Further characterisation of pathogenicity genes from Leifsonia xyli subsp. xyli, causal organism of ratoon stunting disease: SRDC final project report BSS203

Brumbley, SM

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FINAL REPORT – SRDC PROJECT BSS203
FURTHER CHARACTERISATION
OF PATHOGENICITY GENES
FROM LEIFSONIA XYLI SUBSP. XYLI,
CAUSAL ORGANISM OF RATOON STUNTING DISEASE

by

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EXECUTIVE SUMMARY

In a previous SRDC/CRCTPP/BSES-funded project, a gene from the sugarcane pathogen *Leifsonia xyli* subsp. *xyli* (Lxx) was tagged by transposon mutagenesis, cloned and sequenced, and, based on DNA homology searches, was found to encode what appeared to be a putative integral membrane protein (PIMP). To try to better understand the role of a PIMP in Lxx pathogenicity on sugarcane, this project was undertaken to sequence the flanking regions to determine if this gene was part of an operon or a pathogenicity island.

Two overlapping cosmids, both with homology to the flanking region of the transposon in mutant Lxx::Tn4431-476, were shotgun cloned into pBluescript (Stratagene) and partially sequenced using both plasmid and Lxx-specific primers. DNA sequences were analysed using BLASTN and BLASTX against non-redundant databases of Bacterial Genbank and Bacterial GenPept, respectively. Contiguous sequences were assembled using AssemblyLIGN (Oxford Molecular). Gene maps were generated in MacVector (Oxford Molecular). A 13.5-kb contig that encompasses this region has been constructed from the shotgun sequence data of the two overlapping cosmids. Two operons were identified, one on either side of the PIMP, encoding an ABC transporter system and cytochrome oxidase subunits.

In addition, a series of experiments was established to try to get marker-exchange mutagenesis working in *Leifsonia xyli* subsp. *xyli*. However, several attempts to generate Lxx marker-exchange transformants that were either tetracycline resistant or produced any GFP failed using a variety of transformation techniques and tetracycline concentrations.
1.0 BACKGROUND

In a previous SRDC/CRCTPP/BSES-funded project, a gene from the sugarcane pathogen *Leifsonia xyli* subsp. *xyli* (Lxx) was tagged by transposon mutagenesis, cloned and sequenced, and, based on DNA homology searches, was found to encode what appeared to be a putative integral membrane protein (PIMP). To understand the genetic basis of ratoon stunting disease of sugarcane, caused by Lxx, it was critical to develop a set of molecular tools to identify the key genes involved in host recognition, colonization, and pathogenesis. This is even more important now that the full genome of Lxx has been sequenced (http://aeg.lbi.ic.unicamp.br/). Some of those tools, such as transformation and transposon mutagenesis, were developed in previous SRDC, CRCTPP and BSES projects (Brumbley et al. 2002). To best utilise the information gained from genome sequencing, it is important to have a system of site-directed mutagenesis developed so that specific genes can be knocked out to study their role in the interaction with sugarcane. One outcome of the genome-sequencing work has been the discovery that the transposon insertions into the Lxx genome were not random and that over 50% of the insertions occurred in a region of the genome representing less than 2% of the total. This makes it even more imperative that a more efficient, and more importantly a site-directed mutagenesis system be developed to study Lxx-sugarcane interactions.

When this project started we had over 700 Lxx transposon mutants from the previous studies (Brumbley et al. 2002). We had data showing that one of these mutants no longer colonised sugarcane and had shown that, when we transformed the mutant with a cloned copy of the wild-type gene, we were able to restore this mutant’s ability to colonise sugarcane xylem vessels. Unfortunately, when we sequenced this gene and used the sequence data to search the databases for similar genes, we found that we had cloned a PIMP. This told us nothing about how this gene was involved in Lxx pathogenicity on sugarcane. Therefore, the first goal of this research was to sequence the regions of the Lxx genome that flanked this gene to see if it was part of an operon. This might give us more clues as to what role this integral membrane protein might have in pathogenesis.

Because of the large size of the Tn4431 transposon, it was necessary to generate some mutants with smaller inserts for marker-exchange studies. There are two ways of doing this. Firstly, use another random-mutagenesis system to tag some of the genes on our cosmid clones and then subclone the new mutant along with some of the Lxx genomic DNA flanking the insertion into a suicide vector. Secondly, sequence some of the other genes, subclone them into the suicide vector and then splice a marker gene into the middle. Both methods were tested in this project.

At the same time, we commenced sequencing the regions of the Lxx genome flanking the PIMP, we attempted to generate some insertion mutants in this region by developing a novel transposon system. This was designed so that the transposon would hop into plasmid regions rather than genomic regions. We were successful at both generating these mutants and subcloning them into the suicide vector, but were unsuccessful at getting them to marker exchange into a wild-type Lxx. Once we had the data from sequencing the flanking regions, and had identified two operons by screening the sequence data against the various databases, the second method of generating a mutant for marker exchange was possible. The first operon was part of an ABC transporter system involved in scavenging and uptake of nutrients into the cell. The other operon encodes proteins that are part of the bacterium’s energy-production system. Genes from both of these operons have been implicated as having roles in pathogenicity.
2.0 OBJECTIVES

The aim of the project was to clone all the genes from the operon from *Leifsonia xyli* subsp. *xyli* containing the pathogenicity gene cloned in BS99S, by the steps:

- Sequence all the genes flanking the pathogenicity gene cloned in BS99S;
- Determine the function of pathogenicity genes by doing marker exchange knock-out mutagenesis studies;
- Look for homologous genes by doing searches through the various DNA databases around the world;
- Make gene-fusion constructs with a reporter gene to determine when these genes are expressed during the interaction with sugarcane.

The long-term objective of this project is to generate novel DNA constructs that may confer resistance to RSD by interfering with the function of the pathogenicity genes or the products from these genes.

3.0 METHODOLOGY AND RESULTS

3.1 Cosmid sequencing and search for homologous genes

Two overlapping cosmids, both containing the PIMP gene identified from transposon mutant Lxx::Tn4431-476, were shotgun cloned into pBluescript (Stratagene) and partially sequenced using both plasmid and Lxx-specific primers. DNA sequences were analysed using BLASTN and BLASTX against non-redundant databases of Bacterial Genbank and Bacterial GenPept, respectively, using WEB ANGIS. Contiguous sequences were assembled using AssemblyLIGN (Oxford Molecular). Gene maps were generated in MacVector (Oxford Molecular).

A 13.5-kb contig that encompasses this region was constructed from the shotgun-sequence data of the two overlapping cosmids. Two operons were identified, one on either side of the PIMP, encoding an ABC transporter system and cytochrome oxidase subunits. Two putative operons were identified, one on either side of the hypothetical integral membrane protein. One has strong homology to the pro operon [proVWXYZ] from *Mycobacterium tuberculosis*, and the other a cytochrome oxidase operon [cydABDC] (Figure 1).
Figure 1 – Map of 13-kb region sequenced in this project. Annotation of the flanking DNA from transposon mutant Lxx::Tn4431-476. orf, open reading frame; pro, proline operon; cyd, cytochrome oxidase; tetR, transcriptional regulator of the tetR gene family; rep, repressor element; PIMP, putative integral membrane protein. This map shows the orientation of the putative operons and genes identified by BlastN and BlastX homology searches of the nonredundant (NR) database of ANGIS. The ball and stick figures show the sites of insertions of some of the transposons in this region. Note that the transposon insertion designated 476 is the one in the PIMP and is from the mutant that is unable to colonise sugarcane.
Based on database searches the four genes in the putative pro operon encode: (1) an ABC transporter ATP binding protein, proV; (2) a probable permease inner-membrane component, proW; (3) a probable transport-system permease protein, similar to osmoprotection proteins (proW, proZ) involved in glycine betaine/L-proline transport proZ; and (4) a secreted substrate-binding protein, proX. The pro operon genes may play a crucial role in pathogenicity. They encode a glycine/betaine transporter system, which in other bacteria are involved in osmoregulation (Cairney et al. 1985) and may be critical for xylem colonisation. In addition, proV, shares homology to the ABC transporter gene attA1 from Agrobacterium tumefaciens, which appears to be involved in signalling between the bacteria and the plant host (Mathysse 1994; Mathysse and McMahan 1998). In the animal pathogen Salmonella typhimurium, cyd mutants appear to play a role in virulence (Zhang-Barber et al. 1997). These genes were tested in marker-exchange mutagenesis studies.

3.2 Marker-exchange mutagenesis

3.2.1 General

Marker-exchange mutagenesis is a form of site-directed mutagenesis (Ruvkun and Ausubel 1981), where a marker (antibiotic resistance, β-glucuronidase, β-galactosidase, green fluorescent protein, etc) is inserted into the gene of interest in vitro, and this construct is then used to transform the wild-type strain. Homologous recombination then occurs between the regions of the gene on the vector and the homologous regions in the genome replacing the wild-type gene with the defective copy containing the marker. If done correctly, the marker can serve two functions: (1) to generate a knock-out mutant to allow study of the role of the gene; and (2) as a reporter to study how gene expression is regulated. Now that sequencing of the genome of Lxx is completed, marker exchange will be a powerful tool for studying the role and expression patterns of genes not tagged by the transposon Tn4431 and to confirm that the transposon mutants are responsible for the mutant phenotypes. In another CRCTPP/BSES/Brazilian-funded project, over 500 of the transposon insertions in the Lxx-mutant library have now been mapped to the Lxx genome. These insertions appear to only be in about 10% of the Lxx genome, leaving most of the 2500 genes identified so far untagged.

We felt that marker exchange should work in Lxx because of the work done on Leifsonia xyli subsp. cynodontis (Lxc), where Haapalainen et al. (1998) developed a vector that integrates, by recombination, into the genome of Lxc. Initial indicators that marker exchange will work in Lxx came from the previous BSES/CRCTPP-funded complementation studies.

Transformation of the two cosmids containing the wild-type PIMP gene into the line QPF110-476 resulted in some of the transformants loosing the tetracycline resistance (Tet^R) encoded in the transposon and gaining kanamycin resistance. This suggested that a recombination had occurred between homologous regions on the vector with those in the genome (Figure 2). In addition, Southern Blot analysis of these complementation isolates showed at least one genetic rearrangement (Figure 3). Note that in Figure 3 complementation isolate 1C2D is missing a band, suggesting that some genetic rearrangement has occurred. In addition, several other isolates appear not to have the bands from the cosmid clones.
Figure 2 - Antibiotic resistance of Lxx complementation mutants. Lxx isolates were replica-plated onto Metricel CN (Gelman Sciences) nylon membranes sitting on three bacto-agar plates containing tetracycline, kanamycin or no antibiotics, respectively.

1 Wild-type strain QPF110-045.  2 Lxx::Tn4431 mutant QPF110-476.  3 Wild-type strain 045 transformed with the cosmid-cloning vector pLAFR7 containing no inserted Lxx genomic DNA.  4 Mutant 476 transformed with the cosmid-cloning vector pLAFR7 containing no inserted Lxx genomic DNA.  5 Four isolates of mutant 476 transformed with Lxx wild-type genomic library cosmid clone pLB1D6.  6 Six isolates of mutant 476 transformed with Lxx wild-type genomic library cosmid clone pLB1C2.
Figure 3 - Southern analysis of Lxx::Tn4431 complementation study. Further evidence of recombination is found in this Southern blot where total genomic DNA from Lxx wild-type strain QPF110-045, Lxx::Tn4431 mutant QPF110-476, and transconjugants of mutant 476 containing either of the two complementary cosmid clones pLB/C2 or pLB1D6, digested with PstI, separated on an agarose gel and transferred to a Hybond N+ membrane.

Probe: 2153 bp EcoRI/PstI fragment from flanking DNA from Tn4431 insertion.

1 Wild-type strain 045 and Lxx::Tn4431 mutant 476 transformed with the cosmid cloning vector pLAFR7
2 Inoculated into sugarcane and reisolated after 3 months.
3 Lxx cosmid clones digested with PstI.
One explanation for this is that these isolates have acquired kanamycin antibiotic resistance spontaneously. However, several of these isolates were shown to complement the mutation by being able to recolonise sugarcane. Therefore, an alternate explanation may be that a single recombination event occurred between other homologous regions on the Lxx cosmid clones and the Lxx genome. In addition, the Brazilians have found many repeat sequences scattered through the Lxx genome and these are all potential sites of recombination if similar regions exist on either of the cosmids in areas outside of the zone that we sequenced. The two cosmid clones cover approximately 45 kb of the Lxx genome and we only sequenced a little over 13 kb of this region. This leaves about 32 kb of the cosmids cloned Lxx genome that could contain some of these sequence data.

3.2.2 Marker-exchange mutagenesis method I: Generation of random mutations in overlapping Lxx genomic-library clones pLB1C2 and pLB1D6 - Tn3HoHo1 style mutagenesis

We expected to take 1 year to sequence the 13-kb region flanking the Tn4431 insertion in mutant QPF110-476. To start the marker-exchange work prior to completion of the sequencing, a method for generating transposon mutants in the cosmid clones was devised. A special vector was designed to generate random insertions in the Lxx cosmid-library clones pLB1C2 and pLB1D6, both of which are kanamycin resistant (kmR). These are the overlapping Lxx genomic-library clones isolated by homology to the flanking DNA from the transposon insertion in the Lxx mutant 476. The promoterless gene encoding green fluorescent protein (GFP) becomes a marker that we can follow through the infection process.

To create this special vector, pUC<TET-1> (Epicentre Technologies) was digested with PvuI and religated to omit ampicillin resistance (apR) but leave tetracycline resistance (tetR). pTn3HoHo1 (Stachel et al. 1985) was then ligated into the EcoRI/SmaI site of this modified vector, thereby restoring the apR and creating pEE5 (Figure 4). Using EcoRI/EcoRV, the lac operon and most of the nonfunctional tnpA (transposase) gene were spliced out of the Tn3HoHo1 insert and a promoterless gfp gene from pGreenTIR (Miller and Lindow 1997) was spliced in its place creating pEE5gfp (Figure 4). Either pLB1C2 or pLB1D6 was then transformed into E. coli strain S17-1 containing pSSHE (chloramphenicol resistant [cmR]) (Stachel et al. 1985) (Figure 5A). pSSHE has the a functional tnpA gene necessary for the transposon to jump. Because this is a Tn3-based transposon, it will jump primarily into another plasmid, pLB1C2 or pLB1D6. Isolates that were cmR/kmR were then transformed with pEE5gfp (tetR). Isolates that were cmR/apR/kmR/tetR were biparentally mated with E. coli (nalidixic-acid resistant [nxR]) strain C2110. To counter select against the S17-1 E. coli strain, transconjugants were selected for nxR and were selected for apR/kmR/tetR (Figure 5A). pC2EEgfp and pD6EEgfp were isolated from strain C2110. To reduce the size of this plasmid, the cosmid cloning vector pLAFR7 was removed by digestion with either SacII or XmaI and a kmR cassette (GenBlock [EcoRI] Pharmacia Biotech) was inserted in its place, primarily as a spacer, to create pME (Figure 5B). The resulting constructs pC2EEgfpΔS, pC2EEgfpΔX, pD6EEgfpΔS and pD6EEgfpΔX (Figure 4) were transformed into E. coli strain GM2163 (dam/-/dcm-) [New England Biolabs], were selected for apR/tetR/kmR, and were screened for GFP activity (Figures 5A and 7). To identify the clones tagged by the transposon in the 13-kb region that we sequenced, a
colony-blot analysis (Figure 6A-B) was done using two overlapping probes that cover 9 kb of this region (Figure 6C). Plasmids were reisolated and purified using Qiagen columns and were introduced into Lxx cells by electroporation (Bio-Rad) using a modification of the method of Lampel et al. (1994).

No Lxx transformants grew from any of the transformation attempts.

Figure 4 - Construction of the suicide plasmid (pEEgfp) containing a promoterless gfp marker
Figure 5A - Development of a Tn3-based mutagenesis system
Figure 5B - Generation of plasmids for marker exchange studies. pBSKN is pBluescript with the kanamycin resistance cassette (GenBlock) inserted into the SalI site. Digestion with SacII will linearise the construct while pvuII will generate a fragment containing the multi-cloning site (MCS) and the KanR cassette.
Figure 6A-B - Colony hybridisation of *E. coli* transformants to identify those containing sections of the 13-kb region from the Lxx genome sequenced in this project. *E. coli* transformants were selected on LB plates containing ampicillin, kanamycin and tetracycline. Individual colonies were picked with a toothpick and replica-plated onto nylon filters on LB plates with the same antibiotics. After 24 hours, the cells were lysed on the membranes and the plasmid DNA was crosslinked to the membrane using UV light. The colony blot was probed with two fragments isolated for the 10-kb region of the 13-kb contiguous region that we sequenced from Lxx (Figure 6C) radiolabeled with 32P ATP. (A) pCDEE clones (Figure 5). (B) pME clones (Figure 5).
Figure 6C - Restriction map showing probes used for 6A above. Probe p61 is a 4400 bp EcoRI fragment and Probe p27 is 6640 bp PstI fragment. These two fragments were labelled with 32P ATP and were used to probe colony blots of *E. coli* transformed pCDEE and pME clones (see Figure 6A).
3.2.3 Marker-exchange mutagenesis method II: Modification of proZ for marker exchange

3.2.3.1 Construction of p1693 and p1694

The gene encoding GFP was cut out of pGREENTIR using EcoR1 and was cloned into the EcoRI site of the pUC<TET-1> vector just upstream of the tetracycline resistance gene generating pUC<TET-GFP>. The fragment containing tet and gfp was then cloned by PCRing the genes from pUC<TET-GFP> using primers (GFPTETf1 Forward: 5’-AAGCTTCTGCAAGGGAGATCTGCTATTACGCCAGCT-3’ and GFPTETr1 reverse 5’-AAGCTTCTGCAAGGGAGATCTGCTATTACGCCAGCT-3’) containing BglII (underlined) sites. The PCR product was then cut with Bg/II and cloned into p61 (4.4 kb EcoRI fragment containing the pro operon cut from Lxx cosmid clone pLB1D6 and cloned into pBLUESCRIPT) in the BglII site in proZ gene. The difference between p1693 and p1694 is the orientation of the gfp/tet genes inserted into the Bg/II site in proZ gene. The difference between p1693 and p1694 is the orientation of the gfp/tet genes inserted into proZ gene. However, p1693 produced less GFP than did p1694.

3.2.3.2 Attempt to marker exchange p1693 and p1694

Several attempts to generate Lxx marker-exchange transformants that were either tetracycline resistant or produced any GFP failed using a variety of transformation techniques and tetracycline concentrations (Tables 1-5). Both of these constructs confered tetracycline resistance and produced GFP in E. coli strain GM2163. However, p1693 produced less GFP than did p1694.
Table 1 – Marker-exchange experiment using a gfp/tet cassette inserted into the BgII site of proZ. No tetracycline (tet') resistant colonies grew for any of the marker exchange transformants whereas 57 tet' resistant colonies were found on the positive control plate using pLAFR5.

**MARKER EXCHANGE EXPERIMENT TABLE 1**

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2.5KV 0.2cm cuvette=1.5k/cm@200 ohms

Lxx cells grown to an OD of 0.8 and prepared for electroporation

¹TET 10 - the concentration of tetracycline in the selection media was 10 micrograms/ml

²T-CONST is the time in milliseconds it take the electrical charge to cross the 0.2cm gap in the electroporation cuvette

³1694 is p61 with a BgIII fragment containing containing tet and gfp inserted in the BgIII site in proZ in the opposite orientation from 1693. H3 indicates the vector was linearized with *Hind* II prior to electroporation into Lxx.

⁴dcm indicates that DNA was prepared from E coli strain lacking the mcrA, hsdR2 and mcrB1 methylation genes.
Table 2 - A range of different conditions were tested to make the Lxx cells electroporation competent including addition of DMSO, CaCl$_2$, CaCl$_2$ plus MgCl$_2$. No transformants were seen, even in the positive controls.

MARKER EXCHANGE EXPERIMENT TABLE 2

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A2-H2 CaCl$_2$ type transformation protocol

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A3-H3 CaCl$_2$ MgCl$_2$ type transformation protocol

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Lxx cells were grown to an OD of 0.6 and prepared for transformation using Chung and Millar DMSO and Calcium Chloride type transformation.

1 TET 10 indicates the selection media contained tetracycline at a concentration of 10 micrograms/ml
2 KAN 50 indicates the selection media contained kanamycin at a concentration of 50 micrograms/ml
3 pLAFR5-Km is pLAFR5 with a kanamycin resistance gene cassette inserted into the middle of the tetracycline resistance gene
4 pLB1D6 is the clone from the Lxx cosmid library containing the region cloned from the Lxx transposon mutagenesis work
5 1693 is p61 (Figure 5C) with a BgIII fragment containing containing tet and gfp inserted in the BgIII site in proZ.
6 1694 is p61 (Figure 5C) with a BgIII fragment containing containing tet and gfp inserted in the BgIII site in proZ in the opposite orientation from 1693.
7 dcm indicates that DNA was prepared from E coli strain lacking the mcrA, hsdR2 and mcrB1 methylation genes.
8 H3 indicated the vector was linearized with HindIII prior to electroperoration.
Table 3 - The concentration of tetracycline in the selection media was reduced from 10 µg/mL to 2.5 µg/mL in an attempt to take some of the pressure off of the marker exchange cell lines. Still no tet^r Lxx cells were seen on any of the plates with the marker-exchange constructs. However, the positive controls grew 100 tet^r colonies after 32 days in culture.

### MARKER EXCHANGE EXPERIMENT TABLE 3

<table>
<thead>
<tr>
<th>SUB DAY</th>
<th>T-CONST</th>
<th>3.9</th>
<th>4.2</th>
<th>4.2</th>
<th>4.2</th>
<th>4.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>13/01/02</td>
<td>noDNA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14/01/02</td>
<td>1694-H3^2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>27/01/02</td>
<td>1694-dcm^3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14/02/02</td>
<td>1694</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>58</td>
<td>0</td>
</tr>
<tr>
<td>14/02/02</td>
<td>pLAfR5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

2.5KV 0.2cm cuvette=1.5k/cm@200 ohms

Lxx cells were prepared for electroporation after reaching an OD of 0.7.

^1 TET2.5 indicated that the selection media contained tetracycline at a concentration of 2.5 micrograms/ml

^2 1694 H3 DNA was prepared for electroporation by linearizing it at the unique Hind III site.

^3 dcm indicated that DNA was prepared from *E. coli* strain lacking the *mcrA*, *hsdR2* and *mcrB1* methylation genes.
Table 4 - Polyethylene glycol (PEG) was tested for its ability to enhance transformation efficiency. No difference was observed between the PEG and no-PEG treatments. After 32 days in culture on plates containing 2.5 µg/mL tet, no marker-exchange transformants grew and the positive controls had 50 and 56 colonies, respectively.

<table>
<thead>
<tr>
<th>TET 2.5</th>
<th>MARKER EXCHANGE EXPERIMENT</th>
<th>TABLE 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x2.5 kV 0.1cm cuvette @200 ohms No PEG</td>
<td>2x2.5 kV 0.1cm cuvette @200 ohms +40% PEG</td>
<td></td>
</tr>
<tr>
<td>STRAIN 129</td>
<td>T-CONST</td>
<td>3.9 4.2 4.2 4.2</td>
</tr>
<tr>
<td>SUB DAY</td>
<td>noDNA</td>
<td>1693-dcm</td>
</tr>
<tr>
<td>17/02/02</td>
<td>0</td>
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</tr>
<tr>
<td>18/02/02</td>
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<td>0</td>
</tr>
<tr>
<td>3/03/02</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>21/03/02</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Lxx cells were prepared for electroporation after reaching an OD of 0.7.

1 TET 1.0 indicated that the selection media contained tetracycline at a concentration of 1.0 micrograms/ml

2 T-CONST is the time in milliseconds it take the electrical charge to cross the 0.2cm gap in the electroporation cuvette

3 1693 is p61 (Figure 5C) with a BglII fragment containing containing tet and gfp inserted in the BglII site in proZ. dcm indicates that DNA was prepared from E coli strain lacking the mcrA, hsdR2 and mcrB1 methylation.

4 1694 is p61 (Figure 5C) with a BglII fragment containing containing tet and gfp inserted in the BglII site in proZ in the opposite orientation from 1693. dcm indicates that DNA was prepared from E coli strain lacking the mcrA, hsdR2 and mcrB1 methylation.

5 pLAFR5-dcm is the cosmids cloning vector and dcm indicates that DNA was prepared from E coli strain lacking the mcrA, hsdR2 and mcrB1 methylation.
Table 5 - A further reduction of tetracycline in the selection media to 1.0 µg/mL was tested. Still no colonies grew for any of the marker-exchange transformants, while the positive control produced 68 tet^R colonies after only 22 days suggesting that: (1) Lxx is extremely sensitive to tetracycline; and (2) that, by reducing the concentration of tetracycline, the Lxx cells that express the resistance gene grow faster.

**MARKER EXCHANGE EXPERIMENT TABLE 5**

<table>
<thead>
<tr>
<th>Strain 129</th>
<th>T-CONST^2</th>
<th>TET 1.0^1</th>
<th>2x2.5KV 0.1cm cuvettes</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUB DAY</td>
<td>noDNA</td>
<td>p1693-dcm3</td>
<td>p1694-dcmH34</td>
</tr>
<tr>
<td>21/03/02</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>23/03/02</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4/03/02</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15/04/02</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

2x2.5KV 0.1cm cuvettes

Lxx cells were prepared for electroporation after reaching an OD of 0.7.

^1 TET 1.0 indicated that the selection media contained tetracycline at a concentration of 1.0 micrograms/ml

^2 T-CONST is the time in milliseconds it took the electrical charge to cross the 0.2cm gap in the electroporation cuvette

^3 1693 is p61 (Figure 5C) with a BglII fragment containing containing tet and gfp inserted in the BglII site in proZ. dcm indicates that DNA was prepared from E. coli strain lacking the mcrA, hsdR2 and mcrB1 methylation.

^4 1694 is p61 (Figure 5C) with a BglII fragment containing containing tet and gfp inserted in the BglII site in proZ in the opposite orientation from 1693. dcm indicates that DNA was prepared from E. coli strain lacking the mcrA, hsdR2 and mcrB1 methylation. H3 indicates the DNA was prepared for electroporation by linearizing it at the unique Hind III site.

^5 pLAFR5-dcm is the cosmid cloning vector and dcm indicates that DNA was prepared from E. coli strain lacking the mcrA, hsdR2 and mcrB1 methylation.
4.0 DISCUSSION

4.1 Sequence analysis of the regions flanking the PIMP tagged in Lxx::TN4431 mutant 476

Over 13000 base pairs, approximately 6,500 on either side of the PIMP, have been sequenced. The genes and operons encoded in this region have been identified, based on DNA homology searches of the databases on the www. Although we found that the PIMP does not appear to be part of an operon, the operons identified on either side have still proven to be important. In both cases, some of the genes encoded in these operons have been implicated in the literature as having a role in the interactions between pathogens and their host organisms. In addition, we have used this 13-kb region to look at genetic diversity between a Brazilian and an Australian Lxx isolate. Over 10 kb in the middle of this fragment has 100% sequence homology. Only a few mismatches, deletions or insertions were found at the ends of this large fragment and we are not positive yet if some or most of those are not sequencing errors in either the Brazilian or our data. One of the genes from the pro operon (proZ) was used to try and generate marker-exchange mutants. This was not successful.

4.2 Possible reasons why marker-exchange experiments failed

Marker exchange requires a double recombination event to occur between homologous regions that flank the marker gene. At best, this is a rare event. One of the problems we are facing is that the transformation efficiency of Lxx is still not very good. To be able to see these rare events, we will need to either increase the transformation efficiency or dramatically increase the number of transformation events. We are confident that it is possible to accomplish marker exchange because of the evidence we already have that some recombination is occurring between Lxx genomic DNA and homologous regions on the cosmid clones from the Lxx genomic library.

4.3 Importance of marker-exchange mutagenesis technology to Lxx

With the completion of sequencing of the Lxx genome and the beginning of the DNA-array work, a number of genes and regulatory elements will be identified that have a potential role in host colonization or in pathogenesis. To determine the importance of these genes, it is essential to have a site-directed knock-out-mutagenesis system in place so that the function of the gene product can be disrupted and the effect that this has on the ability to cause RSD tested. It appears that the transposon mutagenesis system that we developed will be inadequate, because the transposon insertions appear to be limited to only 10% of the genome with the vast majority located in one 40-kb region of a genome that is 2.8 Mb long.

5.0 IMPROVED TECHNIQUES

5.1 Sugarcane inoculation

A range of techniques was tested to improve the colonisation assay. Sugarcane inoculation methods included: (1) immersion of 1- or 2-eye setts into Lxx cell suspensions
for various times (1, 5 or 10 minutes), then planting the setts into steam-sterilized potting soil; (2) a time-course study by cutting tops off 4-12-week-old sugarcane seedlings just above the apical meristem and applying 100 µL of Lxx cell suspension to the cut surface; or (3) dipping secateurs into a suspension of Lxx cells just prior to cutting one- or two-eye setts or cutting the tops off of plantlets (Figure 8). In addition, Haapalainen et al. (2000) demonstrated that growth of the closely related Lxc is enhanced by components of xylem exudate, so filtered xylem extract was added to some of the cultures 24 hours before inoculation (Figures 8-9).

Figure 8 - Lxx inoculation trials using Lxx wild-type isolate QPF110-129. Lxx cells were inoculated into 100 mL of S8 broth and were grown with constant shaking for 10-14 days until the cultures were in mid log-phase growth. Twenty-four hours prior to inoculation onto sugarcane some of the Lxx cultures were supplemented with filter-sterilised xylem extract from Lxx-infected sugarcane cultivar Q110. Normal, no added xylem extract; Healthy, xylem extract from healthy Q110; and Infected, xylem extract from Lxx-infected Q110. One- or two-eye setts were inoculated by immersing them into the Lxx culture for 1, 5 or 10 minutes before planting them in soil. Alternatively, secateurs were dipped into the Lxx culture prior to cutting the one- or two-eye setts. Lastly, 100 µL of inoculum was pulled into the one- or two-eye setts using negative pressure (vacuum). In addition, 8- to 10-week-old plantlets grown from one-eye setts were inoculated by decapitating just above the apical meristem, either with sterile secateurs and then placing a 100-µL drop of the Lxx culture on the cut surface, or by using secateurs dipped in the Lxx cultures. The results shown are the average of three replicates.
Figure 9 - Effect of plantlet age on Lxx colonisation of sugarcane. One-eye setts of sugarcane cultivar Q110 were planted in potting soil at 2-week intervals. When the oldest plants were 14 weeks old, plants were inoculated with Lxx wild-type strain QPF110-129 prepared as described for Figure 8. The plantlets were inoculated by decapitating plantlets just above the apical meristem and placing a 100-µL drop of the culture on the cut surface. The results shown are the average of three replicates.

Our preliminary results from the sett, secateurs and cut-stem inoculations all indicate that: (1) filter-sterilized xylem extract from either healthy or Lxx-infected sugarcane cultivar Q110 added to Lxx broth culture 24 hours prior to inoculation onto sugarcane had a significant affect both on the ability of isolates to colonize and the overall numbers of Lxx cells reisolated from plants after 10 weeks (Figures 8-9); (2) two-eye setts are more readily colonised than one-eye setts (Figure 8); (3) the 5- or 10-minute dip worked better than a 1-minute dip (Figure 8); and (4) plantlets that were 6-10 weeks old prior to decapitation and inoculation were more easily colonised than younger or older plantlets (Figure 9).

5.2 Lxx transformation

Recently, we reported the first successful transformation and transposon mutagenesis of Lxx (Brumley et al. 2002). In that study, we reported that the cells of Lxx transformed with the broad host-range cosmid cloning pLAFR-5 required 6-8 weeks before single colonies could be observed on MS9 plates supplemented with 10 µg/mL tetracycline.
Here, we show a substantial improvement in the transformation of Lxx by reducing the regeneration time of Lxx transformed with the cosmid vector pLAFR-5. Furthermore, we demonstrate, for the first time, stable transformation of Australian, Indonesian and Brazilian isolates of Lxx (all foreign isolates were destroyed by autoclaving at the end of the experiment). We show that 100% of untransformed Lxx growth can be inhibited after 25 days on MS9 media supplemented with 0.5 µg/mL tetracycline. We demonstrate that Lxx transformed with pLAFR-5 grows significantly faster (within 25 days) on MS9 media supplemented with tetracycline concentrations ranging between 1.0 µg/mL and 2.5 µg/mL. This is in contrast with a 45-55-day period reported previously. The present selection protocol for the transformation of Lxx provides a 3-4 week time saving. Thus, the method provides a means for the rapid genetic transformation of Lxx when pLAFR-5 or other cloning vectors that confer tetracycline resistance are used.

Initially, we tested the transformation efficiency of three isolates of Lxx selected from the Lxx collection held by BSES, Indooroopilly. Lxx strain 118 was provided by Dr. Eugenio Ulian of Copersucar in Brazil, strain 1688 was provided by the Indonesian Department of Agriculture, and strain 129 is an Australian isolate of Lxx used extensively in previous transformation studies. Conditions for the culture of Lxx strains have been described previously (Davis 1980). Briefly, each strain was grown at 26°C in standard, rich S8 liquid medium with shaking at 150 rpm or on MS9 agar plates. Plasmid DNA pLAFR-5 was prepared from a *dem/dam* E. coli strain GM 2163 as previously described (Brumbley et al. 2002).

We prepared electro-competent Lxx cells essentially as described previously (Brumbley et al. 2002) with the following modifications. Lxx strains were grown on MS9 plates for 14-21 days at 26°C to obtain single colonies of Lxx. Single colonies of each isolate were used to inoculate liquid cultures that were grown to an OD550 nm of between 0.6 and 0.9 in S8 medium. Lxx cells from a 1-L S8 liquid culture were centrifuged at 7,500 rpm for 5 minutes in 250-mL bottles in a Sorval GSA rotor and resuspended by gentle agitation in 200 mL of ice-cold sterile H2O. Resuspended cells were centrifuged at 7,500 rpm for 5 minutes to produce a loose bacterial pellet. In second and third sterile H2O washes, additional centrifugal force, 12,000 g (~ 8,500 rpm for the Sorval GSA rotor), and increased centrifugation time, 7.5 minutes, were necessary to pellet the Lxx cells. Finally, cells were resuspended in a final volume of 3 mL by the addition of 1.5 mL of filter sterile 10% glycerol. Electro-competent Lxx cells were frozen on dry ice in 200-µL aliquots and stored at -80°C until required.

To determine the relative electro-competent efficiency of the Lxx preparations, 10-fold serial dilutions of each Lxx strain were plated on MS9 plates and the total number of cells present in our electro-competent Lxx preparations was estimated (Table 6). 50 µL of electro-competent cells contained between 1 x 10^10 and 1 x 10^12 colony-forming units (cfu) capable of growing on MS9 after being prepared for electroporation (Table 6). Single colonies of Lxx were observed on MS9 media between 17 and 22 days after spread plating.
For transformation, frozen Lxx cultures were handled as previously described (Brumbley et al. 2002) with the following differences: 100 ng of pLAFR-5 plasmid DNA were added in 2 µL of sterile TE buffer and allowed to rest on ice for 5 minutes. The mixture of cells and DNA was then transferred to a pre-chilled BioRad GenePulsar™ electroporation cuvette with a 0.1-cm electrode gap. The cells were exposed to two 2.5-kV pulses, with a 20-second interval between pulses, with a capacitance of 250 mF and 250 MΩ resistance using a BioRad GenePulsar™. After the application of two pulses, 50 µL of cells were resuspended in 400 µL of recovery media (S8 media supplemented with 4 g/L NaCl and 83.7 g/L sorbitol) in 5-mL sterile tubes and were allowed to incubate at 26°C with agitation at 150 rpm for 24 hours. Lxx transformations were plated onto 70-mm diameter METRICEL CN™ nitrocellulose filter-discs with a 0.2-µm pore size, placed on the surface of MS9 agar plates supplemented with 10 µg/mL tetracycline. Lxx strains were grown to similar optical densities (Table 7) and gave similar time constants upon electroporation (Table 7). Results demonstrate that the Lxx isolates tested can be transformed with frequencies ranging from 50 to 100 colony-forming units (cfu) per microgram of transforming plasmid DNA (Table 7).

One striking observation was that untransformed colonies of Lxx appeared rapidly (~ 25 days) when on plates with no antibiotics compared with transformed colonies of Lxx when plated on 10 µg/mL tetracycline (~ 55 days) (compare Table 6, column 3 with Table 7, column 5). Recently, stable transformation of Clavibacter michiganensis subsp. sepedonicus with vectors that confer tetracycline resistance was achieved using 1.0 µg/mL tetracycline (Laine et al. 1996). To determine the sensitivity of Lxx to tetracycline (Figure 10), a cell-density assay was established on increasing concentrations of tetracycline. Lxx growth was characterized by quantifying the growth of Lxx on Metricel CN filters (Gelman Sciences). To measure the antibiotic effect of tetracycline on the growth of Lxx, 75-mm² sections were removed from nitrocellulose filters on which Lxx cultures were growing. The 75-mm² nitrocellulose sections were washed to resuspend Lxx cells in 1 mL of MS9 media. The OD_{550} of the resuspended cells was measured and growth of Lxx on filters was expressed as an optical density OD_{550} nm / 75 mm² of nitrocellulose filter. Surprisingly, non-transformed electro-competent Lxx cell preparations did not grow at tetracycline concentrations above 0.5 µg/mL tetracycline after 25 days (Figure 10).

### Table 6 – Growth of colonies

<table>
<thead>
<tr>
<th>Strain</th>
<th>OD_{550}</th>
<th>Lxx cells / 50 µL</th>
<th>Days to obtain single colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1688</td>
<td>0.80</td>
<td>~ 1 x 10^{12}</td>
<td>~ 21</td>
</tr>
<tr>
<td>118</td>
<td>0.75</td>
<td>~ 1 x 10^{11}</td>
<td>~ 17</td>
</tr>
<tr>
<td>129</td>
<td>0.70</td>
<td>~ 1 x 10^{10}</td>
<td>~ 22</td>
</tr>
</tbody>
</table>

### Table 7 – Growth of transformed Lxx

<table>
<thead>
<tr>
<th>Strain</th>
<th>OD_{550}</th>
<th>Time constant</th>
<th>Transformants per µg pLAFR-5</th>
<th>Days to single colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1688</td>
<td>0.80</td>
<td>4.2</td>
<td>100</td>
<td>~ 56</td>
</tr>
<tr>
<td>118</td>
<td>0.75</td>
<td>4.4</td>
<td>50</td>
<td>~ 48</td>
</tr>
<tr>
<td>129</td>
<td>0.70</td>
<td>4.1</td>
<td>70</td>
<td>~ 45</td>
</tr>
</tbody>
</table>
5.3 Improved genomic DNA isolation

To analyse the various Lxx mutants that we generate, whether it is by transposon, chemical, UV or marker-exchange mutagenesis, it is necessary to isolate reasonable quantities of genomic DNA. To visualise the modifications that we have made, we routinely digest 1 µg of genomic DNA per reaction and may need to do analysis with multiple restriction digests to determine precisely which mutation has been generated. One problem with Lxx is that it is a gram-positive bacterium and, therefore, has a very tough cell wall. Lxx appears to be even more difficult to work with than even its closest relative Lxc, where routinely, using the same concentration of cells, we are able to get 10 times the amount of genomic DNA. Therefore, a new rapid method was devised to isolate genomic DNA from Lxx.

Our method is to inoculate a single Lxx colony into either 50 or 100 mL of S8 broth and incubate with constant shaking to late log phase (10-21 days). Then pellet the cells by centrifugation at 8000 g in a Sorval rotor for 10 minutes, pour off the supernatant and scrape cells into a sterile mortar. Liquid nitrogen is added to thoroughly cover the cells and the material is ground with a pestle. It is important to not let all of the liquid nitrogen boil off and the cells to thaw, so more liquid nitrogen should be added as required. Then 2 mL of Lxx lysis buffer is added and the normal genomic-DNA extraction is proceeded with as described by Lampel et al. (1994).

This protocol reduces the amount of time for a genomic DNA preparation from Lxx by several hours per sample and gives better yields of genomic DNA (data not shown).

6.0 OUTPUTS

Key outputs from this project are:

- sequence data from an Australian Lxx isolate that has been used by the Brazilians in their genome-sequencing work to: (1) confirm their sequence data; and (2) confirm
other work that we are doing showing a lack of genetic diversity in world Lxx populations.

- sequence data that has been used to develop reporter-gene constructs that knock out genes in the *pro* operon from Lxx. These will be important as we optimise the transformation of Lxx to get transformation frequencies high enough so that we can detect the rare double cross-over event necessary for marker exchange to work.
- a new protocol for improving transformation frequencies Lxx.
- a technique for rapid genomic DNA isolation from Lxx.
- protocols to improve the sugarcane pathogenicity assay were assessed.

These outputs will be reported in three publications in refereed journals.

### 7.0 EXPECTED OUTCOMES

The ultimate prize of this type of work will be to identify and characterise a gene(s) that encodes a protein that is critical for Lxx’s interaction with sugarcane. Any interference with this protein’s function in Lxx would eliminate Lxx’s ability to cause disease on sugarcane. These genes might encode products such as outer membrane or transmembrane proteins that are either directly or indirectly involved in the interaction between the xylem vessels and Lxx or are necessary for uptake of nutrients from the xylem fluid. The ways we might be able to interfere with these is by engineering sugarcane to produce a monoclonal antibody that is specific for a sight on these proteins that is accessible in the xylem or even better, is produced in large quantities on the wound surfaces where Lxx first colonises sugarcane. Because Lxx does not have any alternate hosts that we know of in Australia and because Lxx cannot survive in the soil for extended periods, a control measure such as this could completely eradicate RSD from Australia saving the industry $10 million per year in yield losses.

Ten years ago we thought we might be able to identify these genes by transposon mutagenesis. We discovered that Lxx was too difficult to work with to be able to accomplish this task by that means. Four years ago we thought that a genomics approach might be the way to get at these genes. Now that the Lxx genome is completed and the annotation is well underway it looks like we were correct in this assumption. However, we still need to advance the molecular tools so that we can prove that any of the genes identified in the genomic analysis of Lxx really do play the critical role in RSD that we think they do. The most powerful tool to do this is marker exchange mutagenesis. If we can get this working in Lxx we should be able to analyse the role of any gene in the Lxx genome in RSD.

### 8.0 FUTURE RESEARCH NEEDS AND RECOMMENDATIONS

A concerted effort needs to be established to improve the efficiency of Lxx transformation. One area that needs to be addressed is the lack of good plasmids for Lxx. In our preliminary work, we have not identified any native plasmids in Lxx. However, several years ago a group in South Africa reported that they had found a plasmid in one of the Lxx isolates in their collection. This work was never carried forward, nor was it ever
I propose that a concerted effort be made to identify a native plasmid or clone an origin of replication from Lxx and generate a plasmid.

The second area that needs attention is to isolate some strong promoters from Lxx so that we can express any genes we want. With the full genome completed and the first set of gene-expression studies on the drawing board, this should be easy to accomplish. Once a decent vector is constructed and a strong promoter cloned, then we can start to analyse whether gfp and other markers function in Lxx.

To improve transformation efficiency, I suggest that a series of experiments be established to determine better mechanisms for getting plasmid DNA into Lxx. This can include technologies like gene compaction, which was developed for eukaryotic transformation systems or restriction enzyme modified insertion (REMI), which has worked in a range of fungal transformation systems. This work will need to be done in conjunction with techniques to loosen up the tough cell wall of gram-positive bacteria of which Lxx is one of the toughest.

I find myself in an interesting position. I am the acting Chairman of the American Phytopathological Societies Bacteriology committee and I have no funding to work on the bacterial pathogen that I have spent the past 9 years putting into the international spotlight. I have taken this bacterium from one where there was no one doing any molecular work on it to getting it listed as one of the top ten phytopathogenic bacteria in the world that should be sequenced and ultimately managed to convince the Brazilians to sequence the Lxx genome. It was because of my activities to get the Lxx genome sequenced that I was given this recognition by colleagues in the US. I believe that I now have an opportunity to raise the profile of this bacterium even higher. As the chairman of this committee I can garner support from my fellow committee members to hold a symposium on phytopathogenic bacterial genomics and can make Lxx one of the key features of this symposia. This could help to get more researchers involved in Lxx research. Therefore, I recommend that funding be made available for me to attend the annual meeting of the American Phytopathological Society in the US in 2002-2003.

The work on sequencing the Lxx genome is now complete and progress towards identifying the genes involved in host recognition, colonization and pathogenicity should be rapid. This requires investment in generation and microarraying oligonucleotides for every gene in the Lxx genome (approximately 2500). Both of these can be outsourced to private companies and there are several microarray readers that we could get access to for data analysis.

9.0 PUBLICATIONS FROM THE PROJECT

Refereed proceedings

Conference papers


Poster presentations and abstracts


Refereed journal papers
No manuscripts have yet been submitted to refereed journals. However, three, which have components of the work done in this project, are in draft form.
Brumbley, S.M, Murphy, R.M. and Petrasovits, L. Molecular analysis of Leifsonia xyli subsp. xyli transposon mutants. (Journal of Bacteriology).


10.0 ACKNOWLEDGMENTS

I thank the large number of people who worked on this project. The project started with Lars Petrasovits as the Research Assistant. He was responsible for the shotgun sequencing of the regions flanking the transposon in the Lxx::Tn4431 insertion mutant 476. He also built the constructs for the Tn3HoHo1 style mutagenesis and screened the shotgun mutagenesis library of the two overlapping Lxx genomic library cosmid clones pLB1C2 and pLB1D6. Rachel Murphy was responsible for much of the annotation of the sequence data from the 13 kb region sequenced by Lars. Steve Bradford spent considerable time trying to get the marker exchange system operational and he built the first of the insertion mutants of pro operon genes. He also participated in some of the work to optimise the inoculation protocol. That work involved a number of BSES staff, including Scott Hermann, Anthony Young and Lars Petrasovits, who helped set up and take down the trials, the BSES RSD diagnostic labs at Tully and Mackay who analysed the samples for the presence of Lxx, and Barry Croft and Andrew Greet at the BSES Woodford Pathology farm and Barry Pearce and Gareth Disley at the Eight Mile Plains Pathology Farm for routine watering and fertilization and pesticide application of the inoculation trials. Lastly, I thank Dr Roland Nagel, who worked with us for 6 months and during that brief period improved the transformation and genomic DNA isolation protocols for Lxx and tried very hard to get some hard data on marker exchange in Lxx.

11.0 REFERENCES


