Establishment of a Functional Genomics Platform for *Leifsonia xyli* subsp. *xyli*

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Leifsonia xyli subsp. *xyli*, the causal agent of ratoon stunting disease in sugarcane, is a xylem-limited, nutritionally fastidious, slow growing, gram-positive coryneform bacterium. Because of the difficulties in growing this bacterium in pure culture, little is known about the molecular mechanisms of pathogenesis. Currently, the genome sequence of *L. xyli* subsp. *xyli* is being completed by the Agronomical and Environmental Genomes group from the Organization for Nucleotide Sequencing and Analysis in Brazil. To complement this work, we produced 712 *Lxx::Tn4431* transposon mutants and sequenced flanking regions from 383 of these, using a rapid polymerase chain reaction–based approach. *Tn4431* insertions appeared to be widespread throughout the *L. xyli* subsp. *xyli* genome; however, there were regions that had significantly higher concentrations of insertions. The *Tn4431* mutant library was screened for individuals unable to colonize sugarcane, and one noncolonizing mutant was found. The mutant contained a transposon insertion disrupting two open reading frames (ORF), one of which had homology to an integral membrane protein from *Mycobacterium leprae*. Sequencing of the surrounding regions revealed two operons, *pro* and *cyd*, both of which are believed to play roles in disease. Complementation studies were carried out using the noncolonizing *Lxx::Tn4431* mutant. The noncolonizing mutant was transformed with a cosmid containing 40 kbp of wild-type sequence, which included the two ORF disrupted in the mutant, and several transformants were subsequently able to colonize sugarcane. However, analysis of each of these transformants, before and after colonization, suggests that they have all undergone various recombinant events, obscuring the roles of these ORF in *L. xyli* subsp. *xyli* pathogenesis.

Ratoon stunting disease (RSD) of sugarcane is caused by *Leifsonia xyli* subsp. *xyli* (Davis et al. 1980), a gram-positive coryneform, xylem-limited bacterial pathogen (Kao and Damann 1980). RSD is the result of vascular plugging that causes general stunting (Kao and Damann 1980), but otherwise,

RSD has no reliable external symptoms (Gillaspie and Teakle 1989). It is currently unknown whether this plugging is bacterially produced, whether it is a plant defense response, or if it is a combination of products from both *L. xyli* subsp. *xyli* and the sugarcane plant. RSD is the most economically important pathogen of sugarcane worldwide, with losses reported ranging from less than 5% to exceeding 30% (Bailey and Bechet 1997; Koike et al. 1982; Steib and Chilton 1968). The effects of the disease and the consequent losses depend primarily on water availability (McDougall et al. 1948; Rossler 1974) and cultivar susceptibility (Gigliotti et al. 1999; Wismer 1971).

L. xyli subsp. *xyli* was originally described in the genus *Clavibacter* (Davis et al. 1984), based on phenotypic characteristics, but recently was reviewed and placed in the genus *Leifsonia* (Evtushenko et al. 2000), based on rRNA gene analysis. It is nutritionally fastidious and grows extremely slow in culture, taking up to four weeks for single colonies visible to the naked eye to appear on complex solid media and two weeks for growth to mid-log phase in liquid culture. As a result, there has been little molecular work on *L. xyli* subsp. *xyli*. Recently, the Agronomical and Environmental Genomes (AEG) program in the state of São Paulo, Brazil, a subprogram of the Organization for Nucleotide Sequencing and Analysis, began sequencing and annotation of a Brazilian isolate of *L. xyli* subsp. *xyli*. Complementary to this work, 712 *L. xyli* subsp. *xyli* transposon mutants were generated (Brumbley et al. 2002) using the transposon *Tn4431*. *Tn4431* is a derivative of *Tn1721* (Shaw et al. 1988), a transposon structurally similar to the *Tn3* family of transposons (Mahillon and Chandler 1998) and carries a promoterless *lux* operon from *Vibrio fischeri*. Luciferase studies on these *Lxx::Tn4431* mutants indicated that 99% of the mutants had insertions into or in frame with functional genes (Brumbley et al. 2002). In this report, we discuss the development of a high throughput polymerase chain reaction (PCR)-based screening method to identify insertion points of the transposon *Tn4431* into the *L. xyli* subsp. *xyli* genome. We also screened the *Lxx::Tn4431* mutants for their ability to colonize sugarcane and have identified two open reading frames (ORF) that may be involved in pathogenicity.

RESULTS

Transposon mutagenesis and mapping of insertion sites.

Of the 712 *Lxx::Tn4431* mutants originally constructed, 383 flanking sequences were PCR-amplified and sequenced using either of the *Tn4431* end-specific primer/probe combinations. Amplification of *Lxx::Tn4431* DNA using the semispecific PCR and 70 cycles of amplification resulted in one or occasionally

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more DNA products (Fig. 1A). The number of specific products did not always equal the number of DNA products amplified, and the specific product was not always dominant (Fig. 1B). PCR products amplified ranged in size from approximately 70 bp to 4 kbp. A total of 251 flanking DNA sequences could not be amplified with the primers used in this study, while another 78 flanking DNA sequences were amplified but could not be sequenced.

Positively hybridizing products (Fig. 1B) were purified and directly sequenced with the respective oligonucleotide probes bss7 or bsl7. PCR reactions that produced two or more hybridizing products were always the result of the single primer

binding at multiple sites downstream from the transposon insertion. Interestingly, when the sequences were compared against those of the AEG *L. xyli* subsp. *xyli* genome project, a 5-bp deletion at the target site was always associated with insertion of Tn4431.

Functional classification was assigned to the 383 flanking sequences, using categories defined by Bolotin and associates (1999) (Fig. 2). Of these, 44% were not similar to any other protein or putative protein currently available (Blast-X). A further 12% of insertions lay within transposon or phage-related genes, while 10% had matches to hypothetical proteins from other members of the class Actinomycetes. The remaining

Fig. 1. Semispecific polymerase chain reaction amplification products of *Leifsonia xyli* subsp. *xyli* Tn4431 mutants using the bss7RM1 primer. **A**, Agarose gel electrophoresis with ethidium bromide stain under UV light. **B**, Southern hybridization of blotted products from A probed with the ^{33}P - γ -ATP labeled primer bss7.

sequences (34%) were distributed across the other categories, with most insertions occurring in genes involved in energy metabolism (Fig. 2). Although there is currently insufficient *L. xyli* subsp. *xyli* sequence data to elucidate Tn4431 distribution across the entire genome, there is evidence that insertion has occurred nonrandomly. Of the 383 Tn4431 mutants sequenced, 54% of insertions were in regions already containing another transposon insertion (determined by sequence overlap or Blast-

X) (Fig. 2). Importantly, nearly all of these sequences had different insertion sites, indicating the insertion events were unique.

Isolation of mutants unable to colonize sugarcane.

To screen the 712 *Lxx::Tn4431* mutants for loss of pathogenicity, inoculations in triplicate for each of the mutants was performed, and after approximately three months, the plants

Fig. 2. Putative functional classification of the flanking sequences identified in this study. All flanking regions were analyzed for matches using Blast-X and then were assigned a functional classification based on the work of Bolotin and associates (1999). The total number of sequences within each category is shown by total bar height. The number of sequences found to have a single Tn4431 insertion is represented by the lower section of each bar. The upper section of each bar shows the number of sequences found to have multiple insertions, although not necessarily at the exact same point along the sequence. Similarities within the no similarity (NS) category were determined using alignment (ContigExpress; InforMax Inc., Frederick, MD, U.S.A.) AA = amino-acid biosynthesis; CIM = central intermediary metabolism; EM = energy metabolism; CPGC = biosynthesis of cofactors, prosthetic groups, and carriers; FA's = fatty acid and phospholipid metabolism; NA's = purines, pyrimidines, nucleosides, and nucleotides; Tr = transport and binding proteins; RTT = replication, transcription, and translation; CE = cell envelope; Other = unassigned functions; Tn = transposon and phage related functions; Hyp = hypothetical; and NS = no similarity.

Fig. 3. Southern hybridization confirming the site of transposon insertion. Total genomic DNA from *Leifsonia xyli* subsp. *xyli* wild-type and Tn4431 mutants was digested with *Pst*I. The inverse polymerase chain reaction product amplified from mutant QPF110-476 was used as a probe.

were harvested, and the presence of *L. xyli* subsp. *xyli* wild-type and *Lxx::Tn4431* mutants was determined. Only one *Lxx::Tn4431* mutant, QPF110-476, failed to colonize in this initial screen. To confirm this, QPF110-476 was inoculated onto sugarcane plants four additional times, twice in replicates of 15. Colonization was not detected in any of these inoculations.

Identification of the genomic region containing transposon insertion and complementation.

The QPF110-476 DNA flanking the transposon insertion could not be amplified using semispecific PCR. However, a 1.3-kbp fragment was amplified using inverse PCR. This was cloned, and its identity as flanking DNA was confirmed by Southern hybridization (Fig. 3). The *Tn4431* insertion disrupted two potential ORF in opposite directions (Fig. 4). The larger of these two ORF (Blast-X) was similar to an integral membrane protein from *Mycobacterium leprae* (E: 2e-18), although we found no evidence of similar domains using PROSITE (Gattiker et al. 2002). The ORF was also similar to a Zn-dependent protease (E: 5e-6) and a peptidase (4e-6) from *Corynebacterium glutamicum*. Similar hypothetical proteins with unknown function were identified in other members of the class Actinomycetes, including *Thermobifida fusca* (E: 1e-31), *Mycobacterium tuberculosis* (E: 2e-24), and *Streptomyces coelicolor* (E: 9e-7) (Fig. 5). From a multiple alignment, it appears likely that this ORF has a Val start codon and encodes a hypothetical protein of 314 amino acids. The smaller of the disrupted ORF (orf24) did not appear to have any similarity to any other public DNA or protein sequences.

To isolate the wild-type gene and to study regions flanking the two ORF, a cosmid library of a wild-type *L. xyli* subsp. *xyli* (QPF110) was constructed. The inverse PCR product was used as a probe, and two unique cosmid clones were identified, one of which (pLB1C2) was shotgun cloned and a total of 11,236 bp was sequenced. Analysis of this region revealed strong homology to the *pro* operon of *M. tuberculosis* and to cytochrome oxidase subunits I and II (Fig. 4). Interestingly, three other mutants contained *Tn4431* insertions within this 11-kbp region; however, these were all still able to colonize sugarcane. Complementation studies were carried out to demonstrate whether these regions were able to restore the ability of mutant QPF110-476 to colonize sugarcane. The mutant was trans-

formed with cosmid pLB1C2 to generate kanamycin-resistant transformants 476-C2a, 476-C2b, 476-C2c, 476-C2d, and 476-C2e. The transformants were analyzed for the presence of the wild-type and mutant ORF by Southern hybridization (Fig. 6A) and PCR (Fig. 6B). In summary (Table 1), the wild-type ORF originally carried on the cosmid could be detected by PCR in all the transformants except 476-C2a, while the cosmid could only be detected in 476-C2a, 476-C2b, and 476-C2c by Southern hybridization. Transformant 476-C2d appeared to lack the larger of the hybridizing bands, suggesting part of this region had been lost, while transformant 476-C2e was no longer resistant to tetracycline.

After three months, fibrovascular fluid was extracted from plants and spread onto kanamycin-containing filters, and a single bacterial colonies were isolated. The presence of *Tn4431* in each of these ex planta bacteria was confirmed by a mutant-specific PCR amplification (Fig 6B). Of the five transformants, only 476-C2d was unable to colonize sugarcane. Interestingly, the wild-type ORF could not be detected in any of the transformants.

DISCUSSION

To complement the *L. xyli* subsp. *xyli* genome sequencing effort carried out by the AEG group, we have produced 712 *L. xyli* subsp. *xyli* transposon mutants and have sequenced flanking regions from more than half of them. The 712 *L. xyli* subsp. *xyli* mutants were generated using a transformation and transposon mutagenesis system developed by Brumley and associates (2002). To identify the *Tn4431* insertion sites for each of the mutants, a rapid, single primer technique based on that of Hermann and associates (2000) was developed. Primers were designed to amplify outward from each end of *Tn4431*. Template DNA was derived from washed *Lxx::Tn4431* frozen stocks and required no further isolation, purification, or modification. Under the amplification conditions used, these primers were able to specifically amplify the *Tn4431* end sequence in 65% of cases. This percentage could probably be increased through the use of different amplification primers and conditions. Amplified products were directly sequenced using an internal primer. Given the fastidious nature of *L. xyli* subsp. *xyli*, its extremely slow growth, and the difficulties encountered with purification of genomic DNA, this high-throughput

Fig. 4. Schematic drawing of 11,236 bp of *Leifosonia xyli* subsp. *xyli* sequence encompassing the *pro* VWZX and *cyd* ABD operons. The position of different *Tn4431* insertions in various *L. xyli* subsp. *xyli* mutants is shown. orf = open reading frame, tetR = transcriptional regulator of the tetR family, pro = proline transport, and cyd = cytochrome *d* oxidase.

technique provided a rapid alternative to either cloning or direct sequencing of the Tn4431 insertion sites.

As the *L. xyli* subsp. *xyli* genome sequence is not yet complete, it is impossible to examine the distribution of Tn4431 insertions across the genome. However, there is evidence that transposition was not completely random. In a 16-kbp contig (data not shown), we identified at least 19 individual Tn4431 insertions, which is 10 times higher than expected (assuming the transposon inserted in a random, nonbiased fashion). Similar results have been found with other Tn3 derivatives in both yeast (Ross-Macdonald et al. 1999) and bacteria (Davies and Hutchison 1995).

Most insertions (171) were in sequences with no similarity to any other putative protein-encoding genes in the databases. The classes with the next highest hits (48 and 40) showed homology to transposon associated sequences and to hypothet-

ical proteins from other members of the class Actinomycetes, respectively. Interestingly, over 50% of the flanking regions sequenced in these categories had multiple insertions, primarily in unique locations along the sequence. The remaining insertions (130) were scattered throughout various categories of genes, with most being in genes encoding i) energy metabolism (31%), ii) transport and binding (18%), and iii) central and intermediary metabolism (17%) (Fig. 2).

One of the 712 *Lxx::Tn4431* mutants was unable to colonize sugarcane. The Tn4431 insertion disrupted two ORF in opposite directions. The larger of these ORF had homology to an integral membrane protein from *M. leprae* and also to a protease and peptidase from *C. glutamicum*. Five transformants were produced containing 40 kbp of wild-type sequence carried on a cosmid vector (pLB1C2), and four of these were able to colonize sugarcane (*ex476-C2a*, *ex476-C2b*, *ex476-C2c*, and *ex476-*

Fig. 5. Alignment of hypothetical proteins showing homology to one of the disrupted open reading frames (ORF) of QPF110-476. The translated product from the larger ORF is compared with putative proteins from: *Corynebacterium glutamicum* (Cg1, accession number NP_599272; Cg2, accession number AAL31539), *Mycobacterium leprae* (Ml, accession number NP_302376), *M. tuberculosis* H37Rv (Mt, accession number NP_216361), *Streptomyces coelicolor* A3 (Sc1, accession number NP_625395; Sc2, accession number NP_628741), and *Thermobidida fusca* (Tf, accession number ZP_00058818). Black boxes are identical residues, Gray boxes indicate conserved substitutions, and dashed lines indicate gaps.

C2e). We performed Southern hybridizations and PCR analysis on these transformants before and after inoculation onto sugarcane. Before inoculation, two transformants appeared to retain the cosmid intact (476-C2b, and 476-C2c), while the other three appear to have undergone recombination events. Transformant 476-C2a may have lost part of the cosmid-borne wild-type ORF, as they could no longer be amplified by PCR, while both 476-C2d and 476-C2e appear to have lost the cosmid but retained kanamycin resistance and the wild-type ORF, suggesting at least part of the cosmid has recombined with the genome. Furthermore, transformant 476-C2d had also lost the larger of its

hybridizing bands, which corresponds to part of the *pro* operon, and transformant 476-C2e had lost resistance to tetracycline, suggesting the recombination event occurred in this region. When the transformants were reisolated, the wild-type ORF could not be amplified from any of the transformants, suggesting that, during colonization or subsequent culture, further recombination events had occurred, resulting in loss of the wild-type ORF from 476-C2b, 476-C2c, and 476-C2e.

What role the disrupted ORF play in sugarcane colonization remains obscure. Mutant QPF110-476, which contained a single *Tn4431* insertion, was unable to colonize sugarcane.

Fig. 6. Molecular analysis of *Leifosonia xyli* subsp. *xyli* transformants. **A**, Total genomic DNA from *L. xyli* subsp. *xyli* QPF110 (*Lxx*), QPF110-476 *Tn4431* mutant (476), and transformants containing the complementary cosmid clone pLB1C2 (C2a to C2e) were probed with a 2.1-kbp ³²P-labeled *EcoRI/PstI* fragment from DNA flanking the *Tn4431* insertion. **B**, The presence of the wild-type and mutant open reading frames (ORF) were identified in *L. xyli* subsp. *xyli* transformants and ex planta isolates, using primer pairs ORF24F and ORF24R (WT), bsI7RM1 and ORF24F (Tn1), and bss7RM1 and ORF24R (Tn2).

Table 1. Summary of characteristics of QPF110-476 transformants before and after colonization

Characteristics	476-C2a	476-C2b	476-C2c	476-C2d	476-C2e	ex476-C2a	ex476-C2b	ex476-C2c	ex476-C2e
Colonization	Yes	Yes	Yes	No	Yes	NT ^a	NT	NT	NT
Cosmid	Yes	Yes	Yes	No	No	NT	NT	NT	NT
Wild-type ORF present	No	Yes	Yes	Yes	Yes	No	No	No	No
Km ^r	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Tc ^r	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	No

^a NT = not tested.

However, one transformant (476-C2a) also apparently lacking the wild-type ORF was able to colonize, while another transformant (476-C2d) that carried the wild-type ORF but lacked at least part of the *pro* operon was not. It is possible that the wild-type ORF in 476-C2a did remain functionally intact after recombination, although we were unable to detect any such event, using several primer pairs (data not shown). Interestingly, loss of the *pro* operon in 476-C2d suggests that this region may also be important in colonization. It encodes a glycine/betaine transporter system that is involved in osmoregulation (Cairnery et al. 1985) and may be critical for xylem colonization and signaling between the bacteria and the plant host (Matthysse and McMahan 1998).

With the full sequence of the *L. xyli* subsp. *xyli* genome about to be completed in Brazil, it is critical to have a set of molecular tools to elucidate the role, if any, individual genes play in host recognition, colonization, and disease induction in sugarcane. Identification of key genes in these various processes could lead to future control measures for this economically important disease of sugarcane. In this paper, we have demonstrated that transposon mutagenesis is one such tool, and in the future, other transposons may be selected for a saturation mutagenesis approach. Alternatively, once the genome is completed, constructs for a more targeted, marker-exchange type mutagenesis can be used on selected genes. Importantly, the recombinations evidenced in the complementation study suggest that a marker selection strategy may be a valid approach for elucidation of the role that the wild-type ORF and genes play, such as in the role of the *pro* operon, in ratoon stunting disease.

MATERIALS AND METHODS

Bacterial strains and cultures.

Bacterial strains and plasmids are listed in Table 2. For inoculation onto sugarcane plants, both the wild-type *L. xyli* subsp. *xyli* and the *Lxx::Tn4431* mutants were grown in 10 ml of modified S8 broth (Brumbley et al. 2002) containing either no antibiotics or 10 µg of tetracycline per ml, respectively. For complementation studies, 25 µg of kanamycin per ml was also included. All cultures were grown to mid-log phase for 10 to 14 days at 28°C with constant shaking (150 rpm). Prior to inoculation, the optical density at 560 nm was adjusted to between 0.5 and 0.6 with sterile S8 broth. *L. xyli* subsp. *xyli* cultures for transformation or genomic DNA isolation were grown as previously described (Brumbley et al. 2002). *Escherichia coli* strains were grown on Luria broth (LB) containing the appropriate antibiotics.

Isolation and sequencing of DNA flanking the *Tn4431* insertion using semispecific PCR.

A semispecific PCR technique based on Hermann and associates (2000) was developed, using primers to each end of the transposon. Two oligonucleotide probes were designed that bound downstream from these primers. In brief, washed cell pellets from frozen *Lxx::Tn4431* cell stocks were used as template. PCR was carried out using a single transposon-specific primer, bss7RM1 (5'-CTGGAAGACGGGAAGTTT CGG-3') or bsl7RM1 (5'-GCTAAAATCTGTGTTCTCTTCG G-3'). These primers are specific to each end of the transposon and are internal to the inverted terminal repeats. Amplifications were carried out in 25-µl reactions containing amplification buffer (buffer 1, 1.75 mM MgCl₂ final concentration; Expand Long Template PCR Kit; Roche Diagnostics, Castle Hill, Australia), 250 µM dNTPs, 10 µM primer (bsl7RM1 or bss7RM1), and 1 U of *Taq* DNA polymerase. Thermal cycling parameters were 1 cycle at 94°C for 5 min, followed by 70 cycles of 30 s at 94°C, 15 s at 60°C (bss7RM1) or 55°C (bsl7RM1), 2 min at 72°C, followed by a final extension step for 7 min at 72°C. No template and wild-type *L. xyli* subsp. *xyli* isolates were used as negative controls. Amplification products were run on a 1% agarose gel, and Southern hybridization was performed essentially as described by Sambrook and associates (1989). The membrane was hybridized with either the ³²P-γ-ATP labeled, transposon-specific probe bss7 (5'-ATGCCCGGAAAACCTTAGCGTACG-3') or bsl7 (5'-AGAACACCGTTAGCTTAGCGTACG-3'). Blots were visualized after 2 h, using a Phosphor-imager STORM 840 (Molecular Dynamics, Sunnyvale, CA, U.S.A.), following the manufacturer's instructions.

Positively hybridized PCR products were purified using MinElute columns (Qiagen, Hilden, Germany), following the manufacturer's instructions. In most instances, the remaining PCR reaction was purified, but when both hybridizing and nonhybridizing PCR products were present, the PCR reaction was electrophoresed (0.8% agarose, 1× Tris-borate-EDTA), and the positively hybridizing DNA product was excised before purification. The purified eluate (2 µl) was used for DNA sequencing using the Big-Dye Terminator kit (Perkin Elmer Applied Biosystems, Foster City, CA, U.S.A.), following the manufacturer's instructions. The oligonucleotide probes (unlabeled) were used in sequencing. Dye terminator was removed using the DyeEx96 kit (Qiagen) or the DyeEx Spin kit (Qiagen), following the manufacturer's instructions. The sequences were run on the ABI 377 automated sequencer at the Australian Genome Research Facility, Brisbane, Australia.

Table 2. Bacterial strains, cosmids, and plasmids used in this study

Strain/plasmid	Relevant characteristics	Source or reference
Strains		
<i>Escherichia coli</i>		
DH5α	deoR, endA1, gyrA96, hsdR17(rk- mk+), recA1, relA1, supE44, thi-1, Delta(lacZYA-argFV169), phi-80deltalacZDeltaM15, F-, Lambda-	Stratagene, La Jolla, CA, U.S.A.
<i>Leifsonia xyli</i> subsp. <i>xyli</i>		
QPF110	Wild-type <i>L. xyli</i> subsp. <i>xyli</i>	Brumbley et al. 2002
QPF110-476	Noncolonizing QPF110 containing a single <i>Tn4431</i> insertion	Brumbley et al. 2002
QPF110 (476-C2a, 476-C2b, 476-C2c, 476-C2d, 476-C2e)	Five QPF110-476 mutants harboring pLB1C2	This study
ex476-C2a, ex476-C2b, ex476-C2c, ex476-C2e	Four colonizing mutants reisolated from sugarcane	This study
Plasmids		
pLAFR5-km	Improved broad host range cosmid cloning vector; 22.7 kbp; RP4 Ori; Km ^r	Brumbley et al. 2002
pBluescript II SK+	Phagemid cloning vector; 2.9 kbp; ColE1 Ori; Ap ^r	Stratagene, La Jolla, CA, U.S.A.
pLB1D6	Cosmid vector containing <i>L. xyli</i> subsp. <i>xyli</i> genomic fragment; 45 kbp; RP4 Ori; Km ^r	This study
pLB1C2	Cosmid vector containing <i>L. xyli</i> subsp. <i>xyli</i> genomic fragment; 45 kbp; RP4 Ori; Km ^r	This study

Analysis of DNA sequence.

Identification of regions into which *Tn4431* inserted was performed using BlastX v. 1.4.9 (Altschul et al. 1990; Gish and States 1993) against the NR Bacterial GenPept database compiled by the Australian National Genomic Information Service (ANGIS, Sydney, Australia). Relative positions of *Tn4431* insertion into the *L. xyli* subsp. *xyli* genome were identified using BlastN v. 2.1.2 (Altschul et al. 1997) against the contig databases of the *L. xyli* subsp. *xyli* Genome Sequencing Project (AEG, Piracicaba, Brazil). Raw sequence data was analyzed in MacVector 6.5.3 (Accelrys, San Diego, CA, U.S.A.) and ContigExpress (InforMax, Bethesda, MD, U.S.A.), and contiguous sequences were assembled using AssemblyLIGN 1.0.9b (Accelrys, Cambridge). BlastN, BlastX, and BlastP (Altschul et al. 1990) searches were carried out using the NR nucleic, bacteria, and *Mycobacterium tuberculosis* databases (ANGIS).

Colonization and complementation assay.

To screen the *Lxx::Tn4431* mutants for loss of colonization, stem sections containing one bud (one-eye set) of sugarcane cultivar Q110 were grown in steam-sterilized soil in 15-cm pots for 6 weeks. The tops of these plants were chopped off above the apical meristem, and a 100- μ l drop of a suspension of the *Lxx::Tn4431* mutants, wild-type *L. xyli* subsp. *xyli* cells, or xylem extract from *L. xyli* subsp. *xyli*-infected sugarcane plants of cultivar Q110 was immediately placed on the cut surface. All inoculations were performed in triplicate. After three months, the plants were harvested by cutting them off just above the soil line. The tops and leaves were removed and discarded, and the fibrovascular fluid was extracted using positive pressure. The presence of *L. xyli* subsp. *xyli* wild-type and *Lxx::Tn4431* mutants in the fibrovascular fluid was determined by evaporative binding enzyme immunosorbent assay (Croft et al. 1994). Any *Lxx::Tn4431* mutants unable to colonize sugarcane after three attempts were reinoculated a fourth time with 15 replicates.

To screen *Lxx::Tn4431* mutant for complementation, either the inoculation method described above was used or an alternative technique using two-eye sets of sugarcane cultivar Q110 was employed. Two-eye sets were inoculated with *L. xyli* subsp. *xyli* in S8 broth supplemented with filter-sterilized fibrovascular fluid from healthy Q110 plants 24 h prior to inoculation. Inoculation was performed by submersion of two-eye sets in cell suspensions for 5 min. Following inoculation, sets were planted in 20-cm plastic pots and were grown in glasshouses for three months. All inoculations were performed in triplicate. Plants were harvested, and fibrovascular fluid was extracted and analyzed for the presence of *L. xyli* subsp. *xyli* as described above.

Isolation and sequencing of flanking DNA from *Lxx::Tn4431* mutant QPF110-476.

Isolation of genomic DNA from *L. xyli* subsp. *xyli* was done as previously described (Brumbley et al. 2002). DNA (1.5 μ g) from *Lxx::Tn4431* mutant QPF110-476 was digested with 20 U of *Pst*I and, of this, 1 μ g was religated using 8 U of *T*₄-DNA ligase. Following phenol-chloroform extraction and ethanol precipitation, ligated DNA was resuspended in 50 μ l of water. Of this, 1 μ l was used as template. Using primers bsl3 (5'-CGTCTCAGTGTCTGCTCTTCG-3') and bsl7 (5'-AGAACACCGTTAGCTTAGCGTACG-3'), which were designed from sequence data from the large *Eco*RI/*Pst*I fragment of *Tn4431*, inverse PCR was performed under the following conditions: 0.26 mM dNTP, 0.1 μ M primers, 3 mM Mg²⁺, 0.5 $\times$ Q-solution, 0.5 U of Taq DNA polymerase (Qiagen) in 25 μ l reactions. The PCR profile was: 95°C for 5 min, 35 cycles of 94°C for 1

min, 54°C for 30 s, and 70°C for 1 min, a final extension step of 70°C for 10 min, and indefinite hold at 4°C. PCR products were digested with *Pst*I and *Eco*RI and were cloned into pBluescript SK II+ (Stratagene, La Jolla, CA, U.S.A.). Correct flanking DNA was confirmed by Southern hybridization against genomic DNA from wild-type and mutant *L. xyli* subsp. *xyli*, using the inverse PCR product as a probe.

A cosmid library was generated from wild-type strain QPF110 by partially digesting 3 μ g of *L. xyli* subsp. *xyli* genomic DNA with *Sau*3AI, followed by ligation into *Bam*HI-cut pLAFR5-Km (Brumbley et al. 2002) for 4 h at 22°C. Following phenol extraction and ethanol precipitation, ligation products were packaged, using Gigapack II XL according to the supplier's instructions (Stratagene). A total of 1,920 colonies were picked and inoculated into 96-well microtiter dishes containing LB with 10 μ g of tetracycline per ml and 50 μ g of kanamycin per ml and were grown overnight at 37°C. Sterile glycerol was added to a final vol/vol of 12%, prior to storage at -80°C. The genomic library was plated onto Hybond N+ membranes (Amersham, Little Chalfont, U.K.) and was grown on LB agar containing 10 μ g of tetracycline per ml and 50 μ g of kanamycin per ml. Colony hybridization was performed as described by Sambrook and associates (1989), using ³²P-labeled flanking DNA, prepared using the Rediprime system (Amersham) in accordance with supplier's instructions. Cosmids with homology to flanking DNA were shotgun cloned into pBluescript SK II+ and were sequenced as described above. Direct sequencing of cosmids using primers designed from previously determined data closed remaining gaps.

Complementation of *L. xyli* subsp. *xyli* mutant 476.

Lxx::Tn4431 mutant QPF110-476 was transformed with cosmid (pLB1C2) containing the complementary DNA from wild-type *L. xyli* subsp. *xyli* strain QPF110 as described (Brumbley et al. 2002). Transformants confirmed to be resistant to kanamycin were analyzed for the presence of the cosmid by Southern hybridization and PCR. Southern hybridization was carried out essentially as described by Brumbley and associates (2002). In summary, total genomic DNA (10 μ g) from *L. xyli* subsp. *xyli* QPF110, QPF110-476 *Tn4431* mutant (476), and transformants containing the complementary cosmid clone pLB1C2 (C2a to C2e) were digested with *Pst*I and probed with a 2.1-kbp ³²P-labeled *Eco*RI/*Pst*I fragment from DNA flanking the *Tn4431* insertion. PCR analysis was carried out to confirm the presence of the mutant and wild-type ORF. The mutant ORF was identified using primer pairs bsl7RM1 and ORF24F (5'-ATGTGTGCGCCGGGGGAGGCTGGTTCA-3') and bsl7RM1 and ORF24R (5'-CTCCTGCGCCTGCTGAGCTCACCGCT-3'), which amplify across the *L. xyli* subsp. *xyli* genome:: *Tn4431* fusion. The wild-type ORF carried on the cosmid was confirmed using primers ORF24F and ORF24R. PCR products were separated on a 1% agarose gel.

Five positive transformants were inoculated into sugarcane, as described above. After three months, fibrovascular fluid was extracted from plants and spread onto filters as described (Brumbley et al. 2002). Single bacterial colonies were restreaked twice and were stored at -80°C. PCR analysis was carried out again to confirm the presence of the mutant and wild-type sequences.

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