

## UNRAVELLING PATHOGENICITY OF *XANTHOMONAS ALBILINEANS*, THE CAUSAL AGENT OF SUGARCANE LEAF SCALD

By

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### Abstract

*Xanthomonas albilineans* is a systemic, xylem-invading pathogen that causes leaf scald in sugarcane. Symptoms vary from a single, white, narrow, sharply defined stripe to complete wilting and necrosis of infected leaves, leading to plant death. *X. albilineans* produces the toxin, albicidin, that blocks chloroplast differentiation, resulting in disease symptoms. Albicidin is the only previously known pathogenicity factor in *X. albilineans*, yet albicidin-deficient mutant strains are still able to efficiently colonise sugarcane. The complete genome of strain *X. albilineans* GPE PC73 from Guadeloupe was recently sequenced and annotated, providing a valuable tool to help identify new pathogenicity factors in this pathogen. In attempts to identify additional pathogenicity genes, site-directed deletions of candidate pathogenicity genes and also random Tn5 insertion mutagenesis were used, both followed by inoculation of sugarcane plants with new strains containing introduced mutations. Knockout mutagenesis of *albXXI*, which encodes a phosphopantetheinyl transferase that activates non ribosomal peptide synthetases (NRPSs), resulted in reduced capacity to colonise the sugarcane stalk by GPE PC73. Knockout mutagenesis of quorum sensing genes, *rpfF* and *xanB2*, did not affect albicidin production nor sugarcane colonisation by *X. albilineans* strain XaFL07-1 from Florida. However, four independent Tn5 insertion mutations of an OmpA family gene strongly affected both disease symptom development and sugarcane stalk colonisation without affecting albicidin production in strain XaFL07-1. Other candidate pathogenicity genes are currently being investigated.

### Introduction

Leaf scald, a vascular bacterial disease, is one of the major diseases of sugarcane (Rott and Davis, 2000). It was first recorded in Australia in 1911 by North, but strong presumptive evidence exists that the disease was prevalent in the Fiji Islands by 1908, if not earlier (Martin and Robinson, 1961). Today, the disease occurs in most sugarcane growing locations. Successful isolation of the causal agent, *Xanthomonas albilineans*, was reported in 1920 by Wilbrink who was also able to reproduce the symptoms of the disease after artificial inoculation (Wilbrink, 1920).

Although *X. albilineans* is thought to be mainly transmitted by infected cuttings, aerial transmission of this pathogen also occurs, and epiphytic life of the pathogen appears to be an important step in the epidemiological cycle of the disease (Autrey *et al.*, 1995a; Champoiseau *et al.*, 2009). Control of leaf scald is achieved by various methods including use of resistant cultivars and prophylactic measures (use of disease-free nursery material, disinfection of harvesting tools, etc.). Successful control based on resistance implies knowledge of genetic diversity and variation in pathogenicity of the pathogen. The objective of this paper is to review the literature on pathogenicity of *X. albilineans* and its genetic basis, and report recent research unravelling pathogenicity of this pathogen.

### Leaf scald symptoms and colonisation of the sugarcane stalk

In the field, the symptoms of sugarcane leaf scald are sometimes classified into two different forms, chronic and acute (Martin and Robinson, 1961; Ricaud and Ryan, 1989). It is unknown if these two forms are caused by entirely different sets of genes in different strains of the pathogen, by gene regulation differences among similar strains, or by external, environmental factors that may act on identical strains. The chronic form is characterised by chlorotic streaks on leaves which are parallel to the main veins. These are generally white to yellow narrow 'pencil-line' streaks, but they can also be several millimetres wide. Their colour often becomes reddish with age. They are the only external symptoms which develop on resistant cultivars (Rott and Davis, 2000). As the disease progresses, a necrosis of the leaf tissue around the chlorotic streaks may be observed which extends progressively from the tip towards the base of the leaf. The streaks also tend to widen and become more diffuse on leaves reaching maturity. The fine central line which is characteristic of the disease can, however, always be seen in the centre of the lesion. The widening of the lines coincides with the chlorosis or the bleaching of the leaf tissue. Chlorosis or bleaching may affect all the leaves, and this discoloration is followed by a desiccation of leaf extremities which curl inwards, giving the shoot or the stalk a tapered aspect. The stalks can be stunted and the leaves wilted, brown, and bent at the ends. This process, which looks like a scalding, explains the name given to the disease.

A common symptom observed in the chronic form of leaf scald in mature cane arises from abnormal development of side shoots on stalks with basal side shoots generally being more developed than those higher up, in contrast to the opposite situation commonly observed in healthy stalks. These diseased side shoots may show similar symptoms to those on the main stalk. Longitudinal sections of diseased stalks show reddening of the vessels near the nodes and sometimes in the internodes. Lysigenous cavities may be observed in severely diseased canes. The whole stalk may die in susceptible cultivars.

The acute form of leaf scald disease is characterised by a sudden wilting of mature stalks, often without previous development of symptoms associated with the chronic form. Previously symptomless sugarcane dies as if it had been killed by drought. This acute form often occurs after a period of rain followed by a period of prolonged dry weather, but seems to be limited to highly susceptible cultivars.

Another important feature of leaf scald is the existence of a latency phase (Martin and Robinson, 1961; Ricaud and Ryan, 1989). Sugarcane plants can be infected by *X. albilineans* for weeks or months without exhibiting symptoms or symptoms are so inconspicuous as to escape detection. This latency phase comes to an end for unknown reasons, and symptoms of the chronic or acute forms of leaf scald disease will then appear.

Regardless of the disease form observed, *X. albilineans* multiplies in the xylem and systemically colonises the entire host plant: the leaves, the stalk and the roots (Champoiseau *et al.*, 2006b; Klett and Rott, 1994). The pathogen can be easily isolated from pencil line symptoms but rarely from chlorotic parenchymatic tissue (Birch, 2001). Pathogen population densities vary according to the sugarcane cultivar and the plant location, and resistance of sugarcane is linked to limited colonisation of the sugarcane stalk (Rott *et al.*, 1997).

### Genetic basis of albicidin biosynthesis and structure of albicidin

Birch and Patil first showed in 1985 that a potent phytotoxin and antibiotic was produced by *X. albilineans* (Birch and Patil, 1985a and 1985b). Albicidin is a small molecule synthesised by a unique, large, and mixed PKS (polyketide synthase)-NRPS (non ribosomal peptide synthetase) gene cluster. Albicidin has been shown to cause leaf scald foliar symptoms in sugarcane by inhibiting the replication of proplastic DNA and consequently blocking the differentiation of chloroplasts (Birch and Patil, 1987a and 1987b). On a molecular level, it has been found to be a potent and novel DNA gyrase inhibitor (Hashimi *et al.*, 2007). The albicidin biosynthesis gene cluster was cloned and

sequenced (Rott *et al.*, 1996; Royer *et al.*, 2004). It contains 20 ORFs including one PKS-NRPS gene (*albI*) and two NRPS genes (*albIX* and *albIV*), as well as several putative resistance, regulatory and modifying genes (Figure 1). *In silico* analyses showed that nonproteinogenic substrates incorporated by AlBI and AlBIX are unique and remain unknown (Royer *et al.*, 2004). Two other genes located in other parts of the genome are also critical for the biosynthesis of albicidin: *albXXI* which encodes a phosphopantetheinyl transferase (Royer *et al.*, 2004) and *albXXII* which encodes the heat-shock protein HtpG (Vivien *et al.*, 2005).

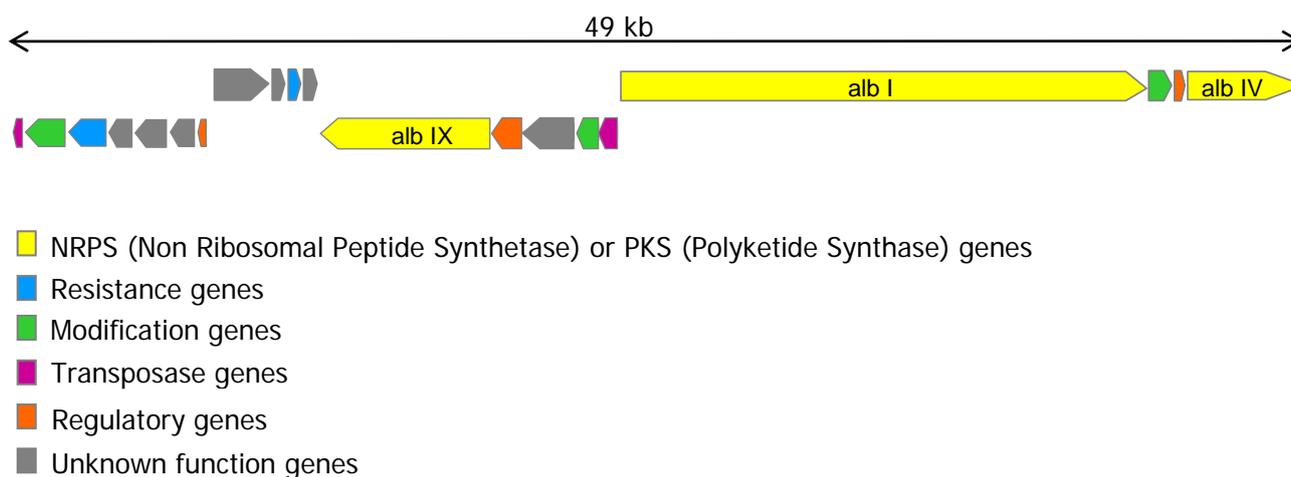


Fig. 1—Physical map of the albicidin biosynthesis gene cluster.

Preliminary analyses by NMR (Nuclear Magnetic Resonance) spectroscopy and mass spectrometry (MS) performed in the 1980s with purified albicidin did not result in the determination of the complex structure of albicidin (Birch and Patil, 1985a and 1985b). However, these structural analyses showed that albicidin has about 38 carbon atoms and an estimated molecular mass of 842 Da. According to these reports, the albicidin structure contains a methyl group, a carboxyl group and at least three aromatic rings (Birch and Patil, 1985a and 1985b).

*X. albilineans* is a slow growing bacterium and production yields of albicidin are low; it was extremely tedious to obtain sufficient amounts of albicidin for structure elucidation. To overcome this problem, Vivien *et al.* (2007) used heterologous expression by transferring all albicidin biosynthesis genes into the fast growing bacterium *X. axonopodis* pv. *vesicatoria* and obtained a significant increase of albicidin production. Even larger quantities of purified albicidin have more recently been obtained (Cociancich *et al.*, 2007 and 2009), which may allow elucidation of the structure of albicidin.

### Variation in pathogenicity and genetic diversity of *Xanthomonas albilineans*

Breakdown of varietal resistance to leaf scald has sometimes been attributed to the development or introduction of new strains but has never been unequivocally proven. Variation in pathogenicity among strains of *X. albilineans* is known to occur, and published data support the possible existence of different races in Mauritius (Autrey *et al.*, 1995b). The outbreak of leaf scald in Florida in the late 1980s was closely associated with the appearance of a genetically new strain of the pathogen (Davis *et al.*, 1997). The capacity of *X. albilineans* to colonise sugarcane stalks and/or to cause symptoms varies according to the strain of the pathogen, indicating the existence of different pathotypes within the species (Champoiseau *et al.*, 2006a; Daugrois *et al.*, 2003; Huerta-Lara *et al.*, 2009; Mohamed *et al.*, 1996).

First evidence of genetic diversity of *X. albilineans* was described by Rott and collaborators who reported the existence of three serovars and six lysovars of the pathogen (Rott *et al.*, 1986 and

1994). Subsequently, existence of different genetic profiles or genetic variation in *X. albilineans* was reported by several authors using various techniques, but the correlation between genetic diversity and variation in pathogenicity was rarely investigated (Alvarez *et al.*, 1996; Huerta-Lara *et al.*, 2009; Jaufeerally-Fakim *et al.*, 2002; Lopez *et al.*, 2001; Shaik *et al.*, 2008; Yang *et al.*, 1993). Existence of at least eight genetic groups (A-H) were identified by restriction fragment length polymorphism with pulse-field gel electrophoresis (RFLP-PFGE), and most strains associated with new outbreaks of leaf scald belonged to PFGE group B (Davis *et al.*, 1997). Fourteen haplotypes and two major genetic groups (ALB-RFLP A and ALB-RFLP B) were identified among 137 world wide strains of *X. albilineans* using DNA probes harbouring the albicidin biosynthetic genes (Champoiseau *et al.*, 2006a).

Not surprisingly, albicidin production *in vitro* varies according to the strain of *X. albilineans*. However, variation in albicidin production and variation in pathogenicity are not necessarily related to the diversity of albicidin biosynthesis genes. High variation in pathogenicity was shown to exist in genetically closely related strains of *X. albilineans* in Guadeloupe (Champoiseau *et al.*, 2006b) and no relationship was found either among the amount of albicidin produced *in vitro* and the pathotypes and genetic diversity of the pathogen (Renier *et al.*, 2007). Albicidin is therefore necessary but not sufficient for leaf scald development, and it appears to be one virulence factor acting coordinately with other virulence factors.

### **The genome of *Xanthomonas albilineans* and pathogenicity**

The complete genome of *X. albilineans* strain GPE PC73 from Guadeloupe has been recently sequenced and annotated (Pieretti *et al.*, 2009). This work revealed that the size of this genome (3.7 Mb) was relatively small in comparison to the size of the genomes of other xanthomonads sequenced to date (approximately 5 Mb). Interestingly, *X. albilineans* possesses 522 genes that are not conserved in other sequenced species of the *Xanthomonadaceae* family, and is missing the **h**ypersensitive **r**esponse and **p**athogenicity (Hrp) secretion system. This type III secretion system (T3SS) is found in most other pathogenic xanthomonads and is used for injection of protein pathogenicity effectors into plant cells. *X. albilineans* also lacks all genes involved in biosynthesis of xanthan gum which are involved in formation of biofilm, an important factor in virulence of plant pathogenic bacteria.

The remarkable absence of a type III secretion system of the Hrp injectisome family in the genome of *X. albilineans* implies that pathogenicity of *X. albilineans* relies on other secretion systems. Small molecules, such as albicidin, which require only pumps to be secreted by the bacterium and to enter the plant cell, may play an important role in pathogenicity of this pathogen.

This assumption is based on findings that the genome of *Xa* contains 12 NRPS genes clustered in four genomic regions potentially involved in the biosynthesis of four small molecules. These 12 NRPS genes are very different from those described in other microorganisms, and cover almost 4% of the bacterial genome.

Three of these 12 NRPS genes were previously described as required for the biosynthesis of albicidin (Royer *et al.*, 2004).

### **Identification of new pathogenicity genes of *X. albilineans***

If albicidin is a major factor in symptom development, it is not the only one involved in disease progress. Mutants of *X. albilineans* that do not produce the toxin are still able to colonise the sugarcane leaf and stalk (Birch, 2001; Marguerettaz *et al.*, unpublished results). Therefore, several studies have recently been started to identify new pathogenicity genes in *X. albilineans*.

### **Quorum sensing genes**

The genome sequence of *X. albilineans* revealed candidate genes potentially involved in pathogenesis. These candidates include a cluster of genes called *rpf* (**r**egulation of **p**athogenicity

factors) involved in the biosynthesis of a small diffusible signalling molecule. This molecule called DSF (**D**iffusible **S**ignalling **F**actor) is synthesised by RpfF which exhibits similarities to long-chain fatty acyl CoA ligases (Barber *et al.*, 1997; Dow 2008). DSF mediates cell-to-cell signalling (= quorum sensing) and disruption of genes *rpfF* or *rpfC* (a hybrid two-component DSF sensor) resulted in reduced or deficient virulence in different xanthomonads, such as *X. campestris* pv. *campestris*, *X. oryzae* pv. *oryzae* and *X. axonopodis* pv. *citri* (Chatterjee *et al.*, 2008; Dow, 2008). However, mutants of *rpfF* and *rpfC* in *X. albilineans* strain XaFL07-1 from Florida were still able to produce albicidin, to colonise the sugarcane stalk and to induce leaf scald symptoms including 'pencil-line' streaks, chlorosis and necrosis of leaves (Rott *et al.*, 2009b). DSF is therefore not essential for albicidin production or sugarcane colonisation by *X. albilineans*.

*Xanthomonas* species also produce another quorum sensing molecule called DF (**D**iffusible **F**actor) encoded by *xanB2* (previously called *pigB*). In *X. campestris* pv. *campestris*, DF regulates the production of both yellow pigments (xanthomonadins) and extracellular polysaccharide (EPS), and these two bacterial products are crucial to the epiphytic survival and pathogenicity of the pathogen on its host plants (Poplawsky and Chun, 1997 and 1998). However, mutants of *xanB2* in *X. albilineans* strain XaFL07-1 from Florida were still able to produce albicidin, to colonise the sugarcane stalk and to induce leaf scald symptoms including 'pencil-line' streaks, chlorosis and necrosis of leaves (Rott *et al.*, 2009a). DF is therefore not essential for albicidin production or sugarcane colonisation by *X. albilineans*, and these two characteristics may not be controlled by quorum sensing or may involve another regulatory pathway. However, it is possible that DF, like in *X. campestris* pv. *campestris*, is crucial to the epiphytic survival of *X. albilineans* during aerial transmission of the leaf scald pathogen.

### NRPS genes

Other genes putatively involved in pathogenicity of *X. albilineans* include NRPS genes that are involved in the biosynthesis of small molecules. As mentioned above, the genome of *X. albilineans* encodes 12 large non-homologous non-ribosomal peptide synthetases (NRPSs). Three of these NRPSs, encoded in the same gene cluster, were previously described as involved in albicidin biosynthesis (Royer *et al.*, 2004). The function of the remaining nine NRPSs is currently unknown but it has been hypothesised that they may be involved in biosynthesis of small molecules playing a role in sugarcane-*X. albilineans* interactions. NRPS enzymes need posttranslational activation by a phosphopantetheinyl transferase enzyme in order to be functional (Lambalot *et al.*, 1996). The genome of *X. albilineans* strain GPE PC73 contains the gene *albXXI* which encodes a phosphopantetheinyl transferase and which was previously shown to be required for albicidin biosynthesis (Huang *et al.* 2000; Royer *et al.*, 2004). This gene may therefore be used for post-translational activation of all NRPSs encoded by *X. albilineans*. Inactivation of *albXXI* in *X. albilineans* strain GPE PC73 resulted in suppression of leaf scald symptoms and reduced capacity to colonise the sugarcane stem when compared to the wild type strain (Marguerettaz *et al.*, 2009). This suggested that at least two secondary metabolites synthesised by NRPSs are involved in virulence of *X. albilineans* strain GPE PC73: (i) albicidin, which causes the white pencil line streaks on the leaves, and (ii) at least another molecule necessary for stalk colonisation.

### Outer membrane protein A gene

Tn5 (transposome) mutagenesis was also used in an attempt to identify additional pathogenicity factors in *X. albilineans*. Sugarcane cultivar CP80-1743, moderately susceptible to leaf scald, was inoculated by the decapitation method (Champoiseau *et al.*, 2006a) with 780 independently derived Tn5 insertions in Florida strain XaFL07-1. Leaf scald symptoms were recorded on emerging leaves one month after inoculation, and stalk colonisation by the pathogen was determined two months after inoculation. In addition to the previously identified albicidin biosynthetic gene cluster mutations, four new Tn5 mutants were identified that produced no or very

few leaf symptoms. These mutants produced albicidin *in vitro* but did not efficiently colonise sugarcane stalks. The transposon insertion site of all four mutants was found to be located in Orf XALc\_0557 of the *X. albilineans* genome. This gene is predicted to encode an OmpA family outer membrane protein, a previously unrecognised and apparently essential pathogenicity factor in *X. albilineans* (Rott *et al.*, 2009a).

### Other genes

Additional Tn5 mutants affected in symptom development or in sugarcane stalk colonisation have been obtained, such as mutants affecting production of exopolysaccharides (EPS) and lipopolysaccharides (LPS) (Rott *et al.*, unpublished data). These mutants will be further investigated and more mutants will also be tested by sugarcane inoculation to identify additional pathogenicity genes in *X. albilineans*.

### Conclusion

The xylem-invading mechanisms of plant pathogenic bacteria are still not well characterised. The *X. albilineans*/sugarcane pathosystem is an original model in plant pathology to study xylem-invading pathogens, and certain aspects of its invasive ability may be unique.

First, *X. albilineans* is constrained within the xylem, but disease symptoms result from changes in chlorenchyma cells; nevertheless, the bacteria are rarely found in chlorotic leaf areas.

Second, *X. albilineans* infections can be latent for many weeks or months before occurrence of acute disease, and this latency phase ends for unknown reasons. It is thought that this phase change is most likely regulated by particular pathogenicity genes of the pathogen.

Third, *X. albilineans* does not possess a Hrp secretion system. Finally, *X. albilineans* produces albicidin, a unique and specific toxin that causes foliar leaf scald symptoms.

Extensive research has been conducted on *X. albilineans* since its first description more than a century ago. Important knowledge regarding the biology of this pathogen has been gained, and genetic diversity studies and pathogenicity studies showed that this pathogen is variable and evolving. However, more studies are needed to understand the *X. albilineans*/sugarcane pathosystem, especially the interactions between the host and the pathogen.

Recent description of the genome of *X. albilineans* will certainly result in new and significant progress, especially regarding the molecular basis of pathogenicity of this pathogen and its interactions with sugarcane.

First genes involved in pathogenicity, such as genes involved in albicidin biosynthesis and in an outer membrane protein have been identified, and more will certainly follow in the near future. Several mutagenesis techniques are already used to identify new candidate genes potentially involved in pathogenesis.

Other methods such as microarray technology will also be used to investigate the full breadth of the response of *X. albilineans* to the sugarcane host environment during colonisation of the xylem, and maybe also during the epiphytic life of the pathogen.

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## DECRYPTAGE DE LA PATHOGENIE DE *XANTHOMONAS ALBILINEANS*, L'AGENT CAUSAL DE L'ECHAUDURE DES FEUILLES DE LA CANNE A SUCRE

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### Résumé

*XANTHOMONAS albilineans* est un agent pathogène colonisant le xylème et l'agent causal de l'échaudure des feuilles de la canne à sucre. Les symptômes varient d'une très fine ligne foliaire blanche à un flétrissement complet et la nécrose des feuilles infectées, conduisant à la mort de la plante. *X. albilineans* produit la toxine albicidine qui bloque la différenciation des chloroplastes, ce qui aboutit à l'apparition de symptômes foliaires. L'albicidine était le seul facteur de pathogénie connu jusqu'à récemment, même si des souches non productrices d'albicidine étaient toujours capables de coloniser la canne à sucre. Le génome complet de la souche de *X. albilineans* GPE PC73 originaire de Guadeloupe a récemment été séquencé et annoté, fournissant ainsi un outil fort utile à l'identification de nouveaux facteurs de pathogénie chez cet agent pathogène. Afin d'identifier de nouveaux gènes de pathogénie, des mutants de délétion dirigée et d'insertion aléatoire (Tn5) ont été produits puis inoculés à des plants de canne à sucre. La mutation du gène *albXXI*, qui code pour une phosphopantetheinyl transférase activant les enzymes NRPS («Non Ribosomal Peptide Synthetases»), a réduit la capacité colonisatrice de la tige de canne à sucre chez la souche GPE PC73 de *X. albilineans*. La délétion par mutagenèse dirigée des gènes de signalisation cellulaire *rpfF* and *xanB2* n'a apparemment pas eu d'effet sur la production d'albicidine ou la colonisation de la tige de canne à sucre par la souche de *X. albilineans* XaFL07-1 originaire de Floride. Cependant, quatre mutations Tn5 indépendantes d'un gène de la famille *OmpA* a fortement affecté le développement de symptômes et la colonisation de la tige, sans modifier la production d'albicidine, chez la souche XaFL07-1 de *X. albilineans*. D'autres gènes candidats de pathogénie sont actuellement à l'étude.

## VARIACIONES EN LA PATOGENICIDAD DE *XANTHOMONAS ALBILINEANS*, EL AGENTE CAUSAL DE LA ESCALDADURA DE LA HOJA DE LA CAÑA DE AZÚCAR

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### Resumen

*XANTHOMONAS albilineans* es un patógeno sistémico, que invade el xilema y causa la escaldadura de la hoja de la caña de azúcar. Los síntomas pueden variar desde la aparición de una sola raya blanca, angosta, bien definida hasta tener una completa marchitez y necrosis de las hojas afectadas, lo que lleva a la muerte de la planta. Además, *X. albilineans* produce la toxina, albicidina, que bloquea la diferenciación de cloroplastos, lo que resulta en la aparición de los síntomas de la enfermedad. La albicidina ha sido conocida como el único factor de patogenicidad asociado con *X. albilineans*, y es así como cepas mutantes con deficiencia en la producción de la albicidina, son capaces de colonizar de manera eficiente la caña de azúcar. El genoma completo de la cepa *X. albilineans* GPE PC73 proveniente de Guadalupe se secuenció recientemente, proporcionando así una herramienta muy valiosa en la identificación de nuevos factores de patogenicidad de este agente causal. En un intento por identificar los genes patogénicos adicionales, se produjeron supresiones de genes candidatos así como inserciones al azar por mutagénesis de Tn5, seguidos por la inoculación de plantas de caña de azúcar con las nuevas cepas que contienen las mutaciones introducidas. El bloqueo por mutagénesis de albXXI, que codifica la transferasa fosfopanteteinil y que activa los péptidos sintetasas no ribosomales (NRPSs), produjo una reducción en la capacidad de la cepa GPE PC73 de colonizar el tallo de la caña de azúcar. El bloqueo por mutagénesis de genes quorum sensing, rpfF y xanB2, de *X. albilineans* cepa XaFL07-1 de Florida no afectaron la producción de la albicidina ni la colonización de la caña de azúcar. Sin embargo, cuatro mutantes por inserción independiente del gen Tn5 de la familia Ompa, en la cepa XaFL07-1, fueron afectados fuertemente tanto en el desarrollo de los síntomas de la enfermedad como en la colonización de tallos de la caña de azúcar sin afectar la producción de la albicidina. Otros genes de patogenicidad están siendo investigados.