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Recent advances in the molecular biology of *Leifsonia xyli* subsp. *xyli*, causal organism of ratoon stunting disease

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Abstract. Twelve years ago our understanding of ratoon stunting disease (RSD) was confined almost exclusively to diagnosis of the disease and control via farm hygiene, with little understanding of the biology of the interaction between the causal agent (*Leifsonia xyli* subsp. *xyli*) and the host plant sugarcane (*Saccharum* spp. hybrids). Since then, research has focused on developing the molecular tools to dissect *L. xyli* subsp. *xyli*, so that better control strategies can be developed to prevent losses from RSD. Within this review, we give a brief overview of the progression in research on *L. xyli* subsp. *xyli* and highlight future challenges. After a brief historical background on RSD, we discuss the development of molecular tools such as transformation and transposon mutagenesis and discuss the apparent lack of genetic diversity within the *L. xyli* subsp. *xyli* world population. We go on to discuss the sequencing of the genome of *L. xyli* subsp. *xyli*, describe the key findings and suggest some future research based on known deficiencies that will capitalise on this tremendous knowledge base to which we now have access.

Additional keywords: genome decay, genome uniformity, lateral gene transfer, pathogenicity, virulence.

Introduction

Ratoon stunting disease (RSD) of sugarcane (Fig. 1), first discovered in 1944 (McDougall *et al*. 1948), is caused by *Leifsonia xyli* subsp. *xyli* (Davis *et al*. 1980), a Gram positive, coryneform, xylem-limited phytopathogen (Fig. 2) (Kao and Damann 1980). *Leifsonia xyli* subsp. *xyli* is the most economically important pathogen of sugarcane worldwide, with losses in infected plants ranging from less than 5% to more than 30% (Steib and Chilton 1968; Koike *et al*. 1982; Bailey and Bechet 1997). Generally, the symptoms of stunting (Fig. 1) and poor ratooning ability are believed to be the result of vascular plugging (Kao and Damann 1980). However, *L. xyli* subsp. *xyli* produces no other reliable external or internal symptoms (Gillaspie and Teakle 1989) and it is currently unknown whether this plugging is caused by something produced by the bacterium, is a plant defence response, or a combination of both.

Initially it was thought that RSD was caused by a virus because axenic culture of the bacterium was unsuccessful (Steindl 1957). It was not until the 1970s that a bacterium was found to be associated with infected plants (Gillaspie *et al*. 1973; Kao and Damann 1978) and not until the 1980s that it was successfully grown in pure culture and Koch's postulate confirmed it to be the causal agent of RSD (Davis *et al*. 1980, 1984) (Fig. 2). *Leifsonia xyli* subsp. *xyli* was originally described in the genus *Clavibacter* (Davis *et al*. 1984), based on phenotypic characteristics (Fig. 3), but recently was reviewed and placed in the genus *Leifsonia* (Evtushenko *et al*. 2000) based on rRNA gene analysis. This classification was recently confirmed by Young *et al*. (2006).

Little is known of the origin and early disseminations of RSD. It is unlikely that *L. xyli* subsp. *xyli* is naturally associated with the main progenitor of modern commercial sugarcane varieties, *Saccharum officinarum*, because the bacterium has previously never been found in populations of

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Fig. 1. Sugarcane variety Q124 infected with *Leifsonia xyli* subsp. *xyli* (left) growing side-by-side with healthy Q124 (right). Ratoon stunting disease losses can be severe when plants infected with *Leifsonia xyli* subsp. *xyli* are used as the plant cane and the cane experiences water stress. The image shows the first ratoon crop; the plant crop was harvested and this is the crop that grows back after the first harvest (ratoon) from the stool left in the ground. In Queensland, the sugarcane crop is normally harvested 4 or 5 times before a plough out and replant is required. However, with losses such as these, the crop may have to be ploughed out and replanted sooner. Photograph taken in Mackay, Queensland and is from the BSES Limited photo archives.

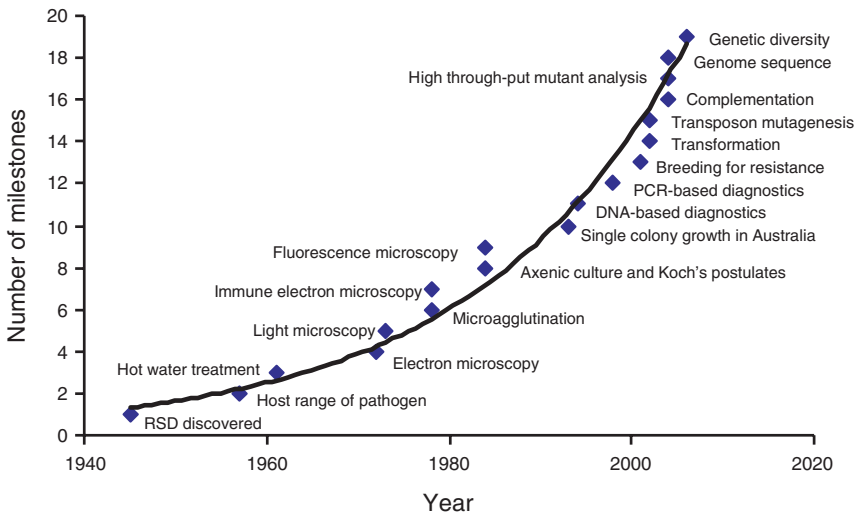

Fig. 2. Significant break-throughs in ratoon stunting disease research since the discovery of the disease in 1944–45.

this grass species throughout its centre of origin, Papua New Guinea (see King 1953; Hughes 1955), and only recently has become established in commercial plantation on the Ramu estate in this country (Magarey *et al*. 2002). Based on its xylem habitat, restricted host range (Rao *et al*. 1990), close relationship with another grass endophyte (*Leifsonia xyli* subsp. *cynodontis*) and certain genomic features (Monteiro-Vitorello *et al*. 2004), the bacterium appears to have had a long association with grasses. Interestingly, clones of the wild cane, *Saccharum spontaneum*, support >10 times the bacterial titres of *L. xyli* subsp. *xyli* than infected *S. officinarum* (Roach 1988, 1990; Roach and Jackson 1992), suggesting that the bacteria are better adapted to the former plant, and may share a longer history of interaction. *Saccharum spontaneum* was hybridised with *S. officinarum* in the 1920s to produce the first of the modern hybrid cultivars, which coincides with the first reports of ratooning problems and varietal yield decline

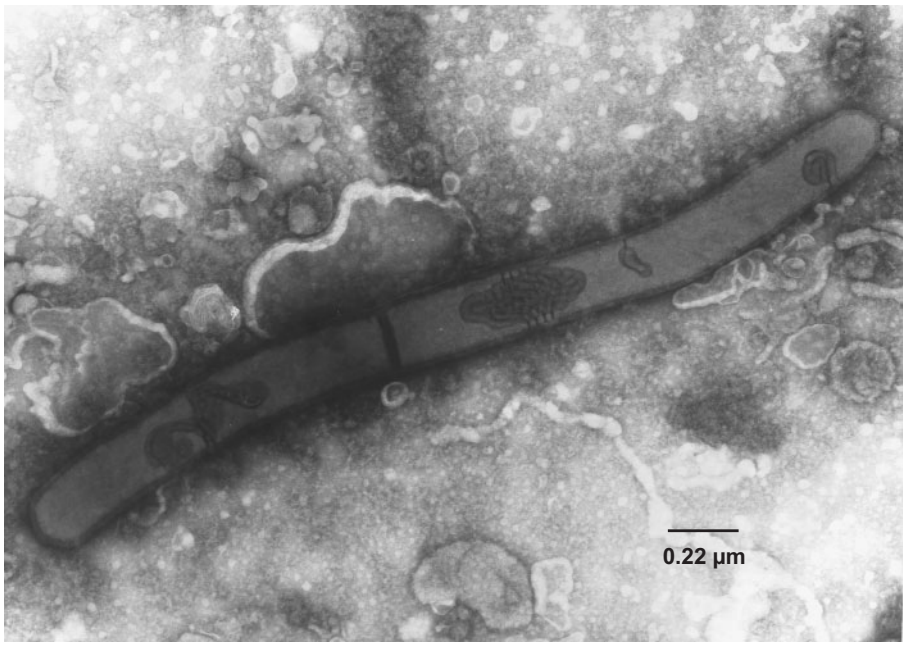

Fig. 3. Electron micrograph of dividing *L. xyli* subsp. *xyli* cells originally isolated from nodal exudate from sugarcane variety Q77 on 22 June 1973. Note the 4 distinct mesosomes, 3 in the longer cell and 1 in the other. *L. xyli* subsp. *xyli* cells measure 195–220 nm in diameter. Length of the two cells together is 2610 nm. Because this sample was dried as part of the fixation process these sizes are most likely smaller than a living bacterium. *L. xyli* subsp. *xyli* cells will not fit through a 0.22-µm filter but will fit through a 0.40-µm filter. These rods can have swelling on one end giving them the appearance of a club-shaped rod, hence the original name, *Clavibacter*. Photograph courtesy of Prof. David Teakle.

(Anon. 1934; King and Steindl 1953). While speculative, the emergence of RSD from *S. spontaneum* endophytes at the dawn of the modern sugar industry would facilitate the world-wide distribution of a single strain of *L. xyli* subsp. *xyli*. Support for this hypothesis has been provided by the genetic diversity studies of Young *et al*. (2006).

An effective control strategy for RSD had been developed even before the causal agent was identified. This involved provision of disease-free planting material and disinfection of cutting implements that could spread the disease (Taylor *et al*. 1988) (Fig. 2). Currently in Australia, disease-free planting material is provided by local productivity services who have established disease-free plots of cane by routine hot-water treatment (for review, see Steindl 1961). This material is sold to growers, who plant the cane into an on-farm nursery from which they plant their commercial fields. Using this system, Australia, which has one of the world's best records at controlling the disease (Bailey and Bechet 1997; Croft *et al*. 2004) has been able to contain the overall incidence of RSD to less than 5% with some districts below 1%. However, a few districts have a 20% incidence of RSD (Croft *et al*. 2004).

Diagnostic methods

There are no reliable internal or external symptoms of RSD. It is readily spread both within a field and between fields and farms on infected planting and harvesting machinery (Taylor *et al*. 1988). In many countries, clean-seed schemes and heat treatment of planting material are important factors in the management of RSD. However, these methods are expensive and labour-intensive and may not always eradicate the causal agent, *L. xyli* subsp. *xyli* (Damann and Benda 1983; Roach 1987). Efficient diagnostic tests are, therefore, crucial to the management of the disease (Gillaspie and Teakle 1989).

Initially, diagnosis of the disease was performed by inoculation of susceptible sugarcane cultivars and/or other grass species with test-plant juice (Steindl 1957; Matsuoka 1971; Mills *et al*. 2001), by electron microscopy (Plavsic-Banjac and Maramorosch 1972) (Fig. 2) and other microscopic techniques (Gillaspie *et al*. 1973) (Fig. 3). Major disadvantages of these techniques include slowness, lack of sensitivity and the requirement for highly trained operators and/or expensive equipment. The identification of *L. xyli* subsp. *xyli* as the causal agent of RSD (Davis *et al*. 1980) greatly facilitated the development of serological (Davis and Dean 1984; Croft *et al*. 1994) and DNA-based diagnostic tests (Chung *et al*. 1994; Pan *et al*. 1998; Fegan *et al*. 1998; Taylor *et al*. 2003) (Fig. 2). Chung *et al*. (1994) used cloned DNA from *L. xyli* subsp. *xyli* as probes in Southern and dot blot hybridisations while Pan *et al*. (1998) and Fegan *et al*. (1998) both used the intergenic

transcribed spacer regions of the 16S–23S rRNA genes to develop diagnostic primers for polymerase chain reaction (PCR)-based assays. Taylor *et al*. (2003) also used a PCR technique but developed *L. xyli* subsp. *xyli* specific diagnostic primers derived from PCR products amplified using random amplified polymorphic DNA primers (OPC2 and OPC11; Operon Technologies, USA). Although these techniques are recognised as being sufficiently sensitive, relatively inexpensive and efficient, due care must be exercised, especially when interpreting negative test results, as current sampling strategies only encompass as little as 0.2% of a given field population (Young and Brumbley 2004). Additionally, all serological techniques for the detection of this bacterium rely on polyclonal antisera raised against whole bacteria. The detection limit is $\sim 10^4$ cells/mL xylem fluid (Davis and Dean 1984), which might be improved by the use of a monoclonal antibody.

Improved culture conditions for axenic growth

In 1993, *L. xyli* subsp. *xyli* was grown in Australia by placing a drop of xylem extract from an infected sugarcane plant on the surface of a MSC-agar plate and incubating at 28°C until the spot was a confluent growth of *L. xyli* subsp. *xyli* cells (B. J. Croft, unpublished data). Cells were propagated by carefully harvesting bacteria from plates, resuspension in sterile water and subsequent spotting of droplets onto fresh MSC-agar plates. For molecular biological investigation of pathogenicity, it was necessary to establish a method for single-colony growth. This was accomplished by growing bacterial cultures on MSC medium (Teakle and Ryan 1992), modified to have increased agar levels so the media was firm enough to spread-plate or streak onto (Brumbley *et al*. 2002) (Fig. 2). Since *L. xyli* subsp. *xyli* is so slow-growing (at 28°C it takes 4 weeks to see visible single colonies after streaking or spread-plating) antibiotic selection pressure was improved by growing cultures on nitrocellulose filters (0.2 µm pore size; *L. xyli* subsp. *xyli* can fit through a 0.45 µm pore) with weekly transfers to fresh medium (twice a week for ampicillin) (Brumbley *et al*. 2002). Although *L. xyli* subsp. *xyli* was resistant to nalidixic acid, it was highly susceptible to ampicillin (100 µg/mL), tetracycline (2 µg/mL, S. M. Brumbley *et al*., unpublished data) and kanamycin (50 µg/mL).

To generate electroporation competent cells and to grow cells for DNA isolation and for inoculation studies, it was necessary to grow *L. xyli* subsp. *xyli* in liquid media. Brumbley *et al*. (2002) demonstrated that for the bacterium to grow efficiently in broth culture it was necessary to increase the concentrations of KH_2PO_4 and sucrose and decrease the concentrations of K_2HPO_4 and cysteine in the S8 medium described in Davis *et al*. (1980). In addition, it was shown that the addition of glycine improved the ability to make electroporation competent cells and to extract high quality genomic DNA from *L. xyli* subsp. *xyli* (Brumbley *et al*. 2002).

Transformation and transposon mutagenesis of *L. xyli* subsp. *xyli*

Brumbley *et al*. (2002) described the transformation of *L. xyli* subsp. *xyli* with the *Clavibacter/E. coli* shuttle vectors pDM302 and pDM306 (Meletzus *et al*. 1993) and pLUX19 (Mudge *et al*. 1996) (Fig. 2). These constructs were highly unstable in this bacterium (Brumbley *et al*. 2002). By contrast, cosmid vectors harbouring the RP4 broad host range origin of replication, derived from pLAFR3 (Staskawicz *et al*. 1987) and pLAFR5 (Keen *et al*. 1988), proved to be highly stable. A pLAFR5 derivative harbouring a kanamycin resistance marker (pLAFR5-km: Brumbley *et al*. 2002) was used for the construction of a genomic library of 1920 clones giving an estimated 15-X coverage of the *L. xyli* subsp. *xyli* genome (Brumbley *et al*. 2002).

Transposon mutagenesis is used to generate knockout mutants. Transposons are introduced into bacteria on a suicide vector incapable of replicating in the host and when they translocate from the vector into the host genome and insert into a gene, its functionality is usually abolished. Brumbley *et al*. (2002) used the suicide vector pUCD623 (Shaw *et al*. 1988) to generate over 700 transposon mutants of *L. xyli* subsp. *xyli*. This vector harbours the transposable element Tn*4431* containing a tetracycline resistance marker gene and the promoterless *lux*-operon from *Vibrio fischeri.*

Since *L. xyli* subsp. *xyli* does not produce reliable external symptoms in sugarcane, a robust colonisation assay was developed (Brumbley *et al*. 2004). A range of inoculation techniques was trialled (Young and Brumbley 2004). Inoculation of 6- to 8-week-old sugarcane plants with *L. xyli* subsp. *xyli* wild-type or mutant cells by decapitation above the apical meristem and transfer of a 100 µL aliquot of bacterial culture to the freshly cut surface worked best. It was also demonstrated that addition of filter-sterilised xylem extract from *L. xyli* subsp. *xyli*-infected sugarcane enhanced colonisation of sugarcane (Young and Brumbley 2004). Xylem fluid was collected 3 months after inoculation and analysed by evaporative-binding enzyme-linked immunoassay (Croft *et al*. 1994; Brumbley *et al*. 2004). Using this technique, three mutants that had lost the ability to colonise sugarcane were identified, one of which was selected for further analysis. Flanking DNA from the transposon insertion in this mutant was cloned, sequenced and used as a probe to isolate two overlapping cosmid clones, pLB1C2 and pLB1D6, from the *L. xyli* subsp. *xyli* cosmid library (Brumbley *et al*. 2004). The insertion of the transposon was in an open reading frame with weak homology to a hypothetical integral membrane protein from *Mycobacterium tuberculosum*. Two putative operons were identified in the immediate vicinity of the insertion site, both of which encode genes implicated in pathogenesis (Brumbley *et al*. 2004). To complement the mutation, we transformed the mutant with the cosmid pLB1C2, which restored the ability of this mutant to colonise sugarcane. However, because a

cosmid clone harbouring multiple genes was used in the complementation studies, it was not possible to determine whether the mutant's inability to colonise sugarcane was the result of transposon insertion or the result of a spontaneous mutation in one of the other genes encoded on the cosmids (Brumbley *et al*. 2004). Further work, such as marker-exchange studies, is required to confirm this.

Genetic uniformity of *L. xyli* subsp. *xyli*

Young *et al*. (2006) have provided a comprehensive study of the genetic variability within *L. xyli* subsp. *xyli*. Using a variety of techniques, no variation was detected among 105 different isolates of the *L. xyli* subsp. *xyli* from nine countries. Furthermore, no diversity was found when the DNA flanking 461 different transposon mutants, all generated in Australian isolates of *L. xyli* subsp. *xyli,* was sequenced. When these were compared to equivalent sequences in the genome of Brazilian isolates (Monteiro-Vitorello *et al*. 2004), they were shown to have 100% homology.

L. xyli subsp. *xyli* genome sequencing project

The genome of a Brazilian *L. xyli* subsp. *xyli* isolate (CTCB07) was sequenced fully by the Agronomic and Environmental Genomics Group (AEG) in Brazil (Monteiro-Vitorello *et al*. 2004). The genome is 2.6 Mb in size, contains ~2022 open reading frames and has a GC content of 68%, (Monteiro-Vitorello *et al*. 2004).

Several interesting characteristics were discovered within the *L. xyli* subsp. *xyli* genome. There is evidence that at one time *L. xyli* subsp. *xyli* could function as a free-living organism, it has a surprisingly large number of pseudogenes and there is evidence that it imported some genes required for its survival in sugarcane from other organisms. In addition, *L. xyli* subsp. *xyli* has genes that help it adapt and survive in its host plant, sugarcane.

Free-living state

The life cycle of *L. xyli* subsp. *xyli* appears to be limited to the xylem vessels of sugarcane plants. As discussed above, the bacterium can colonise other grass species in experimental systems, but it has never been found to naturally colonise any other grass or been found living free in the environment (Gillaspie and Teakle 1989).

Evidence that *L. xyli* subsp. *xyli* is, or used to be, able to survive outside of sugarcane xylem vessels, free-living in the environment, is shown by the fact that there are a large number ($n = 30$) of ABC transporter homologues, indicating it can utilise a large range of carbohydrates (Monteiro-Vitorello *et al*. 2004). In addition, this bacterium harbours homologues of photolyase and flagellar operons, two ABC glycine and betaine transporters, genes that protect against changes in the osmotic condition and genes for carotenoid biosynthesis. These all suggest that at one time it had a free-living life form (Monteiro-Vitorello *et al*. 2004).

Genome decay and pseudogenes

There is evidence that *L. xyli* subsp. *xyli* is undergoing genome decay, based on what appear to be 310 pseudogenes (i.e. gene fragments arising from frameshifts or deletions or caused by point mutations that introduce or remove stop codons, or incomplete genes), comprising 13% of the total number of predicted genes in this organism's genome (Monteiro-Vitorello *et al*. 2004) (Table 1). Other actinomycetes also undergoing genome decay, such as *Mycobacterium leprae*, causal organism of leprosy, have more pseudogenes (905, or 34.1% of its genes) (Cole *et al*. 2001), but *L. xyli* subsp. *xyli* has the highest level seen in any of the plant-associated bacteria whose genomes have been sequenced. Monteiro-Vitorello *et al*. (2004) believe this decay is brought about by the process of host adaptation and narrow host range (Table 1). This large number of

Table 1. Number of pathogenicity genes and pseudogenes in six species of bacterial phytopathogens
All data compiled from Monteiro-Vitorello *et al*. (2004). Rs, *Ralstonia solanacearum*; Xf, *Xylella fastidiosa* 9a5C; Xcc, *Xanthomonas campestris* pv. *campestris*; Xac, *Xanthomonas axonopodis* pv. *citri*; Atu, *Agrobacterium tumefaciens* C58; Lxx, *Leifsonia xyli* subsp. *xyli* CTC B07

Category	Species					
	Rs	Xf	Xcc	Xac	Atu	Lxx
Type III secretion system related function	34	0	35	35	0	0
Toxin production and detoxification	133	69	111	134	93	27
Host cell wall degradation	13	6	24	20	4	6
Exopolysaccharides	0	9	14	15	3	3
Surface proteins	35	6	11	14	28	31
Adaptation to atypical conditions	22	16	26	25	21	36
Other relevant genes[A]	33	28	66	59	57	2
Total pathogenicity and virulence	270	134	287	302	206	105
Pseudogenes	38	66	73	85	13	307
Protein-coding genes	5036	2249	4181	4427	5415	2326

[A]Genes involved in pathogenicity or virulence not listed in the above categories.

pseudogenes is an indicator of *L. xyli* subsp. *xyli* adapting to living exclusively in the xylem vessels of sugarcane. Examples of *L. xyli* subsp. *xyli* pseudogenes include 51 transposase-encoding genes, 4 of 28 flagellar operon genes, 14 of 30 ABC transporters, genes for metabolism of galactose and glutarate and for the synthesis of cysteine and methionine, and, most importantly, 20 of 105 putative virulence and pathogenicity genes (Monteiro-Vitorello *et al*. 2004). These 105 genes for virulence and pathogenicity found in *L. xyli* subsp. *xyli* present by far the smallest number found in any of the phytopathogenic bacteria sequenced: 20% smaller than *Xylella fastidiosa*, which colonises the xylem of citrus, and 60% smaller than two *Xanthomonas* species (Monteiro-Vitorello *et al*. 2004) (Table 1).

The large number of dysfunctional genes explains the nutritional fastidiousness of *L. xyli* subsp. *xyli* and indicates why it has only been found living in the xylem of sugarcane. Monteiro-Vitorello *et al*. (2004) suggest that if these pseudogenes were intact, *L. xyli* subsp. *xyli* would be able to live on plant debris and the flagella would allow it to move about more freely.

Lateral gene transfer and genomic islands

Within the genome of *L. xyli* subsp. *xyli*, there are several regions with altered GC content, and in some of these regions there are also altered codon bias and dinucleotide signatures, suggesting that these regions (genomic islands) may have been acquired from other organisms (Monteiro-Vitorello *et al*. 2004). Three of these islands contain possible pathogenicity genes, including pectinase and cellulase homologues, both of which would be involved in cell wall degradation. This may be one way that *L. xyli* subsp. *xyli* extracts some of its nutrients from sugarcane, although there is no evidence of this in any of the electron micrographs shown in the literature (Kao and Damann 1978, 1980).

In addition to these cell wall-degrading enzymes, one of the genomic islands also has a gene similar to *Pat-1* (Monteiro-Vitorello *et al*. 2004) of *Clavibacter michiganensis* subsp. *michiganensis*, which probably plays a significant role in plant wilting (Dreier *et al*. 1997).

The other potential pathogenicity genes residing in these genomic islands may be involved in the production of abscisic acid, a plant growth inhibitor which may play a role in the stunting from which the disease derives its name (Monteiro-Vitorello *et al*. 2004).

Host adaptation

There is another sugarcane pathogen, X*anthomonas albilineans*, which also colonises the vascular system of sugarcane and produces a toxin responsible for the primary symptom, leaf scald (Rott *et al*. 1994). The *L. xyli* subsp. *xyli* genome encodes a multi-drug efflux pump similar to that found in *X. albilineans*, which would allow *L. xyli* subsp. *xyli* to survive in sugarcane plants also infected with the leaf scald pathogen (Monteiro-Vitorello *et al*. 2004). The genome of *L. xyli* subsp. *xyli*, similar to those of other plant pathogens, encodes several gene products to allow it to survive host defence mechanisms such as reactive oxygen species. (Monteiro-Vitorello *et al*. 2004) (Table 1).

Discussion

Leifsonia xyli subsp. *xyli* is an extremely difficult bacterium to study. Not only does it not produce reliable external symptoms on or in its host plant sugarcane, but under irrigation or adequate rainfall, the stunting symptom can be reduced to the point where it is imperceptible. When this is compounded with the difficulties of growing this bacterium in axenic culture, it is not surprising that so little work was done previously to understand how this insidious pathogen interacts and causes disease on sugarcane. Biotechnology has allowed us to dramatically reverse this situation, to the point now where we can start to design key experiments to understand how this bacterium causes disease on sugarcane and to start devising strategies to control and even eradicate this costly pathogen from sugarcane growing regions around the world.

Our ability to transform and generate mutants of *L. xyli* subsp. *xyli* were critical first steps to understanding the functions of key genes and with the full genome sequenced we now have a good idea of what some of those key genes are. However, we still lack at least one critical tool to help us advance this work. It is possible to do random mutagenesis on *L. xyli* subsp. *xyli*, but site-directed mutagenesis techniques, such as marker exchange (He and Collmer 1990) have not been successful. Random mutagenesis is too slow; it took over a year to generate the 700 random mutants with Tn*4431* (Brumbley *et al*. 2002), whereas marker exchange should allow the knock out of specific genes selected from the genome database almost on a monthly basis: the time it takes to grow *L. xyli* subsp. *xyli* in axenic culture on solid media. To help select genes for knockout mutagenesis studies, oligonucleotide microarrays could be generated, which would include targets for either every putative gene in the *L. xyli* subsp. *xyli* genome or selected subsets of genes (Okinaka *et al*. 2002; Hinton *et al*. 2004; Lu *et al*. 2005). To generate the probes for these microarrays, mRNA would be isolated from *L. xyli* subsp. *xyli* grown under various conditions. For instance, S8 broth cultures of *L. xyli* subsp. *xyli* could be spiked with filter-sterilised xylem extract from healthy and *L. xyli* subsp. *xyli* infected sugarcane plants and these arrays compared to ones probed with non-spiked cultures and with *L. xyli* subsp. *xyli* isolated from xylem of infected sugarcane plants (Okinaka *et al*. 2002). In addition to microarrays, analysis of *L. xyli* subsp. *xyli* wild-type and various knock out mutants could include any of the powerful ‘omics’ tools, and in particular proteomics tools. To date, most proteomics used in plant–microbe interaction studies have focused on the host plant; however,

these technologies have excellent potential in pathogen identification (Padliya and Cooper 2006). Comparison of total cell proteins from *L. xyli* subsp. *xyli* cultures by two-dimensional gel electrophoresis could help identify key proteins that are differentially produced during the interaction between *L. xyli* subsp. *xyli* and sugarcane.

Proteomics could be used to study the impact of individual mutants on overall protein production, especially in relation to the regulatory networks that help trigger host plant recognition and colonisation. Again, pure cultures of *L. xyli* subsp. *xyli,* spiked with xylem extracts collected from healthy and infected sugarcane, could be compared to non-spiked cultures and with *L. xyli* subsp. *xyli* total proteins from cells isolated from infected sugarcane xylem vessels. Controls would include protein extracted from a similar volume of the culture media and of xylem extract from healthy sugarcane plants.

Comparative genomics is another tool that is already being used to help us understand pathogenicity, host range and virulence (Toth *et al*. 2006). The power of this, combined with functional genomics, will only be enhanced as more genomes are added to the databases. Several genes have already been identified in the *L. xyli* subsp. *xyli* genome data that would be worth further study. The xylem vessels of sugarcane do not contain high levels of nutrients. A study of the composition of xylem fluid in healthy sugarcane compared to that of xylem fluid from plants infected with *L. xyli* subsp. *xyli* should give us clues as to which of the functional ABC transporters would be critical for the survival *L. xyli* subsp. *xyli* in this environment. *Leifsonia xyli* subsp. *xyli* has only 105 genes categorised as pathogenicity related and 20 of these are predicted to be pseudogenes (Monteiro-Vitorello *et al*. 2004). A careful analysis of these genes should reveal candidates for knock out mutagenesis studies. There is also evidence of quorum sensing capabilities within the genome (von Bodman *et al*. 2003). Transgenic plants expressing enzymes that break down the quorum-sensing signal have shown significant resistance to infection by bacterial pathogens (Dong *et al*. 2000, 2001; Zhang 2003). If genes encoding these enzymes were under the control of a wound-inducible promoter that switched on when the crop is harvested, this could prevent one of the main forms of transmission. Something similar could be done with a *L. xyli* subsp. *xyli*-specific antibody such as the ones already used in our ELISA screening or one made specifically for one of the putative cell surface proteins identified in the genome database (Monteiro-Vitorello *et al*. 2004). This antibody could also be used as an improved diagnostic tool.

There are several virulence factors identified in the *L. xyli* subsp. *xyli* genome database that are also worth closer scrutiny. Two are cell wall-degrading enzymes, a pectinase and a cellulase (Monteiro-Vitorello *et al*. 2004), which may be involved in nutrient extraction from the xylem vessels and also may trigger the host defence, the production of the gelatinous material found to plug xylem vessels of sugarcane plants infected with *L. xyli* subsp. *xyli* (Kao and Damann 1978). The third, and one that Monteiro-Vitorello *et al*. (2004) state deserves special attention, may be involved in the synthesis of the plant hormone abscisic acid, which has been reported to be a plant growth inhibitor. This gene could be involved in the stunting and reduced tillers seen in sugarcane infected with *L. xyli* subsp. *xyli*.

Conclusions

The tools have now been assembled to rapidly advance our understanding of how *L. xyli* subsp. *xyli* can recognise sugarcane as a host, colonise its xylem vessels and cause RSD, and there are new tools on the horizon. Unlike most plant pathogens, the *L. xyli subsp. xyli* genome has been sequenced and is in the public domain. Establishment of the *L. xyli* subsp. *xyli* transcriptome and proteome databases will be major milestones for this pathogen and will be valuable tools for researchers, with practical uses for the sugarcane industry. At the gene expression level, genome and transcriptome tools can be used to study how *L. xyli* subsp. *xyli* responds to first contact with sugarcane as it moves from cutting and planting machinery to the cut surface of a sugarcane stalk. The proteome, will allow studies of how *L. xyli* subsp. *xyli* responds at the protein level and can be used to develop highly sensitive diagnostic tools. Because *L. xyli* subsp. *xyli* does not appear to colonise other plant species and it does not appear to be able to survive in the soil, it may be possible to devise a strategy to eradicate this disease from our sugarcane industries. This is a worthwhile goal, because RSD is still considered to be one of the most economically important diseases of sugarcane worldwide.

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