a specific phenotype, particularly when the function of the genes is not readily apparent. Further, isolation of suppressor mutants is a well-established strategy for identifying gene partners and elucidating genetic networks (Clementz et al., 1997; Driscoll & Finan, 1993; Li et al., 2004; Ogura et al., 1991; Oresnik et al., 1994; Wells & Long, 2003). Suppressor mutants were isolated in a cmdB non-polar mutant background at a frequency of $10^{-6}$ c.f.u. ml$^{-1}$ on both TY and VMM with 3 mM glycine. Suppressor strains were confirmed through PCR to maintain the original in-frame deletion to cmdB, confirming that a secondary site mutation is the result for the restored growth on TY and VMM with 3 mM glycine phenotype. We were able to isolate suppressor mutants for cmdA, cmdB and cmdC non-polar mutants, as well as a polar mutant at similar frequencies, of $10^{-5}$, $10^{-6}$, $10^{-6}$ and $10^{-5}$, respectively. cmdB suppressors were selected for further study, as it is a likely partner to cmdA based on canonical moxR descriptions (Snider & Houry, 2006; Wong & Houry, 2012), and we were interested in identifying genes that could compensate for loss of cmdB in this partnership. Future studies into the functional partners interacting with the other operon genes will be undertaken to provide a more complete picture of the overall operon function. However, a substantial effort is required to identify each specific unmarked suppressor locus and was therefore beyond the scope of this investigation.

Transposon mutagenesis was conducted in an attempt to identify the suppressor locus in strain 38cmdB-S by screening for a loss of growth on TY media phenotype (Fig. 1). This screen led to the isolation of a mutant with a Tn5 insertion in a broadly conserved gene, within the alphaproteobacteria, coding for a tetraricopeptide-repeat-containing protein (ttpA). Genes containing this motif are predicted to code for proteins involved in a broad range of protein–protein interactions involved in diverse cellular functions such as cell cycle regulation, transcription and protein folding (Cerveny et al., 2013). The conservation of ttpA was similar to the conservation of the cmdA–cmdD operon where both loci are conserved within the Rhizobiales and in other Alphaproteobacteria classes such as the Rhodobacteraceae and Rhodospirillaceae (Fig. S1, available with the online Supplementary Material, and Vanderlinde et al., 2011).

The transposon mutant strain, 38cmdB-S/ttpA::Tn5, was complemented with a plasmid carrying WT cmdB, which restored an ability to grow on TY and VMM with 3 mM glycine plates, suggesting that mutation of the ttpA gene did not create a loss in TY growth that was independent of the cmdB mutation in 38cmdB-S. Furthermore, insertional mutation of ttpA in a WT background does not result in an inability to grow on TY media or VMM supplemented with 3 mM glycine. These combined results suggested ttpA as a putative candidate for the suppressor locus. However, ttpA and the promoter region were PCR amplified and DNA sequenced in all relevant strains, and no polymorphisms were identified between the suppressor strain, the original cmdB mutant strain or the WT strain (data not shown). This result implies that the specific secondary site mutation is located elsewhere in the genome. Nonetheless, the above phenotypic data suggested a link between ttpA and cmdB. Furthermore, bioinformatic analysis supports a predicted interaction of ttpA with genes from the cmdA–cmdD operon. The STRING (http://string-db.org/) algorithm explores predicted protein interaction networks, using data extracted from curated functional genomic and protein structure databases. STRING weakly predicted ttpA to interact with cmdA–cmdD (score of 0.56). It is also interesting to note the STRING analysis predicted interaction between RL0936 and ctpA (score of 0.65), a gene that is known to be associated with cell envelope function in R. leguminosarum and is required for growth on TY agar (Gilbert et al., 2007).

ttpA expression is increased in cmdB mutant backgrounds

To examine the expression levels of ttpA in the various mutants, a transcriptional fusion containing the promoter region of ttpA cloned upstream of the promoter-less gusA reporter gene in pFus1par1 was constructed. GusA assays were performed, and it was noted that expression of ttpA was 3.5-fold higher in the original suppressor when compared to the WT and 1.5-fold higher in 38cmdB (Table 2). The increased expression of ttpA was exclusive to the 38cmdB-S suppressor strain. Expression levels of ttpA in the cmdA, cmdC or cmdD suppressors did not significantly increase relative to the level of expression in the original mutant strains. Since there was no sequence difference for ttpA nor in its promoter region, the actual suppressor locus may be involved in ttpA regulation.

Addition of a broad-host-range plasmid containing the WT ttpA rescues the TY minus phenotype in a cmdB mutant

A broad-host-range plasmid containing ttpA and its promoter region (pRL0936comp) was conjugated into 38cmdB. This strain was able to grow on both TY and VMM with 3 mM glycine, while the control of 38cmdB carrying the empty vector pH41 was unable to grow on TY or VMM with 3 mM glycine. RT-qPCR confirmed that the mRNA concentration of ttpA was a mean of 12-fold higher in strains carrying the pRL0936comp plasmid compared to strains carrying the empty pH41 vector. These data, along with the data in Table 2, support the hypothesis that an increase in expression of ttpA in 38cmdB can lead to restoration of growth on TY and
VMM with 3 mM glycine. Notably, the ttpA containing plasmid did not rescue growth on TY or VMM with 3 mM glycine (Gly+ ); the dashed lines indicate the region of the gene deletion. The suppressor mutation is unmarked. (d) Transposon mutagenesis that was performed on 38cmdB-S to screen for a TY and 3 mM VMM with glycine minus phenotype (TY−, Gly−); the Tn5 labelled arrow indicates the location of the transposon insertion site. The transposon insertion site was mapped to ttpA, a TPR-containing protein. Schematic is not drawn to scale.

**Table 2.** ttpA gene expression measured by gusA fusion in various strains

Expression of ttpA is 1.5-fold upregulated in 38cmdB and 3.5-fold upregulated in 38cmdB-S and 38cmdB-S/ttpA::Tn5.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype and phenotype</th>
<th>Miller units (mean ± sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3841</td>
<td>WT</td>
<td>1954 ± 53</td>
</tr>
<tr>
<td>38cmdB</td>
<td>RL3500-, TY minus, VMM 3 mM glycine minus</td>
<td>2911 ± 71</td>
</tr>
<tr>
<td>38cmdB-S</td>
<td>RL3500-suppressor, TY plus, VMM 3 mM glycine plus</td>
<td>6943 ± 40</td>
</tr>
<tr>
<td>38cmdB-S/ttpA::Tn5</td>
<td>Tn5 in RL0936 in cmdBS background, TY minus, VMM 3 mM glycine minus</td>
<td>6911 ± 90</td>
</tr>
</tbody>
</table>

**cmdB** codes for a conserved hypothetical protein with a domain of unknown function (DUF) 58 and a von Willebrand factor type A (vWFA) motif. vWFA domains are broadly conserved and are found in both prokaryotes and eukaryotes. Proteins that contain VWA domains in eukaryotes are associated with many cellular functions such as cell adhesion and signal transduction (Colombatti et al.,).
Bioinformatic description of \textit{ttpA}, a gene coding for a TPR-containing protein

\textit{ttpA} is annotated as coding for a conserved hypothetical uncharacterized protein. The Pfam (http://pfam.xfam.org) and cdd (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) databases identify a conserved TPR motif. TPR-containing proteins are found in a diversity of organisms ranging from bacteria to humans. They are predicted to be involved in protein–protein interactions for a number of different cellular functions, including cell-cycle regulation, transcription and protein folding. \textit{TpA} contains a TPR structural motif. The basic function of these TPR folds is to mediate protein–protein interactions, which can often be promiscuous (Cerveny \textit{et al.}, 2013). Since \textit{CmdB} is predicted to be involved in a protein–protein interaction with \textit{CmdA}, it is interesting to find a link to \textit{TpA}, which is also predicted to be involved in protein–protein interactions. TPR motifs occur in minimally conserved 34-residue-long regions, centred around the consensus residues W_{4}-L_{7}-G_{8}-Y_{11}-A_{20}-F_{24}-A_{27}-P_{32} (Lamb \textit{et al.}, 1995). There is 3D structural data available for the TPR motif, which contains two antiparallel \( \alpha \)-helical subdomains (helix A and helix B), which are arranged in tandem arrays of three to sixteen TPRs that can accommodate the complementary region of a target protein (Cerveny \textit{et al.}, 2013; Das \textit{et al.}, 1998). There are three predicted TPRs found in RL0936, one TPR-16 at aa 362, one TPR-11 at aa 429 and one TPR-11 at aa 510. Since growth on TY and VMM with 3 mM glycine can be restored through creating a higher copy number of \textit{ttpA} in 38cmdB, and since \textit{ttpA} is overexpressed in 38cmdB-S, we suggest that an increase in expression of \textit{ttpA} can compensate for the loss of \textit{cmdB}. Future collaborative work with protein structural biologists will help to investigate potential interactions between the \textit{TpA} protein with protein components of the \textit{cmdA–cmdD} operon, and in particular the protein coded by \textit{cmdB}.

**Outer membrane integrity is not restored in the suppressor mutant**

Mutations in the \textit{cmdA–cmdD} operon are known to increase the mutant’s sensitivity to hydrophobic antibiotics.
and membrane disruptors (Vanderlinde et al., 2011). To determine if the suppressor’s restoration of TY growth is also correlated with restored outer membrane integrity, we examined 38cmdB-S’s sensitivity to erythromycin, tetracycline, SDS and NPN uptake into the outer membrane. The suppressor mutation did not restore outer membrane stability, and 38cmdB-S remained more sensitive to the antibiotics, SDS and NPN uptake when compared to the WT (Fig. 3a, b). Notably, 38ttpA also experienced increased sensitivity to these stressors and NPN uptake, further supporting a role of ttpA in a cell envelope network.

It is interesting that an inability to grow on TY often correlates with cell envelope deficiencies (Gibson et al., 2008; Vanderlinde et al., 2009, 2010, 2011) yet we observe here that suppressors that restore growth on TY remain sensitive to membrane disruptive agents. It is also interesting to note there is an increase in sensitivity when both genes are mutated in the strain cmdB-S/ttpA::Tn5, when compared with the WT and other mutants. The increased sensitivity to multiple cell envelope stressors when both cmdB and ttpA are mutated suggests their importance in proper cell envelope function in R. leguminosarum. The uncoupling of the two phenotypes in the suppressor strain also suggests a parallel genetic network for the two phenotypes. Supporting this observation, we are able to isolate suppressors in cmdA–cmdD operon mutants that restore resistance to membrane disruptive agents but do not restore growth on TY media. Future work is planned to characterize these suppressors, and by comparing and contrasting the two families of suppressors we hope to gain further insight into branches within the complex genetic networks.

**The suppressor mutant and associated mutant backgrounds with mutation of ttpA display reduced biofilm formation and desiccation tolerance**

Biofilm formation in all mutant backgrounds was compared using the biofilm quantification assay described in the Methods. Fig. 3(c) demonstrates that the TY suppressor strain and strains carrying mutations in ttpA produced significantly (Student t-test, \( P = 0.008 \)) less biofilms than the WT; 38cmdB-S/ttpA::Tn5 had the greatest decrease in biomass with a 50 \% decrease, followed by 38cmdB-S with a 38 \% decrease, 38ttpA with a 33 \% decrease and 38cmdB with a 14 \% decrease in biomass, when compared with 3841 (Fig. 3c). Desiccation sensitivity was also investigated, and 38ttpA had a mean percentage cell survival of only 4.25 \% when compared with the WT of 10.36 \%. 38cmdB had a cell survival mean of 8.92 \%, 38cmdB-S had a mean of 7.03 \% and 38cmdB-S/ttpA::Tn5 had the lowest cell survival value of 1.85 \%. These data, as well as data from biofilm assays, illustrate that mutation of ttpA alters phenotypes related to cell envelope structure.

In conclusion, the gain-of-function suppressor isolation approach has not only provided experimental evidence in...
support of a putative interaction between two relatively uncharacterized genes, but has also provided phenotypic data to suggest that \textit{tppA}, previously annotated as coding for an uncharacterized hypothetical conserved protein, is involved in cell envelope related physiology.

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**REFERENCES**


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